A REVIEW: MOLECULAR BASIS OF NITRIC OXIDE-MEDIATED BIOFILM DISPERSION AS A FUTURISTIC STRATEGY TO ERADICATE BIOFILM-DERIVED INFECTIONS

Abstract

In order to infect people, pathogenic bacteria can use a wide variety of tactics. The ability to switch between single-celled, motile existence and group-based, biofilm existence is a potent tactic. Biofilms are notoriously resistant to antibiotics and are known to attach to both biotic and abiotic surfaces. Biofilm dispersal results in germs that are both more resistant to antibiotics and abler to infect a new host. Biofilm production has been shown to be modulated by the diatomic signaling molecule nitric oxide in a wide variety of species. Although studies on the effects of nitric oxide on bacterial biofilm are still in their infancy, we examine the literature reporting such an effect here. Evolutionarily, dispersal via nitric oxide has been conserved, and its signaling cascade involves cyclic di-GMP. NO is a crucial dispersal signal in numerous bacterial species, initiating a molecular cascade that includes c-di-GMP level modulation. This interaction between NO and c-di-GMP is essential for biofilm dispersal, shedding light on the regulation of bacterial behavior and biofilm-associated infections. Understanding this evolutionarily conserved process may lead to novel biofilm control and chronic infection-fighting strategies. The formation of biofilms, in which bacteria form complex communities with enhanced resistance to antimicrobial treatments, highlights the importance of understanding these quorum-sensing processes. Biofilm development, control, and communication among bacterial populations are all significantly aided by quorum sensing. Because biofilms are linked to both persistent infections and treatment resistance, focusing on quorum-sensing

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pathways may be an effective way to rein in and eliminate these infections. Opportunities to improve patient outcomes, antibiotic efficacy, and the disruption of biofilm formation can be seized by capitalizing on our growing understanding of the intercellular signaling systems that underlie these processes.

Keywords: Nitric oxide, Signaling molecule, Biofilm, Quorum-sensing, Biofilm dispersion

I. INTRODUCTION

Nitric oxide (NO) is characterized by its diminutive molecular size and uncomplicated structure, rendering it a highly volatile gaseous free radical. The substance exhibits solubility in both aqueous and lipid environments, enabling efficient diffusion through biofilms[1]. The substance exhibits a brief half-life of only seconds within a living organism. NO is shown to be prevalent in numerous tissues, particularly within the neurological tissues of animals. NO is a byproduct resulting from the oxidation process of L-arginine through the activity of nitric oxide synthases (NOS). The aforementioned molecule is classified as a new biological messenger and holds significant importance in the control of cardiovascular, nervous, and immune systems[2]. In 1992, Science magazine designated it as a "star molecule."

The pathways of the nitrogen cycle are accountable for the circulation of both inorganic and organic molecules containing nitrogen in the natural environment. Within the realm of metabolic processes in microbes, pathways related to amino acids, N-oxides, and specifically nitric oxide (NO) assume significant roles[3]. These pathways are crucial in both the natural conditions where microorganisms thrive and in the interactions between hosts and pathogens. In addition to their involvement in the nitrogen cycle, amino acids and NO serve as signaling chemicals that facilitate collective behavior in microbes and enable intercellular communication in multicellular creatures, including humans[4]. This study focuses on elucidating the impact of specific amino acids, namely glutamate, glutamine, and arginine, as well as NO, on the signaling pathways associated with the metabolism of 3',5'-cyclic diguanylate acid (c-di-GMP). c-di-GMP serves as a crucial regulator of motility, attachment, and group behavior in bacterial organisms[4]. The investigation of the metabolic pathways associated with these nitrogen-containing chemicals presents a compelling subject for the identification of targets for biofilm regulation in both natural and medicinal environments.

II. NO AS A SIGNALING MOLECULE

NO is produced by the enzymatic conversion of the amino acid L-arginine by a group of enzymes known as NOS. The synthesis of this compound occurs within the neurons of the central nervous system and it operates as a neuromodulator, exerting many physiological effects such as memory formation, synchronization of neuronal activity and blood flow, and pain modulation[5].

Within the peripheral nervous system, NO is released through a complex network of neurons. These nerves play a crucial role in facilitating various forms of neurologic vasodilatation and regulating specific activities related to the gastrointestinal, respiratory, and genitourinary systems[6]. Furthermore, it is produced in substantial amounts during the host's defense mechanisms and immunological reactions, hence playing a crucial role in exerting cytotoxic effects against various entities such as tumor cells, bacteria, viruses, and other types of invading pathogens[4]. NO is classified as a free radical, which refers to an uncharged molecule possessing an unpaired electron. This molecular entity has the capacity to engage in several processes, exhibiting characteristics of both a weak oxidant and a potent chemical[7]. There have been three distinct redox forms of NO that has been identified as physiologically active, specifically NO, nitrosonium (NO^+), and nitroxyl anion (NO^-). Nitric oxide (NO) has the ability to undergo a reaction with oxygen free radicals, resulting in the formation of peroxynitrite ($ONOO^-$), a potent oxidant[8]. This compound plays a role in protein oxidation

events that occur within physiological settings. The NO or NO^+ ion has the capability to generate S-nitrosothiols (RSNO) by interaction with protein sulfhydryl groups, which have strong inhibitory effects on platelet aggregation[8].

