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ADVANCED REVIEW

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Nanomedicine against biofilm infections: A roadmap of challenges and limitations

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Abstract

Microbial biofilms are complex three-dimensional structures where sessile microbes are embedded in a polymeric extracellular matrix. Their resistance toward the host immune system as well as to a diverse range of antimicrobial treatments poses a serious health and development threat, being in the top 10 global public health threats declared by the World Health Organization. In an effort to combat biofilm-related microbial infections, several strategies have been developed to independently eliminate biofilms or to complement conventional antibiotic therapies. However, their limitations leave room for other treatment alternatives, where the application of nanotechnology to biofilm eradication has gained significant relevance in recent years. Their small size, penetration efficiency, and the design flexibility that they present makes them a promising alternative for biofilm infection treatment, although they also present set-backs. This review aims to describe the main possibilities and limitations of nanomedicine against biofilms, while covering the main aspects of biofilm formation and study, and the current therapies for biofilm treatment.

This article is categorized under:

Therapeutic Approaches and Drug Discovery > Nanomedicine for Infectious Disease

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KEYWORDS

antimicrobials, bacteria, biofilm, infectious diseases, microorganisms

1 | INTRODUCTION

Antimicrobial resistance (AMR) is on the top three health threats identified by the Health Emergency Preparedness and Response Authority (HERA) of the European Commission, posing one of the highest risks to human health. In 2019, 4.95 million deaths were associated to bacterial AMR globally (Murray et al., 2022). According to the Center for Disease Control and Prevention (CDC), more than 2.8 million AMR infections occur each year throughout the United States, with more than 35,000 deaths as a result in 2019 (CDC, 2019). AMR is the result of natural genetic mutation and gene acquisition processes which have been accelerated due to the misuse and overuse of antimicrobial agents. In addition, the majority of microbes have the ability to form biofilms, a complex three-dimensional structure that increases their antimicrobial tolerance. To tackle this situation, it is clear that novel antimicrobial therapies are needed, but the development of new antibiotics by the pharmaceutical industry is diminishing due to their low profitability, although the European Union, the United Kingdom and the United States have initiated substantial efforts to fund antibacterial research and development (Årdal et al., 2020).

In this context, the application of nanotechnology into biofilm research and treatment has gained significant attention in recent years. The use of nanoparticles (NPs) as antibiofilm agents offers several advantages. Their small size (from 1 nm up to 1 μm) facilitates penetration into biofilms through matrix pores, ensuring a higher biofilm eradication efficiency. Additionally, NPs possess a high surface area-to-volume ratio, making them highly reactive, and capable of carrying a high drugs or enzymes load (Al-Wrafy et al., 2022; Birk et al., 2021; Dos Santos Ramos et al., 2018; Eleraky et al., 2020). Encapsulation of antibiofilm agents, attachment or adsorption onto supramolecular agents forming NPs improves drug pharmacokinetics and pharmacodynamics while modifying solubility, surface properties, and architecture to stabilize and protect from degradation, thus preventing potential side effects. Moreover, functionalizing NPs with ligands targeting biofilms can increase drug concentration in the biofilm surroundings, enhancing penetration inside the biofilm (Choi et al., 2023). However, the use of nanomedicine in the biofilm field presents many limitations, which hinder the translatability between in vitro biofilm models and clinical applications. This review will provide an overview of different antibiofilm strategies, as well as the main constraints and difficulties in implementing the use of NPs in biofilm treatment.

2 | BIOFILMS AND ANTIBIOFILM THERAPIES

Biofilms account for ~80% of bacterial and archaeal cells on the surface of Earth and represent the dominant way of active bacterial and archaeal life (Flemming & Wuertz, 2019). Interestingly, biofilm-forming microbes display different properties compared to when they are found in their planktonic, free-living form. In the clinical context, they show survival-promoting phenotypic traits, including drug resistance mechanisms and defense strategies against the infected host immune system, and they become extremely difficult to eradicate, as it is considered the most important passive resistance to antimicrobials (Vandeplassche et al., 2020). Thus, biofilm-related infections contribute to the severity and chronification of life-threatening diseases including, but not limited to, infective endocarditis, wounds, mastitis, otitis media, urinary tract infections, inflammatory bowel disease, cystic fibrosis, and chronic obstructive pulmonary disease (Vestby et al., 2020; Welp & Bomberger, 2020).

2.1 | Biofilm definition and composition

Biofilms are three-dimensional structures consisting of sessile bacterial cells attached to each other and embedded in a self-synthesized matrix composed of extracellular polymeric substances (EPS), mainly polysaccharides, proteins, and extracellular DNA (eDNA), but it also includes lipids and non-soluble compounds such as cellulose (Flemming & Wingender, 2010). This extracellular matrix (ECM) structures the microbial community, and its composition, properties and dynamics influence the biofilm's mode of life. In addition, it can present different physical states ranging from dissolved to dense gels depending on the degree of biofilm maturity and environmental factors such as pressure, salt content, temperature, and hydrodynamic shear stress, among others (Flemming et al., 2023).

Originally, for a microbial structure to be considered as a biofilm, it had to be attached to biotic or abiotic surfaces, such as dental surfaces and medical device surfaces, respectively (Muhammad et al., 2020). However, non-attached microbial aggregates have recently been recognized as biofilms, and they have been clinically associated to respiratory

tract infections with impaired host mucociliary clearance and to persistent soft tissue infections (Kragh et al., 2023; Perez & Patel, 2015; Staudinger et al., 2014). Also, biofilm models have originally focused on a single microbial species, although the relevance of polymicrobial biofilms in chronic infections has been widely described over the last decade. Polymicrobial biofilms are aggregates consisting of different microorganisms interwoven or closely distributed, allowing potential inter-species context-dependent interactions (Cendra & Torrents, 2021; Luo et al., 2022). Specific compositions and particular spatial distribution of bacteria in polymicrobial biofilms can increase the tolerance toward antimicrobial therapies, the microbial virulence in infection contexts and the persistence of the infection, as well as the repression of the host's immune system (Anju et al., 2022; Cendra et al., 2019; Lopes et al., 2021).

2.2 | The biofilm life cycle

Over the course of biofilm formation and development, microbes undergo a series of highly regulated phenotypic changes that allow them to display different metabolic and structural characteristics. Those changes are strongly microbe and environment dependent, and thus biofilms exist in a wide variety of shapes and compositions (Ruhal & Kataria, 2021). In an effort to describe the biofilm life cycle in a general way, researchers have proposed a model consisting of three stages: aggregation and attachment, growth and accumulation and disaggregation and dispersion (Figure 1) (Sauer et al., 2022).

