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Pharmaceutical strategies for the treatment of bacterial biofilms in chronic wounds

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Biofilms are sessile communities of microorganisms, mainly bacteria, that grow on biotic and abiotic surfaces. These microorganisms are embedded within an extracellular polymeric substance that provides enhanced protection from antimicrobials. Chronic wounds provide an ideal habitat for biofilm formation. Bacteria can easily attach to wound debris and can infect the wound due to an impaired host immune response. This review highlights the mechanism of biofilm formation and the role of biofilms in the pathophysiology of chronic wounds. Our major focus is on various formulation strategies and delivery systems that are employed to eradicate or disperse biofilms, thereby effectively managing acute and chronic wounds. We also discuss clinical research that has studied or is studying the treatment of biofilm-infected chronic wounds.

Keywords: Biofilms; Chronic wounds; Antibiofilm agents; Antimicrobial resistance; Bacterial infections; Drug delivery systems; Nanomaterials

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Introduction

The serendipitous discovery of penicillin by Sir Alexander Fleming in 1928[1] transformed medicine and spearheaded the era of antibiotic discovery. The average life expectancy in the U.S.A alone rose from 47 years to around 78 years.[2] Nevertheless, bacteria have developed resistance to antimicrobials as a natural coping mechanism that enables their survival, and thus, the menace of antimicrobial resistance (AMR) threatens to return human beings to a time when infectious diseases could again become the leading cause of mortality. The rise of AMR has been influenced by the overuse and misuse of antibiotics. It is estimated that, by 2050, 10 million people may die from diseases caused by AMR strains alone, with an accompanying economic burden that could amount to US$100 trillion.[3] The formation of biofilms by bacteria is a significant contributor to the development of AMR. According to the National Institutes of Health and the Center for Disease and Prevention, it is estimated that 65–80% of human infections are caused by biofilms.[4] A biofilm is an organized community of microorganisms, most commonly bacteria, attached to an abiotic or biotic surface. The community develops embedded within an extracellular polymeric substance (EPS) made of polysaccharides, proteins and DNAs that are secreted by the microorganisms themselves. The EPS accounts for 90% of the biomass of a biofilm.[5]

Infections that result from biofilm formation are a serious threat to patients globally. Biofilm formation mediates a diverse range of diseases, such as cystic fibrosis, wound infections, otitis media, pneumonia, and osteomyelitis, that are formed on tissues and implanted devices.[6] Chronic wounds, such as diabetic foot and pressure ulcers, are hotspots for biofilm growth because necrotic tissue and debris permit the attachment of bacteria. This impairs the healing process and exacerbates the patient's condition.[7] The treatment of all biofilm-dependent clinical presentations is particularly problematic because bacteria that are encased within a biofilm may be 1000-fold more resistant to antibiotics than their planktonic counterparts.[8]

Various antibiotics, such as fusidic acid, mupirocin and silver sulfadiazine, have been used to treat wound infections, but biofilm formation hampers their antibacterial activity. In this regard, antibiofilm agents can offer exceptional advantages as they can be useful in disrupting the biofilm matrix and in enhancing the efficacy of antibiotics. The formulation of efficient delivery systems that incorporate antibiofilm agents can provide appropriate control of wound infections with better clinical outcomes. Following a brief introduction to the processes of wound healing and biofilm formation and to the interplay between them, this review focuses on antibiofilm agents, drug delivery strategies, and clinical trials relating to the treatment of biofilm-infected chronic wounds.

Chronic wound pathophysiology

A wound is any break or damage in the surface of the skin. It can range from a small cut to a large surgical or burn wound. Wound healing is a process that commences naturally and immediately in response to an injury. It is a well-coordinated and a complex process that involves a cascade of physiological responses elicited by various cell types.[9] Acute wounds undergo uninterrupted repair, whereas 'chronic wounds' fail to heal due to interference in the orderly process that result from an infection or an underlying serious illness. The wound healing process is summarized schematically in Fig. 1.

When a wound fails to heal in the normal timeframe due to various factors such as vascular insufficiency, diabetes, ageing or infection, it is said to have become chronic.[10] Chronic wounds are stuck in a vicious loop of hyperinflammation, evidenced by a 2–3-fold increase in the accumulation of neutrophils and macrophages and in the expression of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL1-β) and other pro-inflammatory cytokines.[11] An excess of neutrophils stimulates the overproduction of reactive oxygen species (ROS), leading to direct damage to the extracellular matrix (ECM) and, eventually, to premature cell death.[12] Moreover, the release of proteases such as elastase and matrix metalloproteinases (MMPs) such as neutrophil collagenase (MMP-8) by neutrophils is enhanced in response to prolonged inflammation signals.[13] This results in the degradation of ECM components and growth factors, and impairs the action of antimicrobial peptides such as cathelicidins.[13] The production of pro-inflammatory cytokines such as IL-1β and TNF-α by neutrophils and activated macrophages also leads to a reduction in the accumulation of tissue inhibitors of MMPs (TIMPs). This exacerbates further degradation of the ECM and decreases fibroblast proliferation and collagen synthesis. Such an exhaustive and undesirable inflammatory response, combined with an impaired host response, perpetuates a deleterious cycle that prevents the healing of chronic wounds.

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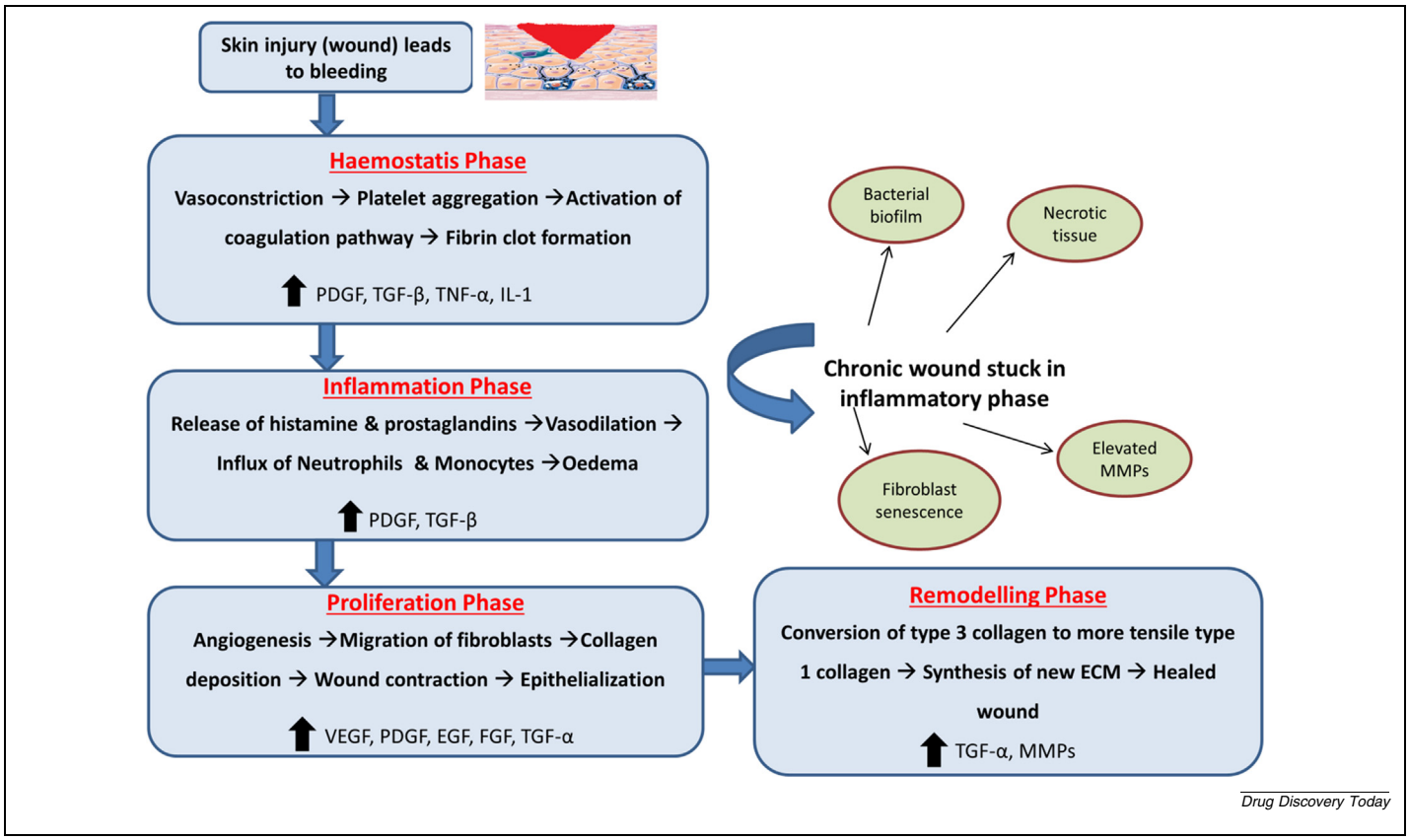