III. NO-MEDIATED IMMUNOLOGICAL RESPONSE

The synthesis and signaling of NO are of utmost importance in the context of immunological responses. NO is produced by various isoforms of NOS, which consist of NOS-1 and NOS-3, all of which are constitutively expressed, as well as NOS-2, which is inducible. Physiological events induce the activation of NOS-1 and NOS-3, resulting in the generation of NO at a quick and transient rate, characterized by low concentrations[9]. On the other hand, NOS-2 is activated in reaction to immunological stimuli, such as bacterial lipopolysaccharide (LPS) or cytokines. This activation leads to the production of consistently elevated amounts of NO, which occurs after a delay that corresponds to the synthesis of mRNA and proteins[10]. The effects of NO are contingent upon its concentration. At lower concentrations, NO generated by NOS isoforms NOS-1 and NOS-3 directly interacts with the iron atom in the heme group of guanylyl cyclase[11]. This interaction subsequently triggers the activation of the enzyme and facilitates the synthesis of cGMP. Consequently, this phenomenon elicits a swift and temporary reaction at the cellular level[12]. Nevertheless, when present in high concentrations, NO produced by NOS-2 undergoes instability and rapidly oxidizes under conditions of sufficient oxygen, resulting in the formation of reactive nitrogen oxide species (RNOS) characterized by the general formula NOx. Reactive nitrogen oxide species (RNOS) have the capability to induce nitrosation of the thiol moiety present in glutathione, resulting in the formation of S-nitroso glutathione (GS-NO)[13]. Additionally, RNOS can also react with thiol groups found in proteins, leading to the generation of protein-S-nitrosothiols (protein-S-NO).

The process of nitrosation has the potential to impede the functionality of diverse proteins, encompassing mitochondrial enzymes and transcription factors, so resulting in enduring physiological consequences[3]. In situations characterized by elevated levels of NO and concurrent heightened oxidative stress, the interaction between superoxide (O_2^-) and NO can give rise to the formation of the considerably hazardous peroxynitrite anion ($OONO^-$)[10]. This chemical exhibits deleterious effects on cellular constituents and has the potential to induce oxidative harm and inflammation[13]. In immunological processes, the significance of NO production and signaling cannot be overstated. Its impact spans a wide spectrum, encompassing both swift and temporary cellular responses at low concentrations, as well as intricate and enduring cellular effects at high concentrations, frequently entailing the generation of reactive nitrogen oxide species. The maintenance of optimal levels of NO is of utmost importance in ensuring the appropriate functioning and regulation of the immune system[5].

IV. NO-MEDIATED VARIOUS PATHOLOGICAL CONSEQUENCES

1. **Vasodilatation:** Vasodilation in blood vessels is mediated by NO, which is influenced by multiple variables. The stimulation of smooth muscle relaxation through the phosphorylation of several proteins is a result of the production of eNOS[3]. The vasodilator action encompasses the management of blood flow, the renal control of extracellular fluid homeostasis, and the maintenance of blood pressure. Following the

process of production, the compound, owing to its notable reactivity, permeates the smooth muscle cells and engages in interactions with soluble guanylate cyclase[4]. The activation of soluble guanylate cyclase is initiated by NO, leading to the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP)[10]. The activation of cyclic nucleotide-dependent protein kinase G (PKG or cGKI) occurs through the soluble form of cyclic guanosine monophosphate (cGMP), leading to the subsequent relaxation of smooth muscles. Furthermore, the role of NO in vasodilation is crucial for both the growth and upkeep of bodily functions[11]. Vasodilation of the blood arteries facilitates augmented blood flow into the corpus cavernosum, hence precipitating the occurrence of an erection. Sildenafil, also known as Viagra, operates by a comparable process whereby it inhibits the enzyme phosphodiesterase-5 (PDE5), resulting in a reduction of cyclic guanosine monophosphate (cGMP) levels through its conversion back to guanosine monophosphate (GMP)[10].

2. **Inflammatory Action:** In humans, inflammatory reactions are characterized by a significant increase in the production of nitric oxide (NO), with the inducible nitric oxide synthase (iNOS) playing a primary role in this biological process. At lower concentrations, NO functions as a pro-inflammatory agent by promoting vasodilation and recruiting neutrophils[13]. Conversely, higher doses of NO have the opposite effect, downregulating adhesion molecules, suppressing activation, and inducing death in inflammatory cells. During the process of inflammatory reactions, pro-inflammatory cytokines induce an upregulation ofiNOS expression in many cell types, such as neutrophil granulocytes and monocytes in macrophages[3]. Nevertheless, the expression of endotoxin is significantly enhanced during bacterial infections. Consequently, there is a substantial overproduction of NO, surpassing the physiological levels by a magnitude of up to 1000-fold[6]. Excessive synthesis of inflammatory mediators can lead to tissue damage, as observed in the context of inflammatory autoimmune disorders. Therefore, NO can exhibit either pro-inflammatory or anti-inflammatory actions, contingent upon its concentration[12]. Numerous studies have elucidated the correlation between cerebral inflammation and the etiology of various neurodegenerative conditions, including Alzheimer's disease, dementia, Parkinson's disease, amyotrophic lateral sclerosis, AIDS, and multiple sclerosis[8].

3. **Lungs:** The lung consists of various cellular components, including macrophages, epithelial cells, endothelium cells, vascular smooth muscle cells, bronchial smooth muscle cells, and neurons. Every cellular type generates NO by means of one or more isoforms of NOS[14]. The lungs are supplied with blood arteries that provide irrigation to all of their components. Within these blood vessels, there are crucial components known as endothelial cells, which express the enzyme NOS-3[15]. NO plays a crucial role in various physiological processes within the lungs. It is implicated in the synthesis of mucin by the bronchial epithelial cells, the involvement of macrophages in phagocytosis, the relaxation of bronchial smooth muscles at the inhibitory non-adrenergic/non-cholinergic nerve terminals, and the dilatation of blood vessels at the endothelium. These functions are regulated and synchronized to facilitate sufficient circulation of blood, as well as in cases of inflammation generated by allergens or immunogens[16].

4. **Hypertension:** The etiology of hypertension involves a crucial mechanism wherein the bioavailability of NO is impaired. According to a recently published article, it has been

observed that rats with hypertension produced by angiotensin II (Ang II) exhibit phosphorylation of endothelial nitric oxide synthase (eNOS) at the threonine 495 residue[14]. Multiple studies have documented elevated BH4 oxidation in rats with salt-induced hypertension, which can be attributed to the activation of the p47phox subunit of NADPH oxidase. A recent study demonstrated the efficacy of oral administration of BH4 in reducing hypertension in spontaneously hypertensive rats. This effect was attributed to the suppression of O_2^- buildup and subsequent lowering of $ONOO^-$ levels[16].