In the first stage of the cycle, aggregation and attachment, planktonic microbial cells come into contact with a surface and adhere to it, or they attach to each other in the case of non-attached microbial aggregates. Then, the cells involved in this process start a notable transformation encompassing global transcriptional changes that include, among others, the cessation of the flagella-induced motility, the production of exopolysaccharides that constitute the biofilm ECM and the expression of antimicrobial resistance-related genes (Sauer et al., 2022). Subsequently, the biofilm becomes a mature and complex 3D structure. Oxygen, nutrient, waste and signaling molecules gradients appear within the biofilm, which promotes the appearance of microbial subpopulations expressing different types of genes depending on their oxygen and nutrient disposition, among others (Serra & Hengge, 2014; Stewart & Franklin, 2008; Yin et al., 2019). A relevant subpopulation is the persister cells, which are slow-dividing or non-dividing cells that are less susceptible to antimicrobial therapies, being responsible for biofilm reconstitution when the antimicrobial treatment is ceased (Ciofu & Tolker-Nielsen, 2019). Finally, the last stage of the cycle, known as disaggregation and dispersion, consists on the separation of planktonic cells or portions of the mature biofilm from the main structure. This stage closes the biofilm life cycle, as those biofilm aggregates or planktonic cells escaping the mature biofilm will disseminate and potentially colonize other surfaces.

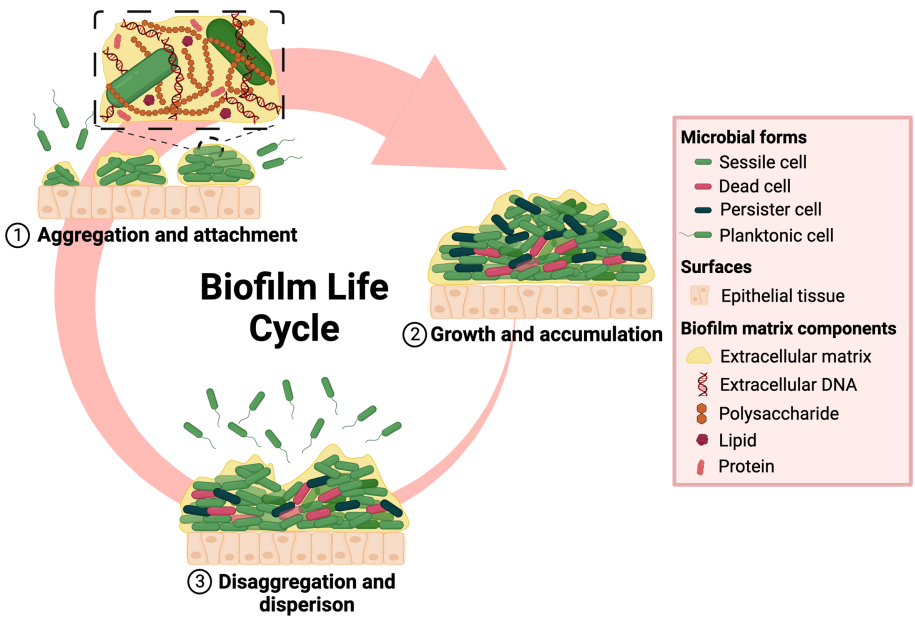

FIGURE 1 Schematic representation of the biofilm life cycle. Created with Biorender.com.

2.3 | Biofilm detection and study: In vitro and in vivo models

Despite the existing guidelines regularly published by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the Infectious Disease Society of America (IDSA), there is no standardized protocol for biofilm diagnosis in clinical practice (Coenye, 2023; Høiby et al., 2015; Paul et al., 2022; Tamma et al., 2023). Biofilm-associated infections are often subclinical for long periods and are only detectable in the case of bacteremia. In addition, when biofilms are located in an implanted medical device, their detection is only possible after a surgical procedure, and even in that case, microbial cells may be difficult to reach (Xu et al., 2019). Moreover, some microbial cells are extremely difficult to culture in an in vitro setting, leading to false negative results in rutinary culturing procedures. Another biofilm-related challenge in clinical practice is the fact that antimicrobial susceptibility is systematically tested in planktonic bacteria, which provides significantly different—and thus, misleading—results compared to biofilm-associated infections (Silva et al., 2021).

In sight of the aforementioned challenges, a wide variety of in vitro models have been ideated with the aim of providing an easy and representative platform for antimicrobial therapy testing and a straight-forward biofilm culture system from clinical samples. Some of the most used in vitro models are denominated static. They often offer the possibility of testing different strains and antimicrobial therapies all at once, are inexpensive and have low technical requirements, although they poorly mimic the infection environment (Alcàcer-Almansa et al., 2023). Some examples are microtiter plates and the Calgary Biofilm Device (Azeredo et al., 2017; Ceri et al., 1999). Other more complex models, denominated dynamic models, require complex technical set-ups and are less throughput, although they closely replicate in vivo conditions. Some examples are flow chamber systems and the Robbins device (Bakker et al., 2003; Gomes & Mergulhão, 2021). Over the past decade, dynamic biofilms have evolved into miniaturized devices based on microfluidic technologies that require lower volumes of reagents and thus have reduced costs and are versatile and easy to fabricate (Alcàcer-Almansa et al., 2023). Some examples are the Bioflux and the BiofilmChip (Benoit et al., 2010; Blanco-Cabra et al., 2021). A comprehensive review of methods for biofilm study with emphasis in microfluidics advantages, limitations and translational challenges can be consulted at Alcàcer-Almansa et al., 2023.

To validate the results obtained in vitro as well as to understand the role of the host's immune system during biofilm-associated infections among other parameters, in vivo models have been key (Guzmán-Soto et al., 2021). A wide diversity of animals and procedures have been used to characterize different biofilm-associated infections in vivo, which complicates the translation of those results in the clinical context. However, there are some well-established models for certain infections such as chronic otitis media (Chaney et al., 2011), chronic wounds (Gurjala et al., 2011), chronic rhinosinusitis (Vanderpool & Rumbaugh, 2023) and osteomyelitis (Funao et al., 2012), among others (Anju et al., 2022; Guzmán-Soto et al., 2021).

2.4 | General biofilm treatment strategies overview

The inherent properties of the biofilm growth mode mentioned in the preceding section have rendered antimicrobial treatments designed against free-living microorganisms ineffective. The development of biofilm eradication strategies involves several concerns. Biofilm cells are more resistant than planktonic cells due to different mechanisms. The efficiency of antibiotics and biocides is reduced by impeded penetration inside the biofilm ECM. Gradients of oxygen and nutrients create microenvironments inside biofilms leading to heterogeneity in the metabolic activity of biofilm cells, which hinders complete responsiveness to antimicrobials. Moreover, changes in cell membrane, enzyme expression, activation of multidrug efflux pumps, and processes of horizontal transfer add complexity to the equation (Zhang et al., 2020).

