# Nanoparticle-biofilm interactions: the role of the EPS matrix

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## Abstract

The negative consequences of biofilms are widely reported. A defining feature of biofilms is the extracellular matrix, a complex mixture of biomacromolecules, termed EPS, which contributes to reduced antimicrobial susceptibility. EPS targeting is a promising, but under-exploited, approach for biofilm control allowing disruption of the matrix and thereby increasing the susceptibility to antimicrobials. Nanoparticles can play a very important role as “carriers” of EPS matrix disruptors, and several approaches have recently been proposed. In this review, we discuss the application of nanoparticles as antibiofilm technologies with a special emphasis on the role of the EPS matrix in the physicochemical regulation of the nanoparticle-biofilm interaction. We highlight the use of nanoparticles as a platform for a new generation of anti-biofilm approaches.

## 1. An introduction to biofilms and nanoparticles

Adhesion of bacteria to surfaces and subsequent production of extracellular polymeric substance (**EPS** – see Glossary), prompts the formation of surface-related bacterial communities called **biofilms** [1]. The negative consequences of biofilms include their defining role in an extensive variety of infections [2] and their role in the **biofouling** of surfaces which has negative impacts in the process industries. The prevalence of biofilms and the pathogenesis of biofilm-related infections, for the most part, originate from the capacity of microorganisms to colonize surfaces. Bacteria are equipped to live at the solid-liquid interface by means of their capacity to use flagella, pili, exopolysaccharides and other adhesive components. Bacterial biofilms have been widely investigated in the context of medical device related infections, dental plaque, cystic fibrosis, marine surfaces, natural aquatic systems, water and wastewater processes and on soil particles [2].

The physical, chemical and biological complexity and dynamics of biofilm behaviour have hampered our full comprehension of why unwanted biofilms are poorly susceptible to **antimicrobial** agents. There are concerns that long-term **antibiotic** use may contribute to the emergence of multi-drug resistant strains [3]. The discovery and development of new classes of antibiotics has been slow [4], hence, the development of alternative approaches is becoming essential. Recently, the potential to exploit **nanoparticles** (NPs) for biofilm eradication or control has taken hold. The advantage of NPs lies in their intrinsic high surface area to volume ratio, providing a platform for the development of materials with a wide spectrum of mechanical, chemical, electrical and magnetic properties [5]. Such materials include metal/metal oxide NPs [6,7], nanocapsules [8], polymeric NPs [9], liposomes [10], nanoenzymes [11], and hydrogels [12]. The description of specific classes of NPs is beyond the scope of this review [13–15].

The development of functional NPs with the capacity to transport antimicrobial agents to the target site and release them in a controlled way is advantageous due to a potential reduction in toxicity and an increase in drug efficacy [16,17]. Furthermore, **nanocarriers** have been shown to enhance the penetration of antimicrobial agents inside the matrix [18]. However, this matrix, the so-called “dark matter” of biofilms” [19] is a complex material and while some progress has been reported in its

characterisation [20,21] a key challenge is its chemical and physical variability and heterogeneity. In the context of biofilm control, while the antimicrobial effects of NPs have been already established, the factors influencing their interaction with the biofilms is poorly understood with respect to the role of the EPS matrix. A fundamental understanding of the NP-EPS interaction would therefore improve our ability to design more effective antibiofilm strategies. This review will describe the interactions between NPs and EPS components of the **biofilm matrix**, considering physicochemical interactions, as well as the applications of nanotechnology in biofilm prevention or eradication.

## 2. Biofilms and the biofilm matrix

Biofilms are widespread, dynamic, structurally complex, integrated multi-cellular communities of surface-adhering microorganisms that are embedded within an extracellular polymeric matrix [22,23]. Biofilm formation can be seen as a mode of growth which provides protection to the cells, therefore allowing them to survive in hostile environments [24]. This formation can be influenced by cellular, surface and environmental factors. These factors include microbial species, the availability of nutrients, surface composition and roughness, cell motility, temperature, hydrodynamics and hydrophobicity [25]. Furthermore, recent advances in molecular biology and microscopy have revealed that social interactions have vital roles in mediating the responses of bacteria to their environment [26].