FIG. 1

Schematic description of the sequence of events in acute and chronic wound healing. ECM, extracellular matrix; EGF, Epidermal growth factor; FGF, Fibroblast growth factor; IL-1, Interleukin 1; MMP, matrix metalloproteinase; PDGF, Platelet-derived growth factor; TGF-β, Transforming growth factor β; TNF-α, Tumor necrosis factor α; VEGF, Vascular endothelial growth factor.

Vasculopathies such as venous hypertension, atherosclerosis or periwound fibrosis lead to local tissue hypoxia, which contributes to the perpetuation of chronic wounds. Enhanced expression of endothelial adhesion molecules in hypoxic tissue leads to extravasation of neutrophils and macrophages, which further promotes the synthesis of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α. Moreover, the production of nitric oxide (NO), an antioxidant, is reduced in the hypoxic state, hindering the regulation of ROS formation.[14] Hypoxia also impairs re-epithelialization and fibroblast proliferation.

Types of chronic wounds

Pressure (decubitus) ulcers

Patients who have lost sensory perception or who are immobile (unconscious or paralyzed) cannot respond in a timely way to the need for repositioning. Therefore, their skin tissue may be susceptible to compression, particularly over bony prominences such as the hips and sacrum.[15] This may lead to tissue necrosis as the result of localized ischemia and reperfusion, sustained cell deformation, and impaired interstitial fluid flow and lymphatic drainage.[15] The skin breakdown is followed by bacterial colonization, which further interferes with angiogenesis, the deposition of ECM and other wound-healing processes, and chronic ulcers persist.[16].

Venous stasis ulcers

More than half of lower-limb chronic wounds are venous stasis ulcers, which have higher prevalence in the elderly and in women.[17] These ulcers manifest secondary to venous hypertension as a result of venous thrombosis or damage in the valves of leg veins.[18] An increase in the venous backpressure causes leakage of plasma macromolecules and fibrin into the perivascular space. Fibrin accumulation impairs collagen synthesis and disrupts the normal function of the vessel by forming pericapillary fibrin cuffs.[19] The fibrosis and oedema retard the circulation of oxygen and nutrients into the wound tissue, creating a hypoxic environment. All of these events lead to skin breakdown and eventually to chronic ulcers.

Arterial ulcers

Arterial ulcers occur due to hypoxic and ischemic damage to distal tissues that results from the narrowing of the arterial lumen due to atherosclerosis and/or embolism.[20] Major risk factors for the peripheral occlusion of arteries in the legs include smoking, hypertension, diabetes and hypercholesterolemia. Ulcers can result from minor trauma to an affected area.

Diabetic foot ulcers

Diabetic ulcers are a grave complication of diabetes that result from the pathogenic triad of ischemia, neuropathy and trauma. Loss of sensation in the feet due to diabetes-associated peripheral

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neuropathy, augmented by disrupted perfusion, enhances the risk of ulceration from recurrent mechanical strain.[21] Moreover, the accumulation of advanced glycation end-products (AGEs) due to metabolic disarray, induces oxidative stress and escalates ECM stiffness.[22] A chronic inflammatory state perpetuates, which most often leads to limb amputations.

Mechanism of bacterial biofilm formation

Within the complex architecture of biofilms, bacteria are shielded and develop adaptive responses to hostile environmental conditions that enable them to endure desiccation or starvation, evade the host immune system, or develop increased resistance to antibiotics.[23] The biofilm matrix that acts as a cocoon for bacteria is composed of exopolysaccharides, proteins and extracellular DNA (eDNA). The polysaccharide composition varies across species, for instance, *Pseudomonas aeruginosa* produces three types of exopolysaccharides: alginate, Pel and Psl. Alginate is responsible for providing mechanical stability to mature biofilms, whereas Pel and Psl take part in the early stages of biofilm formation by non-mucoid strains. Poly-N-acetylglucosamine (PNAG) provides structural integrity to biofilms of *Staphylococcus epidermidis*.[5]

Cell-surface-associated proteins and extracellular carbohydrate-binding proteins (lectins) are a few of the many proteins found among the EPS. The galactose-specific lectin LecA and the fucose-specific lectin Lecb of *P. aeruginosa*, as well as glucan-binding proteins of *Streptococcus mutans*, have been linked to biofilm formation.

Another significant and integral component of the EPS is eDNA, which serves various roles in biofilm formation in different bacterial species. eDNA serves as an intercellular connector in *P. aeruginosa* biofilms, whereas it provides structural integrity to *Staphylococcus aureus* biofilms. In *Bacillus cereus* biofilms, eDNA acts as an adhesion molecule.[5] The significance of eDNA for structural stability is typically shown by the DNase-mediated disintegration of biofilms.[24]

The formation of a biofilm is a multistep dynamic process comprising of four stages: (i) attachment of bacteria to a surface, (ii) microcolony formation, (iii) biofilm maturation, and (iv) detachment or dispersal (Fig. 2).[25]

The initial attachment of bacteria consists of reversible and irreversible stages. The free-floating planktonic bacteria loosely adhere to the substratum through the weak van der Waals, hydrophobic and/or electrostatic forces.[26–27] Bacteria that utilize flagella to overcome the hydrodynamic and repulsive forces that may act against attachment have a competitive advantage. Adhesins secreted by bacteria, which are present at the tips of extracellular appendages (pili, flagella and fimbriae), mediate adherence