Consequently, there is a significant interest within the medical community to develop biofilm treatment strategies that can either independently address these challenges or complement conventional antibiotics. This section aims to contextualize the reader providing a concise description of the primary approaches currently employed or under investigation for treating established biofilms, categorized according to their mechanisms of action, and Figure 2 illustrates them. Detailed description can be found in Asma et al., 2022 and Singh, Padmesh, et al., 2022 (Asma et al., 2022; Singh, Amod, et al., 2022). In addition, a list with some examples and references including more detailed information about specific biofilm treatment strategies is presented in Table 1. Regarding combined therapies, Hawas et al. (2022) and Bari et al. (2023) presented a review and comparison of the current ones used on biofilm forming pathogens (Bari et al., 2023; Hawas et al., 2022).

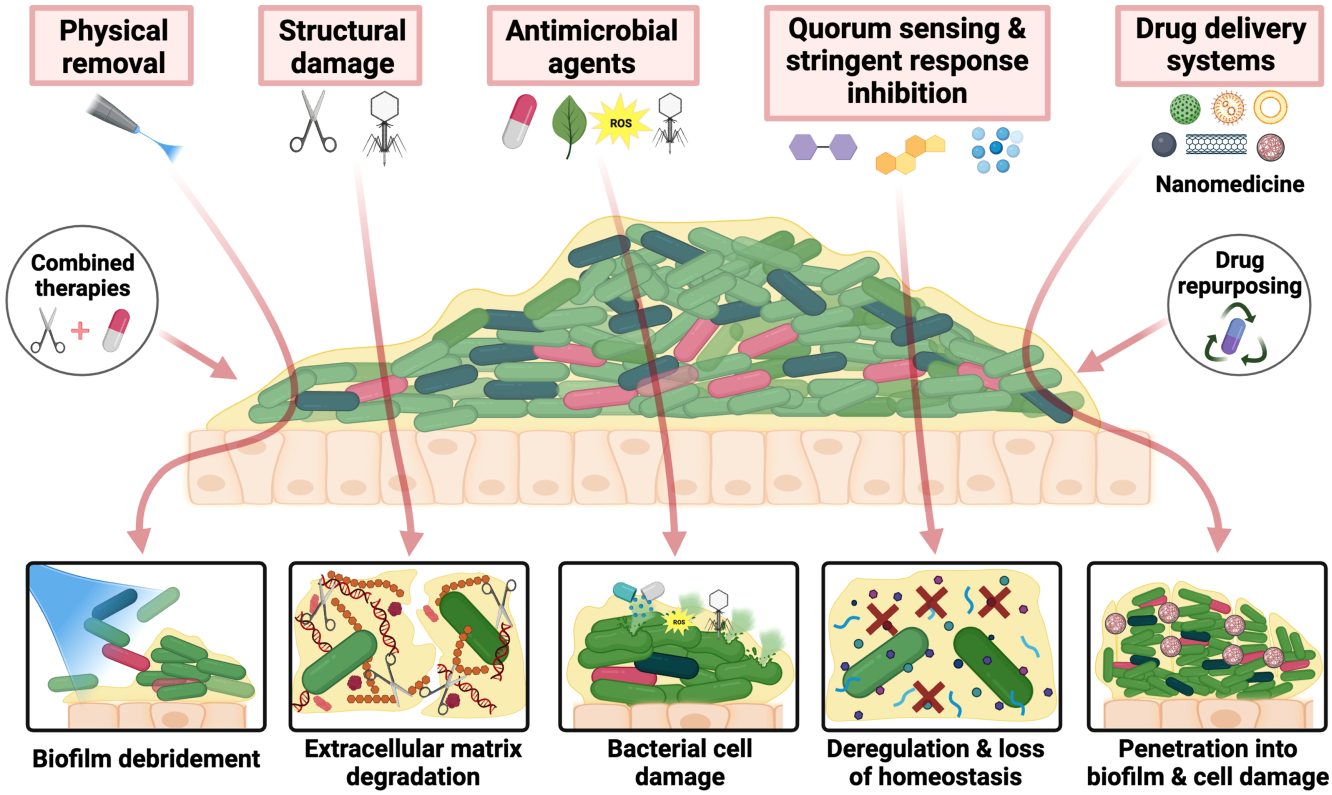

FIGURE 2 Biofilm treatment strategies and their mechanism of action. Created with Biorender.com.

2.4.1 | Biofilm physical removal

The first and most intuitive strategy is the eradication of biofilms through physical removal. This step is critical in biofilm infections associated with medical devices, where the removal of the foreign body precedes antibiotic treatment whenever is possible (Wolfmeier et al., 2018). In other scenarios, such as oral, wound, and joint biofilm-related infections, the use of irrigation with water jets and debridement before antimicrobial therapy is also common (Zhang et al., 2020). However, the implementation of this approach is very limited and greatly depends on the accessibility of the infection site and patient case.

2.4.2 | Biofilm structural damage

To overcome the limitations of physical biofilm removal, alternatives that affect the integrity of the biofilm structure have been proposed. These strategies consider targeting the ECM, leading to impairment of biofilm development, destabilization, and detachment of biofilms, sensitization of biofilm cells, and increased permeability to drugs (Wolfmeier et al., 2018). Several enzymes specific for ECM components have been successfully tested, including phage-derived enzymes (Ferriol-González & Domingo-Calap, 2020).

Enzymes can be produced at high concentrations in a laboratory setting, are specific and effective with relatively low concentrations, and have less resistance issues than antibiotics (Wang, Zhao, et al., 2023). Nevertheless, it is important to consider that ECM composition varies greatly according to the strain, maturity, and environmental conditions of the biofilm (Srinivasan et al., 2021). A similar limitation is found when using phage-derived enzymes because although their specificity is an advantage, this could require knowing the causative agent of the infection before treatment (Ferriol-González & Domingo-Calap, 2020). To overcome this issue, the combination of enzymes specific for different ECM components or the formulation of phage cocktails, plus the inclusion of conventional antibiotics has been proposed, which could increase treatment efficacy, especially in polymicrobial biofilms (Ferriol-González & Domingo-Calap, 2020; Wolfmeier et al., 2018). Currently, the ECM-targeted enzymes dispersin B and Pulmozyme have made it into clinics, but the cytotoxicity of some others may complicate their transition into the clinical context (Wolfmeier et al., 2018).