5. **Diabetes:** Diabetes is characterized by a diminished synthesis of NO generation. According to the findings of many studies, it has been observed that the activity of eNOS was diminished in the obese mice model due to heightened phosphorylation at threonine 495 mediated by protein kinase C (PKC)[17]. Reseachers have demonstrated a reduction in NO generation and diminished levels of tetrahydrobiopterin (BH4) in rats with comorbid diabetes and hypertension[18]. The study conducted by Heitzer et al. revealed that the administration of BH4 resulted in enhanced vasodilation in individuals with type II diabetes, specifically targeting the endothelium-dependent pathway. However, this improvement was not observed in the control group. The observed advantageous outcome was entirely impeded by N(G)-monomethyl-L-arginine, a widely recognized inhibitor of NOS, indicating that it relied on the augmentation of NO synthesis[14].

V. TWO FACES OF NO: INTRACELLULAR SIGNALING MOLECULE AND BACTERICIDAL AGENT

Initially classified as a toxic substance and environmental contaminant, NO, a gaseous molecule consisting of two atoms, has gained recognition as a widely present signaling molecule in several eukaryotic and bacterial organisms[19]. It possesses the ability to permeate cell membranes and acts as a free radical. When NO is present in higher quantities, typically exceeding approximately 1 μM, it has cytotoxic effects[13]. Nevertheless, when present in lower concentrations, generally below 1 μM, NO assumes the role of a mediator in subsequent signal transduction pathways, hence governing several physiological processes. NO functions as an intracellular cytotoxic agent by undergoing conversion to reactive nitrogen species when exposed to oxygen or superoxide radicals. This conversion leads to the induction of damage to several biological macromolecules, including lipids, DNA, and proteins[3]. Due to the capacity of nitrogen radical generation facilitated by NO, humans employ it as a crucial constituent of the immune system. Specifically, dendritic cells, neutrophils, and macrophages utilize iNOS to manufacture NO, thereby safeguarding against detrimental entities such as bacteria, viruses, and malignant cells. The practical uses of NO's cytotoxic qualities have also been utilized. One such example is the utilization of nitrite (NO2–), which dismutates to NO under low pH conditions, in meat packaging to inhibit bacterial deterioration[10].

Paradoxically, it has been observed that bacteria may exhibit a defensive response in certain instances when exposed to elevated levels of NO. For instance, the bacterium's defense against antibiotic-induced oxidative stress may involve two mechanisms: chemical alteration of drugs or stimulation of aerobic respiration[14]. This particular approach has been demonstrated in several bacterial species, predominantly gram-positive pathogens, which possess the capability to internally generate NO through bacterial nitric oxide

synthases (bNOS). Moreover, it has been demonstrated that the elevated levels of NO generated during inflammatory reactions in mammals can promote the adherence of biofilms to human tissue and intravenous medical devices. This is noteworthy considering that biofilm development serves as a defensive mechanism in bacteria[17].

VI. BIOFILMS: MAJOR CONTRIBUTOR TO MYRIAD PERSISTENT INFECTIONS AND UNSUCCESSFUL ANTIBIOTIC TREATMENT

Biofilms are complex assemblages of bacteria that exist as multicellular communities. These communities are surrounded by a matrix of extracellular polymeric substances (EPS) that they create themselves[20]. Biofilms have been found to have a substantial impact on chronic infections and contribute to the ineffectiveness of antibiotic treatments. The conventional assessment of antibiotic tactics often focuses on uniform planktonic bacteria[14]. However, in natural environments, bacteria mostly live in diverse biofilm communities, which display much-heightened resistance to immune responses, biocides, and antibiotics. The significantly increased tolerance observed in biofilm infections, which can be up to 10,000 times higher than that of planktonic counterparts, presents a formidable challenge in terms of eradication[21]. As a result, the creation of biofilms frequently gives rise to enduring and long-lasting infections, hence exerting a substantial influence on rates of morbidity and death. Remarkably, it has been estimated that a significant proportion of clinical illnesses, specifically 80%, can be attributed to bacterial biofilms[22].

The comprehensive comprehension of the underlying mechanisms governing biofilm tolerance is of utmost importance, while it still remains partially unresolved. The observed phenomena appear to be the result of a confluence of physical mechanisms and a mix of both particular and non-specific genetic factors[23]. The extracellular polymeric substance (EPS) matrix of biofilms serves as a defensive barrier, which hinders the entry of antibiotics and facilitates the accumulation of extracellular defense chemicals, including β-lactamase enzymes. In addition, the matrix provides protection for biofilm bacteria by preventing their engulfment and subsequent elimination by macrophages[14]. Bacteria within the biofilm demonstrate elevated levels of tolerance, which can be attributed, in part, to the development of biofilm-specific characteristics such as periplasmic antibiotic-binding polysaccharides, activation of enzymes to counteract endogenous oxidative stress and genetic alterations[24]. The heightened occurrence of mutations within biofilms has the potential to give rise to novel resistance characteristics, such as the continuous activation of efflux pumps, or the transfer of resistance genes from commensal communities to invading pathogens through horizontal gene transfer[11]. Moreover, the prompt and efficient acquisition of antibiotic-resistance genes in the outer regions of biofilms functions as a means of safeguarding the entire community of microorganisms within the biofilm. The biofilm formations exhibit an increased tolerance due to the presence of a large number of persister cells that undergo a transition to a temporary antibiotic-tolerant phenotype in response to nutritional gradients. The aforementioned persister cells possess the ability to endure antibiotic treatments and afterward recommence their proliferation upon cessation of therapy[14].

Moreover, the efficacy of antibiotic therapies in managing biofilms is limited, and exposure to antibiotic concentrations below inhibitory levels might paradoxically stimulate the growth of biofilms. This phenomenon carries substantial therapeutic consequences for several infectious disorders[9]. As a result, there exists a pressing need for the advancement

of innovative therapeutic approaches and methods that may efficiently manage biofilms and surmount their intrinsic resistance to existing treatments. The identification of effective strategies to address biofilm-associated infections is of utmost importance in enhancing patient outcomes and mitigating the impact of persistent infections within healthcare environments[3].