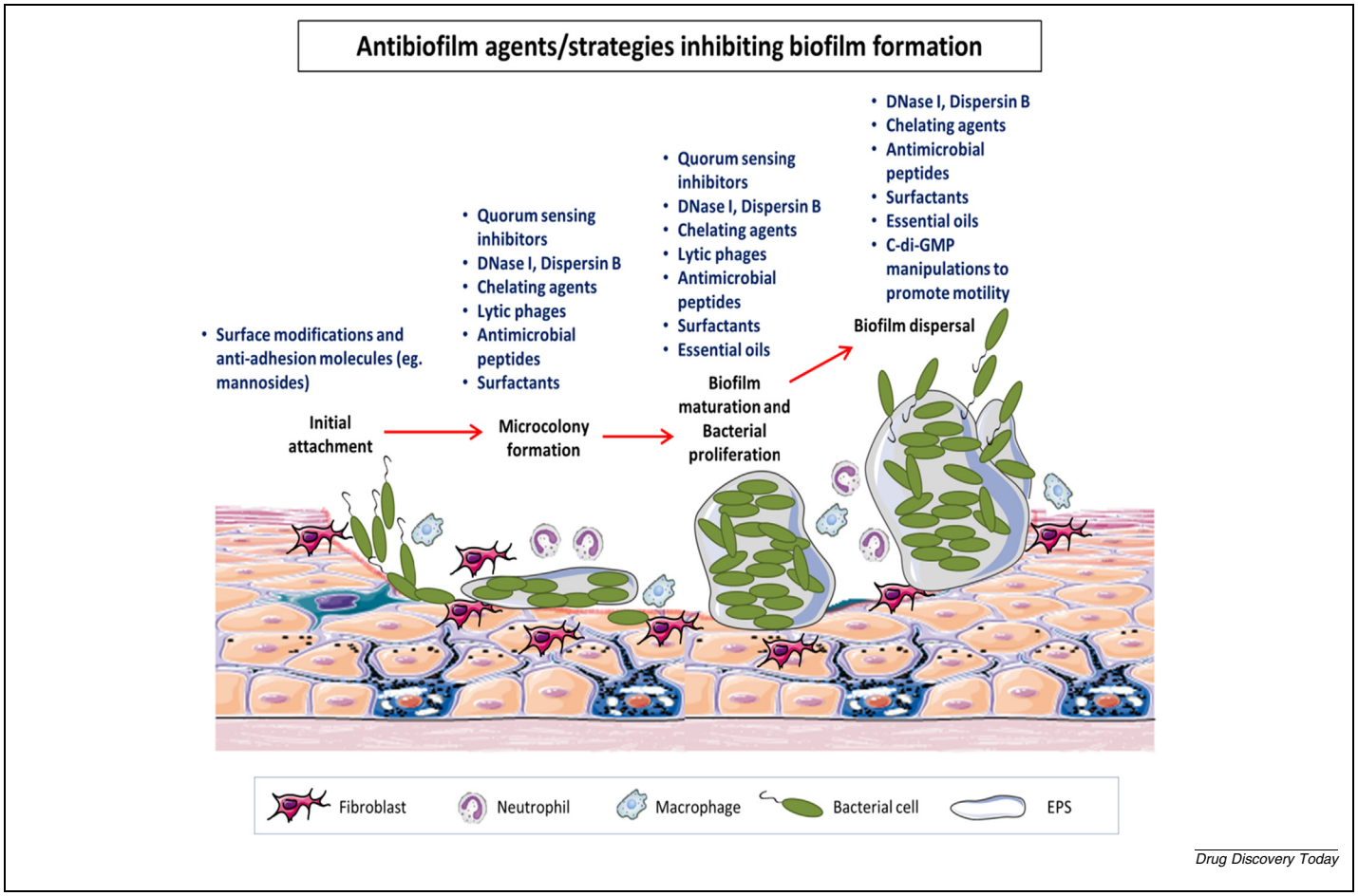

FIG. 2

Stages of biofilm formation on the wound surface, and antibiofilm agents and strategies for biofilm inhibition and/or dispersal. C-di-GMP, cyclic dimeric guanosine monophosphate; EPS, extracellular polymeric substance.

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to the surface.[28] Once the attachment is secured, signals for a change in gene expression are triggered, leading to the upregulation of factors that are implicated in the formation of EPS. Bacteria begin to produce EPS, which further strengthen their attachment to each other and to the substratum. They start to divide and form microcolonies in response to cell–cell communication. This cellular crosstalk is referred to as quorum sensing (QS). The QS mechanism involves signaling molecules that bind to response regulators and lead to the regulation of many genes, including those encoding for bacterial virulence factors.[29] QS molecules are called autoinducers (AIs). Gram-negative bacteria produce N-acyl-homoserine lactones (AHLs) as AIs, whereas Gram-positive bacteria use autoinducing peptide (AIP), and both bacteria also use AI-2-based signaling.[30–31] With the inception of colonization, the microcolonies further enlarge and enhance the production of EPS.[5] Further recruitment of microbial cells, along with deposition of organic and inorganic solutes, results in the biofilm maturation.

After complete establishment of a biofilm, detachment and dispersal take place to allow the colonization of new niches by the bacteria. The dispersal process, which can be active or passive, involves environmental signals, signal transduction pathways and effectors. Active dispersal is mediated by the bacteria themselves, whereas passive dispersal is mediated by the external forces such as fluid shear.[32] Bacteria produce matrix-degrading enzymes, such as glycosidases and proteases, that support the active dispersal of the biofilm. One of the most-studied intracellular signaling molecules is cyclic-diguanyl monophosphate (c-di-GMP), reduced levels of which induce biofilm dispersal.[33] Daughter cells may detach individually or as clumps containing thousands of bacteria due to the shearing of surrounding fluids or changes in the surface properties of substratum. The released cells carry and retain the characteristics of the parent biofilm and reform on a new or existing substratum.[34] In this way, the process of new biofilm formation begins, and the cycle continues.

Biofilms in chronic wounds

Many factors which can delay wound healing, but biofilm formation is of utmost importance. In 2008, James *et al.*[7] identified biofilms in clinical specimens from chronic and acute wounds using both light microscopy and scanning electron microscopy (SEM). Evidence of the presence of biofilms in ulcerated burn wound areas was found by Kennedy *et al.*[35] in 2010 using electron and light microscopy. In 2011, Neut *et al.*[36] found dense aggregates of bacterial colonies surrounded by EPS in patients with diabetic ulcers. Chronic wounds provide an ideal habitat for biofilm formation. Bacteria can easily attach to wound debris and infect the wound due to impaired host immune response.[37] The presence of biofilm affects the function of cutaneous-cellular and immune cells. It acts as a physical barrier to neutrophil and macrophage penetration and prevents the active killing of bacteria. *P. aeruginosa* bacteria have been reported to lyse the neutrophils that come into close contact with them via QS system factors.[38] In addition, rhamnolipids that are produced by bacteria have been reported to prevent the phagocytosis of bacteria by neutrophils.[39] Ciszek-Lenda *et al.*[40] demonstrated that *P. aeruginosa* (PAR5 strain) biofilm contained high concentrations of extracellular DNA and lipo-polysaccharide (LPS), and that phagocytes (neutrophils and macrophages) that were exposed to this microenvironment secreted inflammatory mediators such as TNF-α, IL-6 and PGE_2, thereby inducing a hyperinflammatory state that caused tissue damage.