TABLE 1 Biofilm treatment strategies by mechanism of action.

Strategy	Examples	References of interest
Physical removal	Irrigation and debridement	(Boutsioukis & Arias-Moliz, 2022) (Ousey & Ovens, 2023)
Structural damage	ECM degrading enzymes: Glycoside hydrolases, proteases, and deoxyribonucleases, phage-derived enzymes	(Wang, Zhao, et al., 2023) (Ramakrishnan et al., 2022) (Pires et al., 2022) (Singh, Padmesh, et al., 2022)
Antimicrobial agents	Natural-product-based antibiofilm agents: Phytochemicals, essential oils, biosurfactants, antimicrobial peptides (AMPs), antimicrobial lipids (AMLs), and so forth	(Shamim et al., 2023)
	Chemical compounds: Small molecules, silver compounds, quaternary ammonium compounds, chelators, and so forth	(Nadar et al., 2022)
	Stress-inducing therapies: Photodynamic therapy, voltage and electric current application, nitric oxide-releasing antibiotics, and so forth	(Ribeiro et al., 2022) (Poh & Rice, 2022)
	Repurposed drugs	(Jampilek, 2022) (Barbarossa et al., 2022)
	Phage therapy and predatory bacteria	(Liu, Lu, et al., 2022) (Mookherjee & Jurkevitch, 2022)
Quorum sensing and stringent response inhibition	Quorum sense quenchers and stringent response inhibitors	(Vashistha et al., 2023) (Wang et al., 2022) (Patel et al., 2023)
Drug delivery systems	Inorganic NPs: Metal based NPs, metal oxide-based NPs, and so forth	(Tong et al., 2023)
	Organic NPs: Dendrimers, micelles, liposomes, polymeric NPs, and so forth	(Fang et al., 2021) (Gao et al., 2020) (Le et al., 2021) (Wan et al., 2020) (Blanco-Cabra et al., 2022)
	Carbon based NPs	(Mohanta et al., 2023)

Besides enzymes, molecules such as exo-polysaccharides and Ca^{2+} ion chelating agents can destabilize the ECM and induce biofilm structural modifications and/or dispersion (Roy et al., 2018).

2.4.3 | Antimicrobial agents

Another approach to biofilm treatment involves reducing the biofilm biomass by inducing cell death. As previously mentioned, the antimicrobial capacity of an agent does not necessarily imply antibiofilm activity. However, various compounds have demonstrated efficacy in eradicating biofilms through the induction of cell damage (Asma et al., 2022). Several of them are naturally produced by plants and microorganisms (Shamim et al., 2023).

Moreover, biofilm cell death caused by stress-inducing therapies has also been explored. Antibiotics, nitroxide hybrids, and local application of external stimuli (photodynamic therapy, voltage and electric current, non-thermal plasma combined with water electrospray, etc.) display cytotoxic effects inside biofilms through the generation of reactive oxygen and nitrogen species (Srinivasan et al., 2021; Verderosa et al., 2019; Wolfmeier et al., 2018; Zhang et al., 2020).

Several other alternatives have been considered in the search for antibiofilm treatments, including drug repurposing exploration. This has led to the identification of agents with high biofilm eradication activity, as in the case of some chemotherapy medicines, although the doses required significantly exceed those approved for cancer treatment (Verderosa

et al., 2019). Another approach is the use of phages and predatory bacteria. Their ability to recognize and lyse bacteria has an impact on biofilm eradication. Currently, the cocktail *PYO* designed to treat chronic wound biofilms is commercially available, while potential adverse effects of predatory bacteria on normal microbiota remain a limitation for their implementation (Ferriol-González & Domingo-Calap, 2020; Zhang et al., 2020).

2.4.4 | Quorum sensing and stringent response inhibition

In addition to disrupting biofilm structure and reducing biofilm cell viability, another strategy against biofilms involves targeting quorum sensing, the intricate communication and behavior coordination system employed by microbes (Srinivasan et al., 2021). Quorum sensing allows the species living inside a biofilm to sense and regulate different mechanisms, such as population density and swarming motility. Blocking or altering these signals can lead to bacterial detachment, prompting biofilm disassembly. In the same line, another approach involves disrupting the stringent response, the system that regulates gene expression for growth and survival under conditions of starvation, which is a crucial process for biofilm homeostasis (Roy et al., 2018).

2.4.5 | Drug delivery systems

Besides the identification of new treatments, the development of strategies to enhance the effectiveness of traditional and new antimicrobial/antibiofilm agents is also a relevant topic. The use of drug delivery systems, which improve their safety, bioavailability, stability, and controlled release, has been proposed (Wolfmeier et al., 2018). In this context, various antibiofilm nanotechnologies have been developed in recent years to target biofilm cells, microenvironment and/or ECM. The small size of NPs facilitates the penetration into the biofilms through the matrix pores. Moreover, these systems are highly efficient, non-invasive, controllable, fast, and have a low potential for resistance development, making them a unprecedentedly promising strategy against biofilm infections (Xiu et al., 2021). However, only a limited number of these approaches are currently available for clinical use, and several challenges must be addressed to overcome translational barriers (Xiu et al., 2021). The following section will provide an overview of the main nanotechnology-based strategies and NPs for biofilm treatment, accompanied by a comprehensive critical review of the primary limitations for their clinical implementation.

3 | NANOMEDICINES FOR BIOFILM TREATMENT: CHALLENGES AND LIMITATIONS

In recent years, a significant number of publications have focused on nanotechnology as a viable treatment for biofilm infections. Thus, a wide variety of NPs with diverse characteristics and specificities are available and several reviews covering this topic can be found. Liu et al., Lv et al., Al-Wrafy et al., and Mohanta et al. have provided outstanding comprehensive reviews of existing NP-based biofilm treatment, including all the information regarding material characteristics and the approach to eradicate biofilms, as well as the NPs classification and the challenges implied (Al-Wrafy et al., 2022; Liu et al., 2019; Lv et al., 2023; Mohanta et al., 2023). Other focused reviews about organic, polymer-based, and metal NPs against biofilms were made by Li et al., Birk et al., and Asare et al., respectively (Asare et al., 2022; Birk et al., 2021; Li et al., 2021). Choi et al. and Dos Santos Ramos et al. discussed the benefits, limitations and future prospects of different drug-delivery systems based on NPs against biofilms (Choi et al., 2023; Dos Santos Ramos et al., 2018), and Wang et al. and Sousa et al. have provided insightful reviews of the strategies to enhance the drug delivery inside biofilms using nanotechnology (Sousa et al., 2023; Wang, Cornel, et al., 2023). In the same direction, Vidallon et al. reviewed the recent developments in nanoencapsulation (Vidallon & Teo, 2020). From a different point of view, Fulaz et al. discussed the antibiofilm function of NPs, focusing on the role of the biofilm ECM in the interaction between NPs and biofilms (Fulaz et al., 2019).