VII. LEVERAGING AN INTERNAL DISPERSAL MECHANISM TO AVERT BIOFILM-RELATED AILMENTS

An encouraging strategy for the development of innovative methods to control biofilms involves investigating and focusing on intrinsic mechanisms that govern the life cycle of biofilms. The process of biofilm development is a complex and multi-stage phenomenon that requires the coordinated differentiation of individual cells[25]. After the initial process of attachment, which is facilitated by bacterial motility and the presence of cell surface appendages like pili and fimbriae that interact with both abiotic and biotic surface materials, bacteria proceed to generate a substantial amount of EPS[14]. These EPS consist of various components such as polysaccharides, proteins, extracellular nucleic acids, lipids, and ions like Ca^{2+}. The production of EPS irreversibly binds the bacterial cells to the surface[26]. During the process of maturity, biofilms develop intricate three-dimensional structures consisting of specialised bacteria, resulting in a highly diverse and heterogeneous environment. This environment is characterised by varying nutrition and oxygen levels, creating steep gradients within the bacterial communities[27]. The ultimate phase of biofilm formation entails the synchronized liberation of specialized, mobile, chemotactic cells referred to as dispersal cells. The aforementioned specialized cells possess the ability to establish colonies on previously unoccupied surfaces, initiating the life cycle of the biofilm anew[28]. In several bacterial species, the process of biofilm dispersal is associated with the planned cell death of a specific group of cells within fully developed microcolonies. The surviving cells then exhibit the capability to exit the biofilm at designated escape spots, resulting in the formation of vacant structures within the biofilm matrix[29].

The dispersal of cells is commonly believed to have a positive impact on the biofilm. This is because it allows for the release of cells with different characteristics, which can then colonise other surfaces. Additionally, dispersal helps prevent overpopulation in a mature biofilm that is both densely populated and genetically varied[14].

Multiple molecular triggers have been identified that can initiate the transition from a sessile, surface-associated, or suspended biofilm phenotype to a free-swimming dispersal phenotype. These triggers include environmental and physiological cues, such as nutrient and oxygen availability, low concentrations of nitric oxide, iron levels, and D-amino acids[30]. Additionally, cell-cell communication signals, such as quorum sensing acyl-homoserine lactone signals, autoinducing peptides, and diffusible fatty acids, play a role in this transition. Furthermore, intracellular messengers, such as cyclic di-GMP and cAMP, are key elements in the regulatory network that controls the switch between biofilm and planktonic bacteria[31]. Cyclic di-GMP is particularly important in this network, while cAMP, previously known for its role in the stringent response, has recently been found to be involved in biofilm formation and dispersal. When bacteria detect a signal indicating the need for dispersal, they have the ability to initiate various cellular mechanisms that result in dispersal[32]. These mechanisms include the release of enzymes and surfactants, which have the capability to dissolve and

break down EPS components[33]. In conclusion, the triggering of the dispersion response leads to the activation of motility mechanisms, including flagella and pili, as well as proteins involved in chemotaxis. Therefore, dispersal is a meticulously controlled phenomenon that necessitates the mobilization of cellular mechanisms and allocation of energy resources in order to liberate from the biofilm[14].

In recent years, there has been a growing preference for the utilisation of dispersal signals to manipulate the endogenous biofilm development programme. This approach has emerged as a favoured strategy for the creation of innovative management strategies[34]. For example, researchers have developed 2-aminoimidazole derivatives that specifically target quorum sensing (QS) and have demonstrated their ability to disperse pre-existing biofilms. Proof of concept studies have demonstrated that manipulating c-di-GMP levels in vivo can effectively eradicate Pseudomonas aeruginosa infections in mouse models by reducing c-di-GMP levels or prolonging the infections by increasing c-di-GMP levels[35]. The BdcA protein was modified in order to improve its binding affinity to c-di-GMP, resulting in a decrease in the intracellular concentration of c-di-GMP. This modification led to the almost full eradication of biofilms through dispersal when tested in a laboratory setting[31]. One aspect that is particularly intriguing is the compound NO, which serves as a straightforward and adaptable dispersal signal that exhibits a high degree of conservation among various biofilm species. Significant advancements have been achieved in recent years in the development of effective ways for delivering NO, thereby establishing it as a very promising candidate for innovative therapeutic approaches. This review centers on the exploration and application of NO as a means to induce biofilm dispersion[14].

VIII. NO-MEDIATED PHYSIOLOGICAL SIGNAL FOR BIOFILM DISPERSION

1. **Transition to a Planktonic Lifestyle Mediated by Endogenous NO and Biofilm Dispersal:** NO has been identified as a significant signaling component in the life cycle of biofilms, playing a crucial role in promoting dispersal and allowing the shift to a planktonic existence. This gaseous compound is highly diffusible and possesses lipophilic properties, allowing it to readily distribute throughout many environments[2]. The identification of NO involvement in the process of biofilm dispersal was achieved by an examination of *Pseudomonas aeruginosa* biofilms. It was shown that the synthesis of NO occurred simultaneously with cell death and the subsequent dispersal of the biofilm (Figure 1)[36]. The identification of NO as the primary mediator of cell death was achieved through the utilization of fluorescent dye studies. This discovery was subsequently validated by genetic investigations, which revealed the role of endogenous NO generation in the regulation of biofilm dispersal[37].