Wound repair depends on a fine balance between the activities of proteases and their inhibitors. MMPs are zinc-dependent proteases that digest ECM and mediate the influx of reparative fibroblasts, called keratinocytes.[41] TIMPs keep the level of MMPs in check by downregulating the levels of proinflammatory cytokines. In chronic wounds, however, this balanced regulatory system goes awry and excessive cleavage of unintended targets, such as growth factors and cytokines, ensues.[42] This significantly hampers the repair process. The presence of biofilm has been shown to augment the production of MMP-1 and MMP-3 by keratinocytes.[43] Concentrations of MMP-1 and MMP-8 have been demonstrated to be increased in chronic venous ulcers that are infected with *S. aureus* and *P. aeruginosa*.[44] Thermolysin protease secreted by *P. aeruginosa* activates MMP-1, MMP-8 and MMP-9.[45]

Antibiofilm agents

Biofilms at the wound sites can be addressed in two ways: either by preventing their formation or by the disruption of already established biofilms. Biofilm formation can be prevented by modifying either the surface or the microbial cells themselves to inhibit the attachment of bacteria. Pre-conditioning of the surface with surfactants or conferring surface hydrophobicity can help to inhibit the biofilm formation.[46–47] Pre-formed biofilms can be damaged by disruption of the biofilm matrix and its subsequent detachment. Biofilms can be detached by three approaches: sloughing, erosion or seeding dispersal.[48] Sloughing involves the removal of lumps of biofilm from the surfaces by stripping-off biofilm mass, whereas erosion is the slow and continuous detachment of small portions of the biofilm. Seeding dispersal is an active process in which single bacterial cells from the central biofilm region are discharged rapidly.[48] Herein, we focus on agents that destroy pre-formed biofilms, with their targets and modes of action listed in Table 1.

Delivery systems for antibiofilm agents to promote wound healing

The main aim of wound treatment is to maintain a moist and clean environment, to manage pain and to address comorbidities that aggravate the wound. The systemic treatment of infection faces complexities such as drug interactions, systemic adverse effects, and insufficient drug concentrations at the local wound site. Moreover, the use of systemic antibiotics to treat certain types of chronic wounds, such as diabetic ulcers or wounds that involve the presence of a biofilm, is not preferred because it is not supported by adequate evidence.[49–50] Topical delivery of antibiotics has been used extensively for wound infections. This delivery strategy provides higher local drug concentrations and avoids the need for high-dose systemic exposure by reducing the amount of antibiotic required to kill the bacteria.[51] Nevertheless, the presence of biofilm in chronic wounds limits the effectiveness of topical antibiotic treatment. The EPS in biofilms act as

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TABLE 1

Antibiofilm agents and their modes of action.

Antibiofilm agents	Mode of action	Examples	References
Quorum sensing (QS) inhibitors	QS signaling can be dampened by the quorum quenching (QQ) of enzymes and molecules. QQ can be achieved by the degradation of QS signals, which in turn would suppress QS-regulated phenotypes such as virulence and biofilm formation.	Azithromycin, bergamottin, usnic acid, quercetin, ellagic acid	80–83
Extracellular polymeric substance (EPS)-degrading enzymes	Dispersin B (DspB), a bacterial glycoside hydrolase produced by *Actinobacillus actinomycetemcomitans*, cleaves poly-β 1,6-N-acetylglucosamine (PNAG), a major polysaccharide of EPS that is involved in the surface attachment, biofilm establishment and antimicrobial resistance (AMR). Deoxyribonuclease I (DNase I) degrades eDNA present in the EPS and cell-surface-associated nucleic acids that function as surface adhesins and that inhibit the initial attachment of bacterial cells.	Dispersin B, Deoxyribonuclease I	84–85
Surfactants	Rhamnolipids that are produced by *Pseudomonas aeruginosa* have been reported to induce the dispersal of biofilm through the formation of central hollow cavities.	Poloxamer-188, Tween 20, sodium dodecyl sulfate, rhamnolipids	86–87
Fatty acids (FAs)	FAs can control biofilm formation by functioning as signal molecules. For example, *cis*-11-methyl-2-dodecenoic acid, a diffusible signal factor (DSF) produced by *Xanthomonas campestris*, is capable of causing biofilm dispersion by endoglucanase synthesis leading to EPS degradation.	Caprylic acid, palmitic acid, myristoleic acid	88–90
Metal chelators	Ethylenediaminetetraacetic acid (EDTA) chelates Mg^{2+} and Ca^{2+} from the outer cell wall of Gram-negative bacteria and destabilizes the negative charge of lipopolysaccharides (LPS).	EDTA	91–92
Nitric oxide (NO)	NO-generating agents, such as sodium nitroprusside (SNP), have been shown to trigger biofilm dispersal by shifting bacterial growth from biofilm mode to planktonic state. This shift is induced by a reduction in the intracellular levels of cyclic dimeric guanosine monophosphate (c-di-GMP).	Sodium nitroprusside, S-nitroso-L-glutathione, S-nitroso-N-acetyl penicillamine	93–94
Antimicrobial peptides (AMPs)	These are small molecules (10–100 amino acids) produced by all living beings that play an important role in innate immunity. AMPs permeabilize microbial membranes mainly because of their cationic nature, which affects the transmembrane potential, resulting in cell death. AMPs can also downregulate QS systems, Las and Rhl.	Nisin A, human cathelicidin LL-37, human β-defensin 3, hepcidin 20	95–97

a physical barrier to antibiotics and immune cells. Furthermore, the bacterial cells that reside within biofilm are in a dormant metabolic state and antibiotics are ineffective against them as these agents only target metabolically active cells.[7] Therefore, the topical delivery of antibiofilm agents alone and/or in combination with antibiotics can be of great therapeutic value in the treatment of chronic wound infections. The presence of antibiofilm agents in the delivery system will disperse the biofilm while simultaneously allowing the antibiotics to target the previously unexposed bacterial cells (Fig. 3). In this section, we highlight various delivery systems containing different bioactive agents for the treatment of chronic wound infections. The delivery systems are classified primarily into hydrogels, nanofibers, films, and nanoscale materials.

Hydrogels

Hydrogels are a popular choice as wound dressings because of their biocompatibility, flexibility, and high-water content (96%). Polymers such as polyvinyl alcohol, poloxamers, chitosan, gelatin, and carbomers are commonly used to prepare hydrogels.[52] These hydrogels do not dissolve in water because of the physical and chemical cross-linking that causes their constituents to form a polymeric network.[53] Hydrogels keep the wound moist, absorb wound exudate and have a soothing effect on painful chronic ulcers.[54] Thus, hydrogels are suitable for the delivery of antibiotics and antibiofilm agents for managing biofilms in chronic wounds. The release of the bioactive agents can be prolonged by modulating the polymeric composition of the hydrogel. This can achieve a sustained effect that could reduce the frequency of dressing change and could regulate the exposure of the bacteria to sub-inhibitory concentrations of antibiotics. Antibiofilm agents can be incorporated directly into the hydrogel or can be delivered via incorporation into other delivery systems that are embedded into the hydrogels.