Here, we have selected as an example some works of nanomedicines against biofilm infections using robust biofilm models and set ups: Tong et al. used Fe_3O_4 magnetic NPs as antibiofilm agents against periodontal bacterial biofilms and enhanced the penetration efficacy through magnetic targeting in an in vivo bacterial-induced periodontal inflammation model in rats (Tong et al., 2023). On the other hand, Fang et al. employed cationic liposomes with enzymes

(DNase I and Proteinase K) to disassemble *Cutibacterium acnes* biofilms in a cutaneous and catheter mouse model (Fang et al., 2021). Nanomedicine based on lipids was also employed by Su et al., where micellar nanocarriers responsive to pH and hypoxia were loaded with antibiotic and applied in treating *Staphylococcus aureus* mature in vivo biofilms (Su et al., 2022). Another example is on the work done by Gao et al., which developed a promising antibiofilm nanoplatform tested in a chronic *Pseudomonas aeruginosa* infection lung model consisting of seaweed alginate beads. They used size and charge adaptative clustered NPs with the antibiotic azithromycin conjugated to a dendrimer, which disassembled upon arrival to the infected tissue to release the antibiotic, increasing its internalization inside the biofilm (Gao et al., 2020). An example of NPs functionalization with ligands is in Le et al. work, that conjugated anti-Staph antibodies into PLGA NPs to target *S. aureus* biofilm in a skin mouse model of biofilm infection, resulting in improved biofilm treatment efficacy (Le et al., 2021). PLGA was also employed by Wan et al. to investigate the interactions between NPs and biological barriers in a cystic fibrosis-related biofilm infection (Wan et al., 2020). Another case of polymeric NPs, using dextran as a biocompatible, biodegradable, and water-dispersible material, were employed by Blanco-Cabra et al. to neutralize the negative charges of the antibiotic tobramycin, increasing its penetration into *P. aeruginosa* biofilms (Blanco-Cabra et al., 2022).

Although the use of NPs in biofilm treatment offers many advantages, the application of nanotechnology in this field also presents several adversities. Here, we will discuss some of the challenges and limitations associated with implementing nanomedicine for biofilm treatment, and Figure 3 illustrates them.

3.1 | Synthesis, good manufacturing practices, and scalability of NPs

There are concerns regarding the synthesis methods of nanomedicine, especially in relation to metal NPs (Pinto et al., 2019). Metal NPs are normally prepared using physical and chemical methods that pose risks to the environment and human health due to the substantial energy consumption and the release of adverse and toxic reagents and solvents (Gallo & Schillaci, 2021; Ying et al., 2022). Furthermore, some of these NPs, like silver NPs (AgNPs) and gold NPs (AuNPs) are more costly to produce than other metal NPs, like the ones made with copper, zinc or titanium, or non-metallic NPs (Armijo et al., 2020; Liu et al., 2019; Zazo et al., 2016). Green-synthetized metal NPs, produced by fungi, bacteria, algae or extracts of plants, allow for the use of less toxic reagents, thereby improving cytocompatibility and reducing toxicity. Moreover, their synthesis uses less energy and generates fewer toxic products (Bertoglio et al., 2018; Gallo & Schillaci, 2021). However, the green synthesis of these metal NPs has certain limitations. Raw materials are not readily available in all environments and seasons, and even though the materials are environmentally friendly, the synthesis process required for some green NPs production can be lengthy (Hajipour et al., 2021). Furthermore, some of the NPs' biosynthesis processes are not totally understood, the yield is low and there are large differences in particle size (Ying et al., 2022) (Figure 3).

Scalability of nanomaterial synthesis also represents a bottleneck for transitioning nanomaterials from the laboratory bench to practical medical applications. An indispensable condition for bringing a product to market is to conduct all production processes under good manufacturing practices (GMPs), ensuring consistency and reproducibility. However, adhering to GMP standards significantly increase the cost of upscaling production processes, and nanomedicines are no exception (Bianchera et al., 2020; Fornaguera & García-Celma, 2017). The primary challenges in their production process pertain to sterilization and storage (Figure 3). Sterilization processes often lead to alterations in certain NPs characteristics, such as toxicity, drug-release, stability, and physicochemical properties. Additionally, the diverse nature of existing nanomedicines implies that each one should undergo validation for the most suitable sterilization method (Bernal-Chavez et al., 2021; Vetten et al., 2014). NPs stability also acts as a barrier to scaling up nanomaterial synthesis. While in research labs, NPs are typically used immediately after synthesis, the storage of the final product becomes crucial during scale-up processes (Fornaguera & García-Celma, 2017). Therefore, characterizing the physicochemical properties of NPs is essential to determine product stability and storage conditions (Elfadil et al., 2022). Among various nanomaterials, liposomes tend to be less stable, with potential cargo leakage during storage (Al-Wrafy et al., 2022; Birk et al., 2021; Makhlouf et al., 2023). Conversely, polymer-based NPs are usually more stable (Al-Wrafy et al., 2022; Elfadil et al., 2022). On the other hand, the stability of nanoscale metals produced via green methods primarily hinges on synthesis materials and techniques (Ying et al., 2022).

In light of these challenges, it is important to consider the cost-effectiveness of nanomedicines, given the substantial expenses associated with scaling up. For instance, processes like freeze-drying must be meticulously developed to avoid substantial alterations in properties, degradation, or aggregation (Fornaguera & García-Celma, 2017).