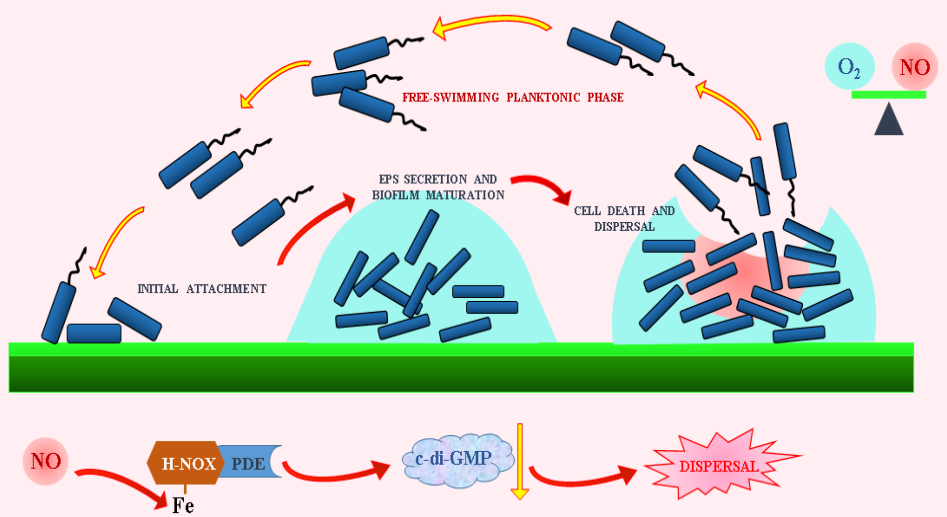

Figure 1: Life Cycle of Biofilm

Cell death and dispersal are triggered by NO signals in the mature biofilm due to O_2 and nutrient gradients in the biofilm's environment.Phosphodiesterase (PDE) activity is increased in response to NO signals, decreasing c-di-GMP levels and facilitating spread. It has been shown that NO directly binds to an H-NOX sensor in *Shewanellawoodyi*, boosting PDE activity.

The mutant strains, which were incapable of producing NO due to defective expression of nitrite reductase (NIR), did not display any cell death or dispersal. In contrast, the mutants lacking the ability to scavenge NO (deficient in NO reductase, NOR) had elevated levels of cell death and dispersal as compared to the wild type[38]. Furthermore, the introduction of NO back into biofilms via donor chemicals that release NO in a solution has been shown to induce dispersal and the shift to planktonic growth at low concentrations of NO within the picomolar to nanomolar range, without causing toxicity[39]. Significantly, the exposure to low concentrations of NO reinstated the vulnerability of both biofilm and disseminated bacteria to a range of antimicrobial treatments, thereby considerably augmenting their effectiveness.The aforementioned findings underscore the significant function of NO in the process of biofilm dispersal and propose its potential as a therapeutic intervention for addressing infections associated with biofilms[40]. The utilisation of NO as a means to induce dispersal and augment antimicrobial activity presents a promising avenue for the development of innovative biofilm management techniques. This strategy holds the potential to facilitate more successful strategies in the fight against chronic infections, ultimately leading to improved patient outcomes[41].

2. **Dispersal Via NO remains an Evolutionarily Conserved Trait:** Dispersal reactions to NO have been documented in various monospecies biofilm communities. For example, the introduction of NO donors has been observed to stimulate the dispersion of biofilms in various bacterial species including *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Bacillus licheniformis*, *Serratiamarcescens*, *Fusobacteriumnucleatum*, *Shewanellawoodyi*, *Neisseria gonorrhoeae*, and a marine Pseudoalteromonas species[42]. Conversely, in Vibrio fischeri, the addition of a NO scavenger has been found to inhibit

the dispersal of aggregates. The presence of nitrite was found to hinder the formation of biofilms by Staphylococcus aureus, most likely by producing NO and triggering dispersal[43]. The reduction of biofilm biomass was seen in Bacillus subtilis due to alterations in the endogenous generation of nitric oxide. The bacterium Legionella pneumophila was revealed to possess a sensor protein that responds to nitric oxide, resulting in a decrease in the amount of biofilm biomass. Nitrifying biofilms have demonstrated a notable sensitivity to NO[44]. For instance, prior studies have seen the dispersal of Nitrosomonas europaea biofilms in response to low levels of NO. In previous research conducted on Pseudomonas putida, it was observed that the introduction of a NOS enzyme by heterologous expression led to enhanced motility and dispersion of biofilms[45].

Moreover, NO has the ability to drive dispersal in biofilms consisting of several species. The introduction of low concentrations (ranging from 20 to 500 nM) of NO donors resulted in the dispersion of microbial biofilms consisting of many species. These biofilms were present in drinking water and recycled-water systems, as well as on reverse osmosis water filtration membranes[46]. Biofilm aggregates that were suspended in expectorated sputum from patients with chronic cystic fibrosis (CF) infection were effectively dispersed through the utilisation of NO donors. Therefore, the process of biofilm dispersal mediated by NO seems to be highly conserved among many bacterial species[47]. Paradoxically, certain investigations have presented contrasting findings, indicating that the introduction of NO actually promotes the development of biofilms. This phenomenon has been observed in specific organisms such as *Shewanellaoneidensis* and the rhizobacterium *Azospirillumbrasilense*. There exists a possibility that NO may not elicit dispersal responses in certain species, particularly within the framework of host-microbe symbiotic or mutualistic associations[48]. Interestingly, the phenomenon of disaggregation, dispersal, and inhibition of attachment caused by NO has been observed in various eukaryotic organisms, such as fungi, amoeba, and algal zoospores. This observation implies that NO might serve as an ancient and well-preserved regulator of dispersal[49].

3. **NO Signaling Molecular Cascade Embraces c-di-GMP:** The molecular cascade of NO signaling in biofilms encompasses the involvement of the secondary messenger c-di-GMP. It serves as a pivotal intracellular signaling molecule that governs the shift from planktonic to biofilm modes of existence in bacterial organisms[50]. The production of NO during the life cycle of a biofilm has the potential to exert an influence on the levels of c-di-GMP within bacterial cells. Elevated concentrations of c-di-GMP have been observed to be correlated with the adoption of a biofilm mode of growth, facilitating the production of EPS and stimulating cellular adhesion to various surfaces[51]. Conversely, diminished concentrations of c-di-GMP promote the planktonic growth mode, characterized by increased bacterial mobility and the ability to migrate and establish in novel habitats[52].

The term "NO" serves as a regulatory cue for modulating the concentrations of c-di-GMP in bacteria that have the ability to build biofilms. The precise methods by which NO induces a decrease in intracellular c-di-GMP levels are incompletely comprehended[53]. The reduction in c-di-GMP concentrations subsequently induces the

dispersion of bacteria from the biofilm, enabling them to transition back to a planktonic mode of existence[33].