Thapa *et al.*[55] formulated a hybrid hydrogel called 'GarKS gel', which was composed of Pluronic F127 (PF127) liposomes loaded with ethylenediaminetetraacetic acid (EDTA), glutathione (GSH), and the bacteriocin Garvicin KS (GarKS). Bacteriocins are antimicrobial peptides (AMPs) that are synthesized by bacteria. The gel was found to inhibit ~ 82% of *S. aureus* biofilm *in vitro*, as determined by crystal violet assay, in comparison to just ~ 18% inhibition by a blank gel. The *S. aureus* Xen-31 infected mouse wound model was subsequently used for *in vivo* evaluation of the hydrogel, with the antibacterial effects being assessed using an IVIS Lumina II imaging system to measure the luminescence of bacteria present in the wound site. A single treatment with the GarKS gel showed an ~ 12-fold reduction in bacterial lumines-

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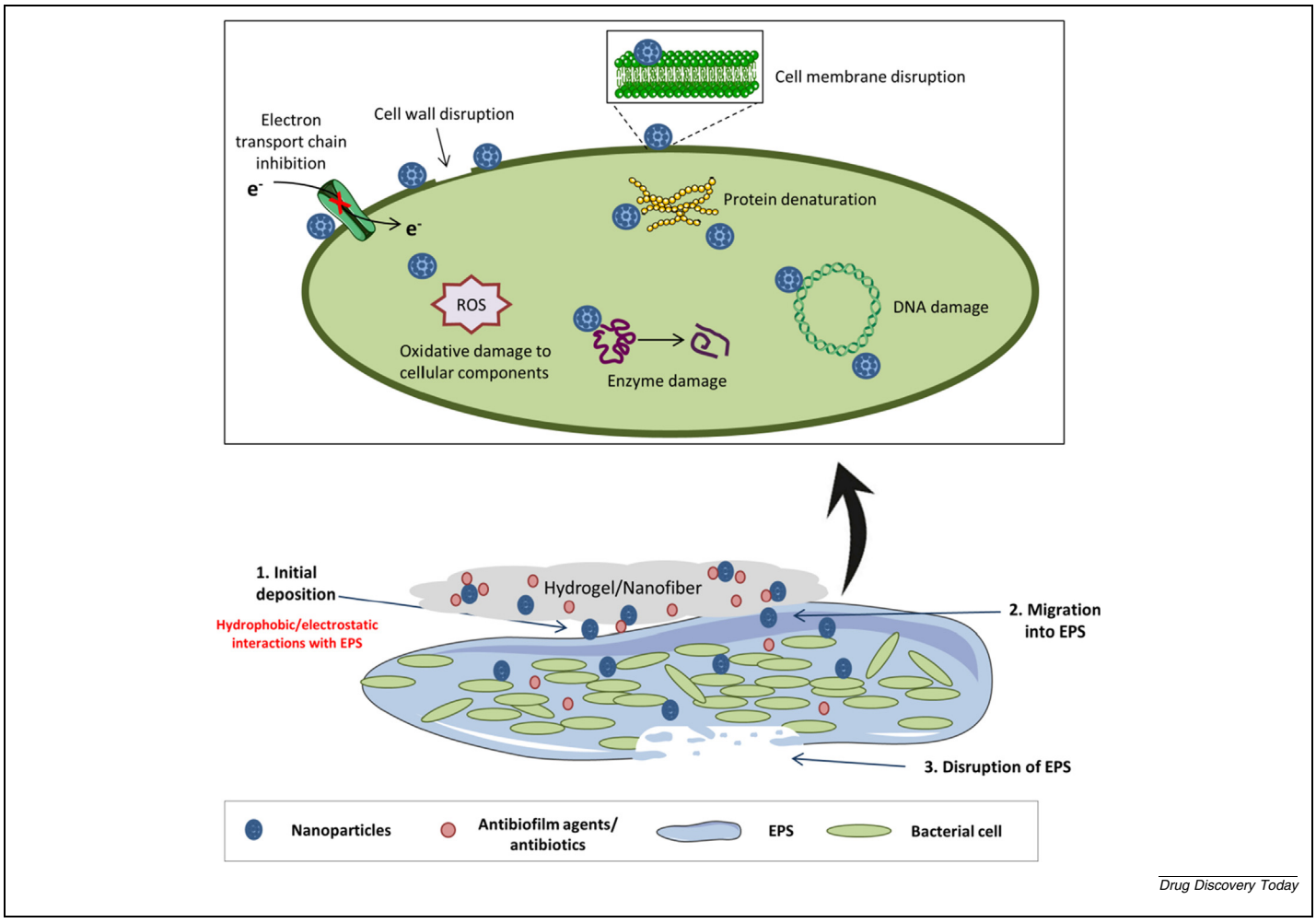

FIG. 3

Antibiofilm agents and antibiotics, alone or incorporated within nanoparticles (NPs), are released from topical carriers such as nanofibers, hydrogels or films. The initial interactions with the biofilm matrix can be electrostatic or hydrophobic. NPs have a large surface area because of their small size, which faclitates their penetration into the biofilm matrix. The transport of the active agents including nanoscale materials can be affected by the viscosity, flow and density of the extracellular polymeric substance (EPS). The antibiofilm agents disrupt the matrix structure and break the biofilm apart. Once within the matrix, the NPs interact with bacterial cells and act upon various targets as determined by their composition.

cence at day 1 post-treatment when treated and untreated groups were compared.[55]

Neff *et al.*[56] assessed the activity of two synthetically engineered cationic AMPs (ASP-1 and ASP-2) loaded in a chitosan matrix against biofilms of *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA), which were grown on polyethylene terepthalate (PET) mesh and porcine skin for 24 h and 72 h, respectively. The ASP-1 gel eradicated *P. aeruginosa* biofilm and both AMP gels eradicated MRSA biofilms from the PET mesh substrate within one day. Both gels decreased MRSA counts in the porcine skin by 4–7 $\log_{10}$ within the first day of treatment, and similar results were obtained over days 2 and 3 of treatment. By comparison, the commercially available silver gel Opticell Ag showed < 1 $\log_{10}$ reduction over a 3-day period. This can be attributed to the sustained release of AMPs from the chitosan matrix and the maintenance of AMP stability.[56]

Chhibber *et al.*[57] prepared a novel topical gel containing the antibiotic moxifloxacin, as well as the antibiofilm agents chitosan and EDTA, and evaluated its efficacy in MRSA-infected burn wounds in mice in comparison to a conventional gel containing moxifloxacin only. The novel gel caused a 3.5 $\log_{10}$ reduction in bacterial count at 24 h post-infection (compared with a 2.8 $\log_{10}$ reduction for the conventional gel) and demonstrated better wound-healing potential.[57]

Deoxyribonuclease-I (DNase-I) has been proven to be an efficacious antibiofilm agent as it hydrolyzes the extracellular DNA found in biofilms. Patel *et al.*[58] prepared silver-sulfadiazine-loaded solid lipid nanoparticles (SSD-SLNs) and incorporated them into a DNase-I-containing chitosan gel. When compared to pure SSD, this combined formulation had reduced fibroblast toxicity in human dermal fibroblast (HDF) cell lines. When used in combination with DNase-I, SSD-SLNs produced 96.8% inhibition of *P. aeruginosa* biofilm, whereas a combination of SSD and DNase-I produced 82.9% inhibition. Confocal microscopic analysis revealed a reduction in the thickness of the biofilm treated with the SSD-SLNs DNase formulation. The incorporation of DNase in the SSD-SLN gel resulted in complete wound healing after 21 days when tested on burn wounds in rats.[58]

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Silver nanoparticles (AgNPs) have been reported to have antibiofilm properties, but their success has been constrained by their toxicity and susceptibility to aggregation in the wound environment. To overcome these shortcomings, Haidari *et al.*[59] developed a biocompatible hydrogel that was loaded with ultrasmall AgNPs that provided favorable controlled-release characteristics. They evaluated this hydrogel using a *S. aureus* Xen-29 infected full-thickness mature biofilm mouse wound model. Xenogen IVIS bioluminescent live animal imaging was used to image the infected wounds. The AgNPs were found to be < 3 nm in size and the AgNP hydrogel showed sustained release of silver ions for up to 24 h. The treatment of infected wounds with the AgNP hydrogel resulted in quicker wound closure (46% vs. 20% for SSD) and accelerated wound re-epithelization (60%) as determined by the histological analysis. Furthermore, the AgNP hydrogel treatment significantly reduced the *S. aureus* bacterial load on day 10 ($\sim 5 \times 10^6$ photons/s for the AgNP hydrogel in comparison to $\sim 2 \times 10^7$ photons/s for SSD or blank hydrogel treatment).[59]