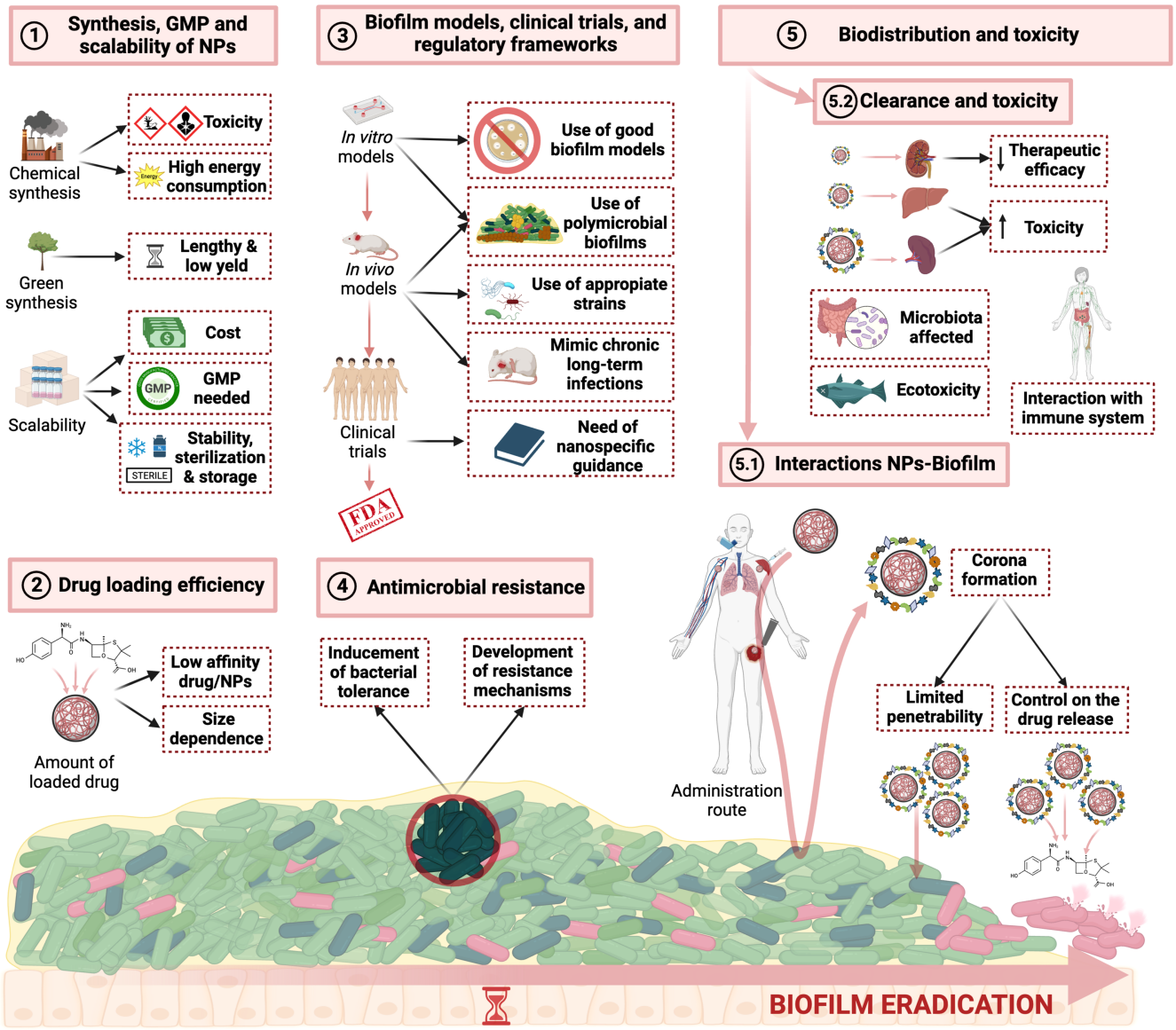

FIGURE 3 Challenges and limitations in nanomedicine for biofilm treatment, highlighted within dashed squares at each stage. Created with Biorender.com.

3.2 | Drug loading efficiency

Encapsulating antibiotics in nanomedicines not only protects the drug from enzymatic degradation but also reduces potential adverse effects. Moreover, the encapsulation ensures a controlled drug release, thereby maximizing therapeutic effectiveness (Marchianò et al., 2020). The drug loading efficiency is a critical consideration in all the nanomedicines used as drug delivery systems, also in the loading of antibiofilm molecules into NPs. In this regard, a distinction should be made between encapsulation and drug loading efficiency. Encapsulation efficiency refers to the amount of loaded drug in relation to the total drug used. This aspect is crucial for avoiding drug wastage during the fabrication process and for scaling up the production of nanomedicines, and it depends mostly on the synthesis method employed. On the other hand, drug loading efficiency focus to the amount of loaded drug relative to the NP's weight. This factor significantly influences the antimicrobial efficacy of the nanomedicine and is primarily contingent on the characteristics of both the carrier and the drug (Birk et al., 2021; Ho et al., 2019).

In this scenario, certain encapsulation processes pose challenges. For instance, encapsulating hydrophilic drugs within a hydrophobic NP-shell can be demanding due to the inherent low affinity between these components (Liu et al., 2019). Furthermore, some NPs composed of PLGA exhibit low drug loading, necessitating larger drug quantities

to achieve therapeutic dosages. This impediment hampers the scalability and clinical applicability of such formulations (Birk et al., 2021; Kim, 2016). Therefore, drug loading efficiency also significantly influences the clinical translation of nanomedicines. For instance, in cases involving the lungs, the amount of nanoformulation that can be applied is limited, making high drug loading imperative. However, many studies only report encapsulation efficiency and overlook drug loading efficiency (Birk et al., 2021; Ho et al., 2019).

In a general context, drug loading capacity tends to increase with particle size augmentation. Consequently, by expanding nanomedicines into the submicron range (100–900 nm), the amount of drug loaded within them can be significantly enhanced. Nonetheless, this characteristic can hinder their efficacy against biofilms, as it leads to reduced penetration of the nanomedicines into these biofilm structures (Ho et al., 2019) (Figure 3).

3.3 | In vitro and in vivo biofilm models, clinical trials, and regulatory frameworks

Resembling the body biofilm infections in the laboratory is an inherent problem when testing any type of antibiofilm medicine, since mimicking the biofilm-life style of bacteria is challenging. Planktonic assays are easy and convenient for measuring antibiotic resistance but unsuitable for testing antibiofilm activity (Birk et al., 2021). Static biofilm assays are more reliable, but only provide an estimation of the nanomedicine's antibiofilm potential, whereas assays performed in dynamic biofilms enable pharmacokinetics and pharmacodynamics simulations and can offer more information on NP-derived biofilm changes, NPs penetration (Birk et al., 2021; Haagensen et al., 2015; Pinto et al., 2019). Given the legal and ethical framework permitting the use of human tissues, ex vivo models allow a more extensive evaluation of the nanomedicine effectivity in the tissue environment (CD-P-TO, 2022; Pinto et al., 2019; Van Gent et al., 2021). However, such simulations are insufficient for comprehending the in vivo behavior of chronic infections, influenced by various factors like site-specific microbial diversity or environmental conditions (Liu et al., 2019; Makhlouf et al., 2023). Therefore, in vivo models are necessary to see in a more general view the pharmacodynamics, pharmacokinetics and toxicity of the NPs, as well as the immunogenic response at a systemic level (Birk et al., 2021; Fan et al., 2023; Van Gent et al., 2021).