The significance of the relationship between NO and c-di-GMP in the management of biofilms lies in its ability to facilitate bacterial adaptation to dynamic environmental conditions and enable coordinated behavioral responses to cues triggering dispersal[31]. The manipulation of the NO-c-di-GMP signaling pathway exhibits potential for the development of innovative approaches to regulating biofilms, augmenting the effectiveness of antimicrobial agents, and addressing persistent infections linked to biofilm communities[33].

IX. CLINICAL RELEVANCE OF NO AS AN ANTIBIOFILM STRATEGY

Biofilm formation is a complex and highly regulatedprocess, which is often an adaptation response to environmentalcues, such as changes in temperature, pH, nutrientlimits, and iron or oxygen levels[54]. There are 3defined stages in a biofilm life cycle: initial attachment, maturation, and dispersal. The initial attachment of planktoniccells to a surface is facilitated by flagella-mediatedmotility and pili-mediated adhesion. After attachment, cells in the biofilm upregulate extracellular polysaccharidesynthesis and continue to grow[55]. The laststep, dispersal, involves the release of cells from the biofilmwhere they are free to colonize another surface or host.

Biofilm production is a protective growth mode forbacteria and a major player in bacterial pathogenicity andinfectious diseases[56]. In a human host, biofilm-derived infectionsare often chronic infections and are the maincontributor to nosocomial infections, such as respiratorypneumonia, bloodstream, and gastrointestinal infections, endocarditis, and infections resulting from contaminated medical devices (e.g., intravenous catheters and heart valves)[55]. Along with the protective barrier provided by extracellular polysaccharides, bacterial cells display increased resistance towards antibiotics and host defenses in part due to the upregulation of efflux pumps. Current biofilm removal and control strategies (e.g., chlorine disinfection and antibiotics) are insufficient for the eradication of biofilm-derived contaminates and infections[54].

X. CONCLUSION AND FUTURE PERSPECTIVES

NO was initially identified in the 1980s for its ability to regulate vasodilation through the activation of soluble guanylate cyclase. Subsequently, NO has been recognized as a ubiquitous signaling molecule that governs a wide range of physiological processes in various living creatures. The significance of NO in the field of human physiology was acknowledged through the receipt of several accolades. In fact, Science magazine bestowed upon NO the prestigious title of "molecule of the year" in 1992. The distinctive features of NO, such as its diffusivity across cell membranes and its reactivity towards various target locations, play a significant role in signal transduction. These properties enable the rapid dissemination and amplification of an initial cue, as well as the coordination of a specific group of neighboring cells. The involvement of NO in the regulation of biofilm dispersal among different microbial species presents a unique and promising avenue for the development of innovative treatments aimed at inducing biofilm dispersal and enhancing the efficacy of therapies for chronic infections. Due to the involvement of non-toxic activation of a signaling pathway, the

mechanisms associated with dispersal result in diminished selective pressure for the evolution and dissemination of variant bacteria. Therefore, it is not anticipated that low-dose NO therapies will result in resistance. As our understanding of the signaling pathways deepens, we can expect to discover new markers originating from both infectious biofilms and host tissues. These markers will play a crucial role in enabling the assessment of novel drugs that have the potential to disperse biofilms in in-vivo experiments.

There exists a variety of NO donor chemicals that can be utilised as supplementary therapies to enhance the effectiveness of antibiotic treatments. Furthermore, researchers are currently exploring innovative delivery systems such as nanoparticles and dual-action hybrid medicines, as well as polymer coatings and prodrugs that are particularly engineered to release nitric oxide (NO) at biofilm infection sites. In the forthcoming era, novel compounds will be formulated with the capability to demonstrate diverse actions. These actions may encompass the release of nitric oxide (NO) signals and/or other substances that impede the functioning of different effectors involved in the signalling cascades responsible for dispersal and virulence. Moreover, these compounds will possess robust antibiotic properties. Moreover, considering the inherent simplicity of NO as a primary component, it is conceivable to explore other specialised chemical formulations that enable the exact administration of NO signals to specific pathogenic bacteria, while preserving the commensal microbial community.

REFERENCES

[1] Baloch, H.A.; Nizamuddin, S.; Siddiqui, M.T.H.; Riaz, S.; Jatoi, A.S.; Dumbre, D.K.; Mubarak, N.; Srinivasan, M.; Griffin, G.J.J.o.e.c.e. Recent advances in production and upgrading of bio-oil from biomass: A critical overview. 2018, *6*, 5101-5118.
[2] Förstermann, U.; Sessa, W.C.J.E.h.j. Nitric oxide synthases: regulation and function. 2012, *33*, 829-837.
[3] Drew, B.; Leeuwenburgh, C.J.A.o.t.N.Y.A.o.S. Aging and the role of reactive nitrogen species. 2002, *959*, 66-81.
[4] Änggård, E.J.T.l. Nitric oxide: mediator, murderer, and medicine. 1994, *343*, 1199-1206.
[5] Ricciardolo, F.L.; Sterk, P.J.; Gaston, B.; Folkerts, G.J.P.r. Nitric oxide in health and disease of the respiratory system. 2004, *84*, 731-765.
[6] Cinelli, M.A.; Do, H.T.; Miley, G.P.; Silverman, R.B.J.M.r.r. Inducible nitric oxide synthase: Regulation, structure, and inhibition. 2020, *40*, 158-189.
[7] Solanki, K.; Rajpoot, S.; Bezsonov, E.E.; Orekhov, A.N.; Saluja, R.; Wary, A.; Axen, C.; Wary, K.; Baig, M.S.J.P. The expanding roles of neuronal nitric oxide synthase (NOS1). 2022, *10*, e13651.
[8] Pérez de la Lastra, J.M.; Juan, C.A.; Plou, F.J.; Pérez-Lebeña, E.J.S. The nitration of proteins, lipids and DNA by peroxynitrite derivatives-chemistry involved and biological relevance. 2022, *2*, 53-64.
[9] Engwa, G.A.; Nweke, F.N.; Nkeh-Chungag, B.N.J.A.T.i.H.; Medicine. Free radicals, oxidative stress-related diseases and antioxidant supplementation. 2022, *28*.
[10] Al-Harbi, N.O.; Imam, F.; Al-Harbi, M.M.; Qamar, W.; Aljerian, K.; Anwer, M.K.; Alharbi, M.; Almudimeegh, S.; Alhamed, A.S.; Alshamrani, A.A.J.S.P.J. Effect of Apremilast on LPS-induced immunomodulation and inflammation via activation of Nrf 2/HO-1 pathways in rat lungs. 2023.
[11] Coleman, J.W.J.I.i. Nitric oxide in immunity and inflammation. 2001, *1*, 1397-1406.
[12] Khatsenko, O.G.; Gross, S.S.; Rifkind, A.B.; Vane, J.R.J.P.o.t.N.A.o.S. Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. 1993, *90*, 11147-11151.
[13] Roy, K. *Nitric Oxide in Chronic Obstructive Pulmonary Disease: Measurement and Role in the Characterisation of Disease*; The University of Manchester (United Kingdom): 2008.
[14] Kobzik, L.; Bredt, D.; Lowenstein, C.; Drazen, J.; Gaston, B.; Sugarbaker, D.; Stamler, J.J.A.j.o.r.c.; biology, m. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. 1993, *9*, 371-371.