In another excellent study, Wang *et al.*[60] developed an innovative pH-switchable supramolecular hydrogel system using an amphipathic synthetic octapeptide (IKFQFHFD). The octapeptide formed a hydrogel at the neutral pH of 7.4 owing to intermolecular electrostatic interactions between the lysine (K) and aspartate (D) residues at opposite ends of the octapeptide, and π-π stacking conferred by the three phenylalanines (F). In a wound environment, with an acidic pH of 5.5, the network was destabilized by the protonation of aspartate. This led to an antimicrobial effect as a result of the release of IKFQFHFD, which has an AMP-like structure. The hydrogel was able to prevent the formation of MRSA biofilm, as confirmed by crystal violet assay, although it was unable to eradicate pre-formed MRSA biofilm. Complete eradication of pre-formed MRSA biofilm was observed when the hydrogel was loaded with cypate, a cyanine dye that possesses photothermal properties when under near-infrared irradiation. Irradiation of the hydrogel system loaded with cypate and proline (hydrogel-Cy-Pro) eradicated the MRSA biofilm and resulted in complete healing of MRSA-biofilm-infected full-thickness wounds in diabetic mice within 20 days, as demonstrated by the Gram-staining of the wound tissues and a reduction in the TNF-α expression. Complete re-epithelialization and enhanced collagen deposition was observed in the infected wounds treated with hydrogel-Cy-Pro, thus showing the potential of this treatment as a future therapy for MRSA-biofilm-infected chronic wounds.[60]

Waite *et al.*[61] evaluated the antibiofilm activity of an advanced device (NOx) for chronic wound treatment. This device comprised a primary mesh layer and secondary hydrogel layer that were capable of generating 3 μmol/cm^2 of nitric oxide when placed in contact with each other for a period of 48 h. The NOx dressing was able to prevent the formation of *P. aeruginosa* and *S. aureus* biofilms on nitrocellulose filters, with biofilm cell densities reduced significantly from 4×10^8 CFU/filter for the placebo dressing to 50 CFU/filter for the NOx dressing. The NOx dressing was also able to disrupt pre-formed biofilms and reduced the bacterial population to levels that were not detectable. When compared to untreated and placebo-treated polymicrobial biofilms, NOx also reduced both MRSA and *P. aeruginosa* populations by 5.3 $\log_{10}$ and 4.1 $\log_{10}$, respectively, at 24 h post-inoculation. In the same comparison, the pyocyanin and elastase activities of *P. aeruginosa* PA5 were also reduced 1.9- and 3.2-fold, respectively. The clinical significance of the reduction in these virulence factors could have significant therapeutic implications for wound healing because these factors promote tissue damage.[61]

Richter *et al.*[62] prepared chitosan-dextran hydrogels containing deferiprone (Def) (an iron chelator) and the heme analog gallium-protoporphyrin (GaPP), either alone or in combination with the antibiotic ciprofloxacin (Cip), and evaluated their activity against biofilms of *S. aureus*, *P. aeruginosa* and a clinical MRSA isolate grown on an artificial wound model. The artificial wounds were composed of hyaluronic acid (HA), collagen, bovine plasma, Bolton broth and horse blood. Reductions of up to 0.5 $\log_{10}$ and 0.2 $\log_{10}$ were observed with the Def gel and the GaPP gel, respectively, at a concentration of 500 μg/ml. When compared to a blank gel, a gel containing a combination of Def, GaPP and Cip showed significant antibiofilm activity, with a 0.7 $\log_{10}$ reduction in both *S. aureus* and MRSA biofilms, as well as a 1.9 $\log_{10}$ reduction in a *P. aeruginosa* biofilm. Thus, a combination therapy involving an antibiofilm agent with an antibiotic holds promise for future therapies against chronic wounds.[62]

Mupirocin is a topical antibiotic that is active against Gram-positive bacteria, including MRSA. Hurler *et al.*[63] prepared liposomal mupirocin, which has been reported to possess antibiofilm activity against *S. aureus*, and enhanced the penetration of the drug through the bacterial biofilm. The liposomal formulation was further incorporated into a chitosan hydrogel, which functioned as a vehicle and also to provide additional wound-healing effect. The liposomal mupirocin was not toxic to HaCaT cells at the highest investigated concentration of 100 μg/ml, and it was able to prevent the formation of biofilm by *S. aureus* as determined by resazurin and crystal violet assay. Nevertheless, liposomal mupirocin was only able to cause a 50% reduction in viability when applied 18 h post biofilm establishment at a concentration of 405 μM. The liposomal mupirocin hydrogel showed faster healing in comparison to a marketed formulation (Bactroban), albeit with similar efficacy. This study shows that chitosan hydrogel embedded with liposomal mupirocin is effective in preventing the formation of biofilms and in enhancing the wound healing process.[63]

Nanofibers

Nanofibers are nanometer-scale fibers that are prepared using various techniques, such as self-assembly, solution blowing and electrospinning.[64] They show promise in wound healing due to their enhanced cell attachment, which is related to their high surface-to-volume ratio and high porosity. Nanofiber scaffolds also aid in cell proliferation as they mimic the structure of the ECM and promote re-epithelialization and wound closure. Moreover, the porous nature of nanofibers allows them to adsorb the wound exudate and provide exchange of the nutrients.[65] They also have the benefit of improved drug loading. A wide range of biodegradable polymers, such as chitosan, polycaprolactone, poly(lactic-co-glycolic acid), polyvinyl alcohol, cellulose and non-biodegradable polymers such as poly(acrylates), have been used to synthesize nanofibers.

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Su *et al.*[66] fabricated Pluronic F127-polycaprolactone (PCL) core–shell nanofibers encapsulating engineered human cathelicidin peptide 17BIPHE2. As this peptide is water soluble, coaxial spinning was used to encapsulate it within the core. The core–shell structure allowed the peptide to retain its biological activity when present in the wound environment. The fibers provided an initial burst and subsequent sustained release of the peptide over a period of 28 days. When full-thickness excision wounds infected with MRSA in type 2 diabetic mice were treated on a daily basis with peptide-loaded fiber dressings for a period of 7 days, there was a 5.01 $\log_{10}$ reduction in bacterial count without debridement as compared to placebo dressings. With debridement prior to administration of the dressing, no colonies were detected in the wounds (10.24 $\log_{10}$ reduction). Similarly, mice infected with *P. aeruginosa* biofilms showed a 3.61 $\log_{10}$ reduction in bacterial count as compared to placebo dressing after daily treatment for a period of 3 days without debridement. With debridement prior to administration of the dressing, no colonies were detected in wounds (10.75 $\log_{10}$ reduction). Moreover, the level of inflammation was substantially reduced, as evidenced by the wound tissue histology. This study thus demonstrated the antibiofilm efficacy and wound-healing potential of nanofiber dressings loaded with an AMP.[66]

An electrospun zein–PCL triple layered (3L) fibrous dressing encapsulating tetracycline (Tet) was developed by Alhusein *et al.*[67] These authors evaluated the dressing against preformed biofilms of MRSA *in vitro* and *ex vivo* using a pig skin model. The triple layer was developed to reduce the burst release observed with single layer matrices. The outer layers act as a diffusion barrier to prolong the drug release. The zein–PCL blend aided in conferring stability to the fibres, as zein fibres on their own lost fibre structure upon coming in contact with the buffer medium. The zein–PCL 3L nanofibres had released 50% of their encapsulated Tet after 24 h and were able to release up to 60% of the Tet after 15 days. The 3L matrix was able to reduce the biomass of the MRSA biofilm *in vitro* by 90% after 24 h of incubation, as determined using a crystal violet assay. When treated with zein–PCL 3L nanofibres for 24 h, pig skin harboring an MRSA biofilm that had been grown for over 5 days demonstrated reduction from 100% (untreated sample) to 19 % of CFU/skin sample. These nanofibres were also found to be non-toxic to FEK4 fibroblast cells.