In vivo animal models offer several advantages over human clinical trials since ethics have become stricter. They provide the opportunity to be infected with specific pathogens, including multiresistant or even modified microbial strains (Liu et al., 2019). However, it remains challenging to find a model that accurately represents human chronic infections, as certain human pathogens cannot colonize some animals (Gabrilska & Rumbaugh, 2015). Moreover, when selecting an in vivo biofilm model, the intended application of the nanomedicine should be considered, as the infection site becomes a crucial factor and it should be available in our chosen organism (Liu et al., 2019). While some burn and wound models are quite well established, other biofilm models are more challenging to obtain, like the cystic fibrosis model, where bacterial strains suffer diverse genotypical and phenotypical changes due to the interplay with the immune system that can last up to 30 years (Bjarnsholt et al., 2013; Van Gent et al., 2021).

Additionally, there is ongoing effort to refine the experimental design to accurately mimic the natural interactions between bacteria in the human body. Thus, another significant challenge of the in vitro and in vivo models is achieving a stable polymicrobial biofilm. Co-culturing different species proves difficult, often leading to the inhibition of one specie, despite their coexistence in their natural habitat. As a result, only a few biofilm models have successfully incorporated multiple species of microbes, despite the fact that most chronic infections in soft tissues are polymicrobial (Cendra et al., 2019; Cendra & Torrents, 2021; Gabrilska & Rumbaugh, 2015; Li et al., 2021). Besides, prioritizing clinical strains and bacteria with acquired antimicrobial resistance instead of acute-infectious laboratory strains should be seriously considered (Lebeaux et al., 2013; Liu et al., 2019; Makhlouf et al., 2023). Moreover, most of the in vivo experiments are conducted using young and healthy animals, whereas actual infection often occurs in elderly or immunocompromised patients and, given that biofilms are found in chronic infections, that endure more than the typical 24–48-h assay duration, experiments should be conducted in a more long-term. However, even in vivo models of chronic infections have a limited lifetime of no more than 3 weeks (Bjarnsholt et al., 2013; Liu et al., 2016; Pinto et al., 2019). Therefore, regardless of the chosen biofilm model, there is a necessity for guidelines for in vitro and in vivo NPs testing (Makabenta et al., 2021; Van Gent et al., 2021) (Figure 3).

Despite some promising in vitro results of many antimicrobial NPs and other types of nanomedicines, there have been few clinical trials conducted in comparison (Aflakian et al., 2023; Al-Wrafy et al., 2022; Birk et al., 2021; Mohanta et al., 2023; Shreffler et al., 2019). Apart from the lack of suitable in vivo models, the absence of precise regulatory frameworks tailored to nanomedicines complicates their clinical use approval. For instance, NPs surface modifications

or polymer alterations can complicate regulatory approval and serve as impediments to successful clinical translation (Zazo et al., 2016). Thus, the establishment of more nanospecific guidance is necessary to streamline the regulatory process in nanomedicine, as well as for assessing nanotoxicity and biocompatibility in clinical trials (Bjarnsholt et al., 2013; Liu et al., 2016; Pinto et al., 2019).

3.4 | Antimicrobial resistance against NPs

One of the concerns regarding the use of NPs against multi-resistant bacteria is the potential development of additional resistance mechanisms against nanomaterials, which could actually impact bacterial response to antibiotics. Nanomaterials have the capability to alter the physicochemical properties of bacteria within biofilms, leading to changes in membrane permeability, efflux pumps, and even genetic modifications that might influence antibacterial resistance, potentially heightening it against new antibiotics (Franco et al., 2022; Lee et al., 2019). Furthermore, instances of bacterial resistance to nanomaterials have been reported, which could potentially lead to the development of novel resistance mechanisms against antibiotics (Butler et al., 2023). On the other hand, it has been demonstrated that certain NPs can induce bacterial tolerance within biofilms by causing a dormant state of the cells due to a hypertonic environment (Zhang, Qiu, et al., 2022) (Figure 3).

Currently, resistance to nanomaterials is not widespread, although NPs have been detected in the soil where many antibiotic-resistant bacteria reside. Thus, there is an expectation that such resistance might develop as the use of NPs becomes more prevalent. This underscores the importance of exercising caution when introducing nanomaterials into the environment without a complete understanding of the potential consequences (Butler et al., 2023; Hajipour et al., 2021).

3.5 | Biodistribution and toxicity of the NPs

Understanding of the NPs' distribution within the body is a crucial factor to ensure the safe use of NPs against biofilms and to predict potential side effects. Biodistribution is closely tied to pharmacokinetics and clearance, which have direct implications for toxicity (Jain et al., 2008; Li & Wang, 2023).

3.5.1 | Interactions between NPs and the biofilm: Protein corona formation and controlled drug release

The transport to the biofilm's proximity is the first step for the NPs to reach the biofilm. The administration route mostly depends on the infection's location and the type of nanomedicine used, significantly affecting the biodistribution of NPs and determining their localized or systemic effects (Chenthamara et al., 2019; Yang et al., 2021). Upon entering the human body, and depending on the administration route, the NPs can interact with different biomolecules including proteins, lipids, polysaccharides, metabolites, and nucleic acids, to form what is known as the protein or biomolecular corona. This event plays a significant role for NPs biodistribution, particularly in their interactions with cells (Kumar et al., 2023). These initial molecules that form the "soft corona" are typically abundant in human biological fluids, such as lung mucus or gastrointestinal fluids (Ernst et al., 2018; Plaza-Oliver et al., 2021). Over time and once the NPs reach the biofilm location, they are replaced by higher-affinity proteins or biomolecules, creating the "hard corona" (Nazarenus et al., 2014). Thus, when NPs get to the biofilm and attach to their surface, the protein corona can also be modified by the biofilm ECM, conditioning the NPs interactions with bacteria (Fulaz et al., 2019) (Figure 3).

The last step for the biofilm treatment is to overpass the biofilm ECM barrier. The limited penetrability of particles within the ECM hinders the delivery of antimicrobial drugs. To address this, engineering the surface of NPs with active targeting can enhance their penetration into the biofilm. (Butler et al., 2015; Forier et al., 2014; Li et al., 2015). This approach employs ligands to modify the NPs surface, enhancing cellular recognition and improving target selectivity. Antimicrobial peptides, among others, are frequently used to facilitate the recognition and disaggregation of bacterial cells. However, by the time these particles reach the infection site, the protein corona has significantly altered their properties, potentially causing disparities between in vitro studies and in vivo results (Zou et al., 2023).