[15] Villanueva, C.; Giulivi, C.J.F.R.B.; Medicine. Subcellular and cellular locations of nitric oxide synthase isoforms as determinants of health and disease. 2010, *49*, 307-316.
[16] Ricciardolo, F.L.; Nijkamp, F.P.; Folkerts, G.J.C.d.t. Nitric oxide synthase (NOS) as therapeutic target for asthma and chronic obstructive pulmonary disease. 2006, *7*, 721-735.
[17] Jamwal, S.; Sharma, S.J.I.R. Vascular endothelium dysfunction: a conservative target in metabolic disorders. 2018, *67*, 391-405.
[18] Subiabre, M.; Villalobos-Labra, R.; Silva, L.; Fuentes, G.; Toledo, F.; Sobrevia, L.J.B.e.B.A.-M.B.o.D. Role of insulin, adenosine, and adipokine receptors in the foetoplacental vascular dysfunction in gestational diabetes mellitus. 2020, *1866*, 165370.
[19] Gupta, A.; Gupta, R.; Singh, R.L.J.P.; future, a.o.e.b.f.a.s. Microbes and environment. 2017, 43-84.
[20] Stoodley, P.; Sauer, K.; Davies, D.G.; Costerton, J.W.J.A.R.i.M. Biofilms as complex differentiated communities. 2002, *56*, 187-209.
[21] Carrascosa, C.; Raheem, D.; Ramos, F.; Saraiva, A.; Raposo, A.J.I.J.o.E.R.; Health, P. Microbial biofilms in the food industry—A comprehensive review. 2021, *18*, 2014.
[22] Yu, H.; Liu, Y.; Li, L.; Guo, Y.; Xie, Y.; Cheng, Y.; Yao, W.J.T.i.F.S.; Technology. Ultrasound-involved emerging strategies for controlling foodborne microbial biofilms. 2020, *96*, 91-101.
[23] Umrao, P.D.; Kumar, V.; Sagar, S.S.; Kaistha, S.D.J.E.p.i.b. Bacteriophage control for Pseudomonas aeruginosa biofilm formation and eradication. 2020, 119-137.
[24] Su, S.; Panmanee, W.; Wilson, J.J.; Mahtani, H.K.; Li, Q.; VanderWielen, B.D.; Makris, T.M.; Rogers, M.; McDaniel, C.; Lipscomb, J.D.J.P.O. Catalase (KatA) plays a role in protection against anaerobic nitric oxide in Pseudomonas aeruginosa. 2014, *9*, e91813.
[25] Parrino, B.; Schillaci, D.; Carnevale, I.; Giovannetti, E.; Diana, P.; Cirrincione, G.; Cascioferro, S.J.E.J.o.M.C. Synthetic small molecules as anti-biofilm agents in the struggle against antibiotic resistance. 2019, *161*, 154-178.
[26] Gulati, M.; Nobile, C.J.J.M.; infection. Candida albicans biofilms: development, regulation, and molecular mechanisms. 2016, *18*, 310-321.
[27] Lee, Y.; Puumala, E.; Robbins, N.; Cowen, L.E.J.C.r. Antifungal drug resistance: molecular mechanisms in Candida albicans and beyond. 2020, *121*, 3390-3411.
[28] Mohammed, Y.H.E.; Manukumar, H.; Rakesh, K.; Karthik, C.; Mallu, P.; Qin, H.-L.J.M.p. Vision for medicine: Staphylococcus aureus biofilm war and unlocking key's for anti-biofilm drug development. 2018, *123*, 339-347.
[29] Pontes, J.T.C.d.; Toledo Borges, A.B.; Roque-Borda, C.A.; Pavan, F.R.J.P. Antimicrobial peptides as an alternative for the eradication of bacterial biofilms of multi-drug resistant bacteria. 2022, *14*, 642.
[30] K Satputea, S.; G Banpurkar, A.; M Banat, I.; N Sangshetti, J.; H Patil, R.; N Gade, W.J.C.p.d. Multiple roles of biosurfactants in biofilms. 2016, *22*, 1429-1448.
[31] Valentini, M.; Filloux, A.J.J.o.B.C. Biofilms and cyclic di-GMP (c-di-GMP) signaling: lessons from Pseudomonas aeruginosa and other bacteria. 2016, *291*, 12547-12555.
[32] Hengge, R.; Gründling, A.; Jenal, U.; Ryan, R.; Yildiz, F.J.J.o.b. Bacterial signal transduction by cyclic di-GMP and other nucleotide second messengers. 2016, *198*, 15-26.
[33] Jenal, U.; Malone, J.J.A.R.G. Mechanisms of cyclic-di-GMP signaling in bacteria. 2006, *40*, 385-407.
[34] Park, S.; Sauer, K.J.P.a.B., Pathogenesis; Strategies, C. Controlling biofilm development through cyclic di-GMP signaling. 2022, 69-94.
[35] Rodesney, C.A.; Roman, B.; Dhamani, N.; Cooley, B.J.; Katira, P.; Touhami, A.; Gordon, V.D.J.P.o.t.N.A.o.S. Mechanosensing of shear by Pseudomonas aeruginosa leads to increased levels of the cyclic-di-GMP signal initiating biofilm development. 2017, *114*, 5906-5911.
[36] Barraud, N.; Hassett, D.J.; Hwang, S.-H.; Rice, S.A.; Kjelleberg, S.; Webb, J.S.J.J.o.b. Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. 2006, *188*, 7344-7353.
[37] Asally, M.; Kittisopikul, M.; Rué, P.; Du, Y.; Hu, Z.; Çağatay, T.; Robinson, A.B.; Lu, H.; Garcia-Ojalvo, J.; Süel, G.M.J.P.o.t.N.A.o.S. Localized cell death focuses mechanical forces during 3D patterning in a biofilm. 2012, *109*, 18891-18896.
[38] Barraud, N.; J Kelso, M.; A Rice, S.; Kjelleberg, S.J.C.p.d. Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. 2015, *21*, 31-42.
[39] Lewis, A.; Matzdorf, S.; Endres, J.; Windham, I.; Bayles, K.; Rice, K.J.M.m. Examination of the S taphylococcus aureus nitric oxide reductase (sa NOR) reveals its contribution to modulating intracellular NO levels and cellular respiration. 2015, *96*, 651-669.