Films

Films are commonly used as dressings for low exudative and postoperative wounds. They are made semi-permeable so as to allow ventilation of the wound. Gaseous exchange across films is measured in terms of moisture vapor transmission rate (MVTR). This exchange avoids the accumulation of moisture under the dressing and prevents tissue maceration at wound sites. Most films are designed to have a high MVTR. Films have the advantages of being transparent and conforming to the contours of flexible body parts such as knees and elbows due to their elasticity. Nevertheless, they are only well suited for shallow wounds as they are too thin to be applied to the deep wounds.[68]

Choi *et al.*[69] evaluated the antibiofilm and wound-healing efficacy of a nitric oxide (NO)-releasing chitosan film (CS–NO film) in MRSA biofilm-infected full-thickness wounds in diabetic mice. The CS–NO film demonstrated 3-fold higher antibiofilm activity *in vitro* in comparison to control and chitosan films. Furthermore, the CS–NO film produced faster dispersal of biofilm (at day 12 post-injury) *in vivo* as compared to control (biofilm growth visible on day 15 post-injury) and CS film-treated groups (at day 15 post-injury). The percentage of initial wound area remaining at day 15 post-injury was 5.8% and 40.6% for CS–NO film and CS film, respectively. This indicates the importance of biofilm dispersal in escalating the wound-healing process.[69]

In another study, Yang *et al.*[70] formulated a benzalkonium chloride (BZL)-loaded novel nanoscale liquid film-forming system (LFFS) using polyvinyl alcohol (PVA) and chitosan (CS). A LFFS formulated using 5% PVA and 1% CS loaded with 5 mg/ml of BZL was able to decrease the minimal inhibitory concentration (MIC) from 5 μg/ml to 1.43 μg/ml. The viable bacteria were reduced by 81%, as compared to 60% by BZL solution, on day 7 of treatment of full-thickness wounds in mice infected with MRSA. Complete wound healing was observed with LFFS on day 14. Moreover, LFFS–BZL treatment markedly damaged the MRSA biofilm, whereas BZL treatment mildly affected the biofilm structure, as determined by crystal violet assay, electron microscopy and confocal microscopy analysis. After treatment with LFFS, 45.33% of live bacteria were found within biofilm, in comparison to 85.82% after BZL treatment, when observed under a confocal microscope using the LIVE/DEAD Baclight kit.[70]

Nanoscale materials

Owing to their size (1–100 nm), nanoscale materials (NMs) can penetrate and deliver antibiofilm agents into tissues and biofilms.[71] Moreover, NMs that bear a positive charge can interact more efficiently with negatively charged bacterial cells. They can be synthesized from wide array of materials such as polymers, metals and metal oxides. NMs can include inorganic nanocomposites, dendrimers, silica nanoparticles, and polymeric micelles. Silver (Ag)-based NMs possess inherent antibacterial properties. NMs can induce DNA damage and membrane disruption through the production of ROS. Metabolic pathways have been reported to be disrupted by interactions between NMs and the thiol groups of proteins.[72]

Metal oxides

Ghaseminezhad *et al.*[73] fabricated Ag–Fe_3O_4 nanocomposites (NCs) that can penetrate and eradicate biofilms upon application of a magnetic field. Ag–Fe_3O_4 NCs were synthesized using starch as a stabilizer and a linker between Ag and Fe_3O_4 NPs that enhances their antibacterial properties and reduces their cytotoxicity. The NCs had a particle size of < 20 nm. As analyzed under a confocal microscope by Live/Dead staining, Ag–Fe_3O_4 NCs eradicated > 90% of biofilm bacteria *in vitro* when a magnetic field was applied. Upon application of a magnetic field, NCs were also able to kill *S. aureus* bacteria and eradicate a *S. aureus* biofilm grown within collagen matrix containing calf serum, which mimicked the chronic wound bed. The magnetic field allows the penetration of NCs within the collagen and biofilm matrix, thus enhancing their efficacy. Ag–Fe_3O_4 NCs were found to be less cytotoxic to human fibroblast cells than Ag-NPs, and thus demonstrate better safety.[73]

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Dendrimers

Dendrimers are synthetic molecules that have a highly branched and star-shaped structure. They have a narrow size distribution and antimicrobial peptides can be used to prepare dendrimers. Dendrimerization of AMPs enhances their activity by protecting the AMPs against proteases and providing a high local concentration of bioactive units. Grassi *et al.*[74] evaluated a dendrimeric derivative of the semi-synthetic antimicrobial peptide lin-SB056-1 [(lin-SB056-1)$_2$-K] against biofilms of *P. aeruginosa* PAO1 and of two clinical burn wound isolates (2091 and 2549). This evaluation was performed using an *in vitro* wound model composed of a dermis-like scaffold embedded with blood components at physiological levels. Dendrimerization of the AMP maintained its activity at a high physiological salt concentration, which would otherwise be a limiting factor for the AMP activity. The scaffold was composed of two layers: the upper layer consisting of high-molecular-weight (HWA) HA and the lower layer made up of HMW HA, low-molecular-weight (LMW) HA and collagen. The dendrimeric derivative exhibited remarkable antibiofilm activity against both *P. aeruginosa* PAO1 and 2091 strains at a concentration of 19.25 μM, decreasing the number of viable bacteria by up to 1 log-unit after 16 h of incubation.[74]

Graphene oxide

Graphene-based nanomaterials have limited water solubility and are usually applied as contact-killing coatings on surfaces. Di Giulio *et al.*[75] evaluated the antibiofilm effect of graphene oxide (GO) dispersion on biofilms formed by clinical isolates of *S. aureus* and *P. aeruginosa* obtained from the wounds of patients suffering from chronic venous leg ulcers. A significant reduction in biomass was observed in viable counts for *S. aureus*, which decreased from 8.63 to 6.89 $\log_{10}$ CFU/ml. The biofilms became detached from the walls of the plate, illustrating the penetrative capacity of GO.[75]

Nanoscale emulsions

'Nanoscale emulsion' (NE) is an umbrella term for emulsions that have globule size in the nanometer range, which include both micro- and nano-emulsions. Microemulsions have been reported to possess antibiofilm activity even without the incorporation of other antibiofilm agents. Razdan *et al.*[76] developed a levofloxacin (LFX)-incorporated clove oil nanoscale emulsion (LFX-NE) and demonstrated its *in vitro* antibiofilm activity against *P. aeruginosa* biofilm. Clove oil was selected for its antibiofilm activity and as the oil phase of the NE. The particle size of the LFX-NE was found to be 18.84 ± 0.5 nm. It showed high antibacterial activity, and field emission scanning electron microscopy and confocal microscopy revealed the destruction of a pre-formed mature biofilm of *P. aeruginosa* by LFX-NE at its MIC and minimum bactericidal concentration (MBC).[76]

Clinical studies

Clinical studies of two of the patented antimicrobial dressings developed by ConvaTec Ltd. UK, which are based on Hydrofiber™ technology, have shown very high efficacy in biofilm-harboring chronic wounds.[77] The efficacy of the AQUACEL Ag + dressing was evaluated for biofilm eradication and wound healing in patients across the UK and Ireland. AQUACEL Ag + is used to manage the exudate and employs ionic silver and antibiofilm agents to control the infection and biofilm. The

TABLE 2

Clinical trials of antibiofilm agents for the management of chronic wounds.