If biofilm penetration is successfully achieved, nanomedicines containing encapsulated drugs must deliver them. Encapsulation of antimicrobials inside NPs is a valuable strategy to modify and improve the pharmacokinetics of the encapsulated drug by controlling its release. The controlled release of the drug is a crucial issue in order to treat biofilms with drug-delivery systems. A sustained drug release to maintain the therapeutic concentrations is necessary to reduce the frequency of doses, so a high release would be necessary in the first moment to diminish the biofilm, followed by a sustained release above the MIC to avoid regrowth of surviving cells (Birk et al., 2021; Choi et al., 2023). In this sense, an equilibrium is needed to prevent both the premature delivery of the cargo and its failure to detach from the carrier. Again, the required procedure mostly depends on the nanomedicine type, for example, polymeric nanocarriers generally control better the release but they have a limited loading capacity of the drug, while it is the opposite for liposomes (Ho et al., 2019). To control the delivery of antimicrobials, some nanomedicines can use physical external stimuli such as magnetic fields, temperature changes, light sources and ultrasound, as well as internal signals like pH or enzyme activity (Yeh et al., 2020; Zaidi et al., 2017; Zazo et al., 2016).

3.5.2 | Clearance and toxicity of NPs

Clearance is the efficiency to metabolize and remove a drug from a system. In the specific scenario of nanomedicine, NPs with a swift clearance offer an advantage in reducing toxicity. However, if the clearance occurs too rapidly, it can significantly compromise therapeutic efficacy (Zhu et al., 2022). In general, the clearance systems vary depending on the NPs size. NPs smaller than 6 nm pass through the kidney filter and are eliminated through the urine with minimal toxicity, while those larger but still under 25 nm are typically captured by Kupffer cells and degraded in the liver. NPs that measure more than 150 nm are often phagocytosed in the spleen (Figure 3). Finally, larger NPs move more slowly, remaining in circulation for longer times. So, it is generally agreed that ideal NPs are between 25 and 150 nm (Li & Wang, 2023; Ngo et al., 2022).

In the NPs route to the biofilm, the protein corona formed can modulate the clearance by interacting with the immune system. For instance, it can activate the complement cascade through macrophage opsonization, resulting in rapid particle clearance from the bloodstream and accumulation within the cells of the reticuloendothelial system (RES). Such premature clearance compromises the effectiveness of nanomedicine therapy (Zaidi et al., 2017). On the other hand, the NPs surface changes can lead to agglomeration and particle accumulation, potentially leading to toxic effects (Du et al., 2018; Sousa et al., 2023).

Toxicity of NPs depends on multiple variables, such as administration route, composition, size, shape, stability, concentration, and surface chemistry of the NPs (Yang et al., 2021). Size-dependent toxicity is well documented, both in vitro and in vivo (Cho et al., 2018; Kim et al., 2012), particularly concerning AgNPs, which their associated toxicity limits their usage to topical applications (Choudhury et al., 2020; Das et al., 2020; Gajbhiye & Sakharwade, 2016; Moya-Andérico et al., 2021). In general, metallic NPs generate reactive oxygen species (ROS) as a bactericidal mechanism, but this same mechanism leads to oxidative stress and subsequent damage in host tissues (Makabenta et al., 2021; Soenen et al., 2011; Yang et al., 2021). Furthermore, metallic NPs can be activated by an energy source, converting it into an increase in heat on their surface (hyperthermia). Since high temperatures inhibit bacterial proliferation, hyperthermia has also gained popularity in the field of bacterial infection treatment. Nonetheless, careful control is necessary to avoid damage to host cells and nearby tissues (Alumutairi et al., 2020; Ibelli et al., 2018; Kim, 2016). Related to polymeric and biodegradable materials such as chitosan, alginate, poly-(lactic-co-glycolic) acid (PLGA) or polyvinyl alcohol (PVA) among others, toxicity has also been reported due to their slow degradation rate, which can lead to particle accumulation (Liu, Long, et al., 2022; Zhang, Bera, et al., 2022). Moreover, the NPs can not only attack the bacteria within the biofilm, but also disrupt the normal microbiota, leading to dysbiosis and other illnesses (Al-Wrafy et al., 2022; Karavolos & Holban, 2016; Van Den Brule et al., 2015) (Figure 3).

Additionally, NPs have also a toxic impact on the environment. A large amount of NPs can be found in the air and soil, where they interact with bacteria and other organisms. NPs found in water were reported to influence the marine ecosystem, especially by affecting microbial plankton and thus disrupting the ecological chain (Hajipour et al., 2021; Soenen et al., 2011).

Having compiled the main adverse effects of NPs, it is clear that further research is essential to minimize both immediate and long-term toxicity, and to fully harness the potential of these therapies. Prior to the clinical trials, NPs should undergo an assessment of their risk–benefit ratios. Instances of failure, such as nanomedicines with surface modifications failing to pass over Phase II clinical trials due to the protein corona formed that alters the antibiotic

release and the immunogenic response, highlight the need of rigorous evaluation. Furthermore, toxicological in vitro studies should be also optimized by employing the most appropriate cellular models (Pircalabioru & Chifiriuc, 2020).

4 | CONCLUSION

Bacterial biofilms causing chronic infections have become a significant burden on the global healthcare system, due to their inherent resistance to the antibiotics and the immune system. Therefore, there is an urgent need for new therapies to combat biofilm infections. Nanotechnology has emerged as a promising strategy for the treatment of these diseases, but certain challenges and constraints must be addressed to streamline the translation preclinical assays to the market for nanomedicines. Difficulties in synthesizing and scaling up NPs attributed to factors such as cost, stability, and storage conditions, have a negative impact on nanomedicine cost-effectiveness. Furthermore, some of the constraints are intrinsic to the field of biofilm studies, such as the requirement for reliable biofilm models in preclinical studies that accurately replicate the biofilm environment, the use of the appropriate bacterial strain, and the challenge of potential resistance development against the nanomedicine. Moreover, it is crucial to consider the biodistribution of NPs within the human body. The formation of a corona when NPs interact with body fluids and the ECM of the biofilm can alter the pharmacokinetics and pharmacodynamics of antibiofilm nanomedicine and this interaction can also introduce biases in NPs clearance from the body and impact its toxicity.

AUTHOR CONTRIBUTIONS

Núria Blanco-Cabra: Conceptualization (lead); data curation (equal); investigation (lead); methodology (lead); project administration (lead); supervision (equal); writing – original draft (lead); writing – review and editing (equal). **Júlia Alcàcer-Almansa:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Joana Admella:** Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Betsy Verónica Arévalo-Jaimes:** Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Eduard Torrents:** Conceptualization (lead); funding acquisition (lead); investigation (equal); methodology (supporting); project administration (lead); resources (lead); supervision (lead); validation (lead); visualization (lead); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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