[40] Bergaust, L.; van Spanning, R.J.; Frostegård, Å.; Bakken, L.R.J.M. Expression of nitrous oxide reductase in Paracoccus denitrificans is regulated by oxygen and nitric oxide through FnrP and NNR. 2012, *158*, 826.
[41] Huang, C.-j.; Haataja, L.; Gurlo, T.; Butler, A.E.; Wu, X.; Soeller, W.C.; Butler, P.C.J.A.J.o.P.-E.; Metabolism. Induction of endoplasmic reticulum stress-induced β-cell apoptosis and accumulation of polyubiquitinated proteins by human islet amyloid polypeptide. 2007, *293*, E1656-E1662.
[42] Schulze, A.; Mitterer, F.; Pombo, J.P.; Schild, S.J.M.C. Biofilms by bacterial human pathogens: Clinical relevance-development, composition and regulation-therapeutical strategies. 2021, *8*, 28.
[43] Yang, L.; Feura, E.S.; Ahonen, M.J.R.; Schoenfisch, M.H.J.A.h.m. Nitric oxide–releasing macromolecular scaffolds for antibacterial applications. 2018, *7*, 1800155.
[44] Sugimoto, S.; Sato, F.; Miyakawa, R.; Chiba, A.; Onodera, S.; Hori, S.; Mizunoe, Y.J.S.r. Broad impact of extracellular DNA on biofilm formation by clinically isolated Methicillin-resistant and-sensitive strains of Staphylococcus aureus. 2018, *8*, 2254.
[45] Arora, D.P.; Hossain, S.; Xu, Y.; Boon, E.M.J.B. Nitric oxide regulation of bacterial biofilms. 2015, *54*, 3717-3728.
[46] McDougald, D.; Rice, S.A.; Barraud, N.; Steinberg, P.D.; Kjelleberg, S.J.N.R.M. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. 2012, *10*, 39-50.
[47] Schlag, S.; Nerz, C.; Birkenstock, T.A.; Altenberend, F.; Götz, F.J.J.o.b. Inhibition of staphylococcal biofilm formation by nitrite. 2007, *189*, 7911-7919.
[48] Trunk, T.; Khalil, H.S.; Leo, J.C.J.A.m. Bacterial autoaggregation. 2018, *4*, 140.
[49] Bardy, S.L.; Briegel, A.; Rainville, S.; Krell, T.J.J.o.B. Recent advances and future prospects in bacterial and archaeal locomotion and signal transduction. 2017, *199*, 10.1128/jb. 00203-00217.
[50] Escobar-Ramírez, M.C.; Castañeda-Ovando, A.; Pérez-Escalante, E.; Rodríguez-Serrano, G.M.; Ramírez-Moreno, E.; Quintero-Lira, A.; Contreras-López, E.; Añorve-Morga, J.; Jaimez-Ordaz, J.; González-Olivares, L.G.J.F. Antimicrobial activity of Se-nanoparticles from bacterial biotransformation. 2021, *7*, 130.
[51] Hall-Stoodley, L.; Costerton, J.W.; Stoodley, P.J.N.r.m. Bacterial biofilms: from the natural environment to infectious diseases. 2004, *2*, 95-108.
[52] Minasyan, H.J.S.j.o.t., resuscitation; medicine, e. Sepsis: mechanisms of bacterial injury to the patient. 2019, *27*, 1-22.
[53] Emerenini, B.O.; Hense, B.A.; Kuttler, C.; Eberl, H.J.J.P.o. A mathematical model of quorum sensing induced biofilm detachment. 2015, *10*, e0132385.
[54] Sauer, K.; Stoodley, P.; Goeres, D.M.; Hall-Stoodley, L.; Burmølle, M.; Stewart, P.S.; Bjarnsholt, T.J.N.R.M. The biofilm life cycle: Expanding the conceptual model of biofilm formation. 2022, *20*, 608-620.
[55] Wani, A.K.; Akhtar, N.; Sher, F.; Navarrete, A.A.; Américo-Pinheiro, J.H.P.J.A.o.M. Microbial adaptation to different environmental conditions: molecular perspective of evolved genetic and cellular systems. 2022, *204*, 144.
[56] Schilcher, K.; Horswill, A.R.J.M.; Reviews, M.B. Staphylococcal biofilm development: structure, regulation, and treatment strategies. 2020, *84*, 10.1128/mmbr. 00026-00019.