ClinicalTrials.gov identifier	Status	Year	Study details	Reference
NCT02228122	Completed	2014	The main aim of this pilot study was to assess the effect of the Aquacel Ag + Extra dressing on biofilms in chronic wounds for a period of 4 weeks in comparison to a non-treatment group.	98
NCT04079998	Completed	2020	A parallel, randomized, controlled clinical study aimed at evaluating the antibiofilm efficacy of the FDA-approved wound dressing Procellera® in comparison to the standard treatment for acute trauma and burn wounds. The dressing contains a silver-zinc electro-couple that generates a weak electrical field upon being activated by a moist wound environment. The efficacy is assessed by determining the bacterial load (colony forming units (CFU)) and by visualization of the biofilm using scanning electron microscopy (SEM) of tissue biopsies taken after a week of treatment.	99
NCT03686904	Active, not recruiting	2018	A parallel, randomized, controlled clinical study designed to examine the effect of benzalkonium irrigation solution and benzalkonium gel (BlastX™) on biofilms in chronic wounds, in comparison to saline irrigation and standard hydrocolloid gel following debridement. The study will be carried out for 12 weeks with at least four follow-up visits, and wounds will be assessed for size and signs of infection (by measurement of CFUs).	100
NCT03248154	Withdrawn (did not receive funding)	2022	A parallel, open label, randomized, controlled clinical study that will determine the implications of biofilm infections in burn wounds. A total of 300 subjects will be enrolled and divided into three age groups: 2–18 years old, 18–49 years old and $\geq$ 50 years. There will be three arms, and subjects in arm 2 will receive standard dressing or Procellera®. At days 0, 3, 7, 14, 21 and 28, burn wound size, blood cytokine levels and laser doppler imaging determination will be carried out on tissue biopsies.	101
NCT03461783	Completed	2019	This parallel, open label, randomized, controlled clinical study aimed to assess the effect of the Zorflex® activated carbon cloth dressing on biofilms in wounds of the lower extremities and feet. The dressing conforms to the contours of the wound and provides an antimicrobial effect for a minimum of 7 days per dressing. (Results not yet available.)	102

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Hydrofiber™ dressing consists of two layers of sodium carboxymethylcellulose strengthened by regenerated cellulose fiber. The Hydrofiber transforms into a gel upon coming into contact with the wound fluid. The dressing incorporates a metal-chelating component (EDTA) and a surfactant component (benzethonium chloride) as antibiofilm agents, allowing ionic silver to access bacteria that are embedded within the biofilm matrix.[78]

The study involved a total of 29 patients (19 males and 9 females) selected from eight healthcare facilities across the UK and Ireland, who were suffering from chronic wounds such as leg ulcers and diabetic foot ulcers. The clinicians were advised to replace the primary dressing with AQUACEL Ag + dressings for up to 4 weeks. At baseline, seven wounds (24%) were found to be deteriorating and 21 wounds (72%) were stagnant. After a median period of 4.5 weeks, the wound status changed from deteriorating/stagnant to improved, and the tissue type of the wound bed improved to mainly granulation tissue (53%) from previously biofilm/sloughy tissue (76%). Out of 29 wounds, 26 reduced in size or healed completely. Hence, the AQUACEL Ag + dressing was found to be highly effective because of the absorption of wound exudate and the subsequent immobilization of bacteria by the gelling of sodium carboxymethylcellulose, coupled with a reduction in wound bioburden caused by ionic silver. Moreover, disruption of biofilm, due to the detergent-like action of benzethonium chloride and metal chelation by EDTA, significantly enhanced the effectiveness of the dressing.

In another study by Metcalf and Bowler,[79] the AQUACEL Ag + Extra™ dressing was evaluated in 65 patients suffering from chronic wounds that were previously managed unsuccessfully using conventional silver- or iodine-containing dressings. This dressing is 9 times stronger and has 50% more absorbing capacity than the AQUACEL Ag + dressing. The wounds ranged in duration from 1 week to 20 years, with median wound duration of 12 months. Of the 65 chronic wounds, 47 (72%) were stagnant and 15 (23%) were deteriorating at baseline. Biofilm was associated with 57% (n = 37) of the chronic wounds. The clinicians were advised to replace the primary dressing with the AQUACEL Ag + Extra™ dressing. After an average of 4.2 weeks, 11 wounds (17%) healed completely, 40 wounds (62%) improved, while 9 wounds (14%) remained as they were and 5 wounds (8%) deteriorated.[79] These studies demonstrate that chronic wounds that harbor biofilms can be managed successfully with the proper use of appropriate novel dressings that are designed to deliver antibiofilm and antimicrobial agents in combination. Other clinical studies related to the treatment of biofilms related chronic wounds that are either completed or still underway or recruiting are summarized in Table 2.

Concluding remarks

It has been a little over a decade since biofilms were found to be present in the clinical wound specimens. Since then, extensive research has been undertaken to reveal the mechanism of biofilm formation and to find treatment strategies for biofilm-infected wounds. Biofilms are particularly important in the context of public health, as conventional antibiotics and treatment strategies are unable to treat such infections. This contributes to the burden on patients, both financially and emotionally. The treatment of chronic wounds constitutes a major part of healthcare budgets, and biofilms are a major obstacle in the progression of non-healing wounds. In addition to debridement, the current approach for treating chronic wounds involves the systemic or topical administration of antibiotics for long periods. Therefore, it is imperative that effective ways are found to fight biofilms and to promote wound healing. Various studies have explored versatile antibiofilm agents, ranging from the simple sugar xylitol to complex natural compounds. Some of these, such as such as xylitol, EDTA and Dispersin B, have been found to be highly effective.

Antibiofilm agents have been reported to be very efficacious when used in conjunction with antibiotics. Even the best of molecule is ineffective, without a proper delivery system. Various pharmaceutical drug delivery strategies have been developed to treat biofilm-infected non-healing wounds. These delivery systems have therapeutic potential to provide a platform for altering the wound care paradigm in the near future. A range of delivery systems, including hydrogels, nanofibers and films, have shown a lot of promise in co-delivering the antibiotic along with an antibiofilm agent. Owing to their prolonged contact and sustained delivery of the drugs, these systems also aid in promoting wound healing by alleviating the impaired host immune response as the biofilm infection starts to get cleared. Some recent clinical trials have shown better management of chronic wounds with these novel dressings. Furthermore, nanoparticle-based systems have shown an enhanced ability to penetrate the biofilms and to eradicate them effectively. Bioactive-loaded nanoparticles that are embedded in hydrogels or nanofibers hold promise as future therapies. Further research and clinical trials should be carried out in this area of unmet clinical need, which continues to pose a significant challenge to healthcare systems. Continually emerging novel wound dressing technologies, combined with nanomaterial-based drug delivery systems, have the potential to improve treatment outcomes in patients who have biofilm-infected chronic wounds.

Conflict of interest

The authors declare that they have no conflict of interest.

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