The production of the biofilm matrix is one of the key stages in biofilm formation (Figure 1). The matrix provides crucial architectural support and protection for the microbial communities that it surrounds and is composed of extracellular polymeric substances (EPS) [20,27]. In the majority of cases, the matrix comprises 90 % of the biofilm mass, with microorganisms accounting the remainder [20]. Although the physical and chemical composition of the EPS varies between species, it is mainly composed of polysaccharides, proteins, lipids and extracellular DNA (eDNA) [28,29] as described in Box 1. The EPS comprises a network of diverse macromolecules.[30] For a more detailed discussion on this specific topic we refer Flemming *et al*.[31] .

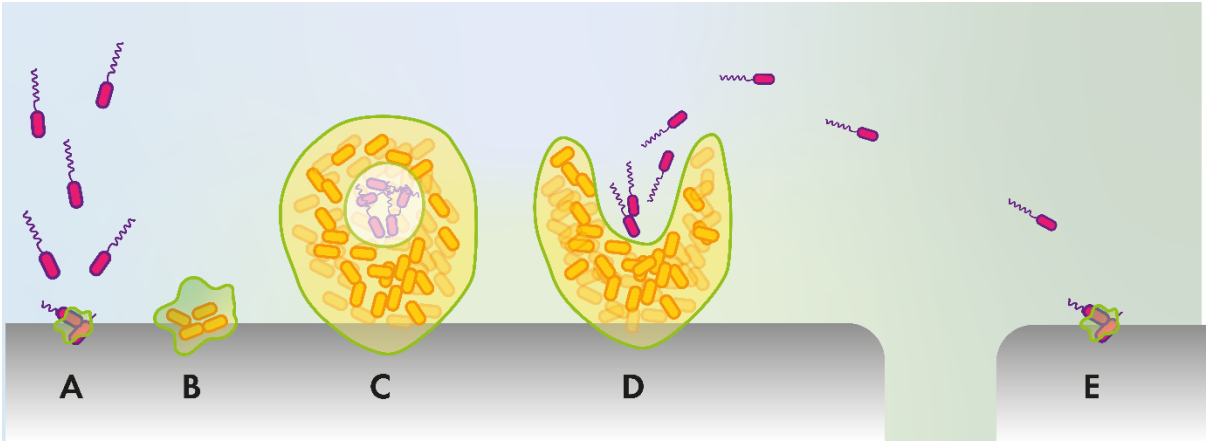


**Figure 1.** Life cycle of biofilm. A) Attachment/Adherence of the bacteria to the surface, B) Formation of monolayer and production of EPS, C) Micro-colony formation and proliferation, D) Biofilm dispersal-detachment and reversion of planktonic cells, E) Start of life cycle of biofilm again.

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**BOX 1. EPS components and their function in the biofilm**

Polysaccharides play a fundamental role in the biofilms matrix [32], some of the most common are cellulose, Psl, Pel, alginate and the staphylococcal polysaccharide intercellular adhesin (PIA) [33–36]. Proteins also play a critical role and in some cases are present at higher concentrations than polysaccharides [37]. Common proteins present in the matrix are amyloid fibers [38]. TasA, Tap A and Sip W which are all functional amyloid fibers aid in the cell-cell adhesion during biofilm formation [49], [50], [41], [52]. BslA is a cell surface associated amphiphilic protein which can form a protective barrier on the surface of *B. subtilis* biofilms therefore providing protection against environmental stressors [42]. A proteomic study of proteins present in *Pseudomonas aeruginosa* biofilm indicated that roughly 30% of the matrix proteins were external membrane proteins, normally found in outer membrane vesicles (OMVs), while some proteins were derived from secreted proteins and lysed cells [43].

Previous studies have suggested that eDNA plays an important role in the formation, structural stability and integrity of bacterial biofilms [44,45].

As previously stated the EPS have been called ‘the dark matter of biofilms’ [19] and therefore can provide a number of challenges in the development of EPS-targeting therapeutics and biofilm eradication methods due to the complexity, variability of the components of the EPS and the interactions between these components. Examples

of the EPS components and their role in biofilm formation are listed in detail in Table I.

***Table I***. Components of extracellular polymeric substances (EPS) in biofilms and their function in biofilm formation and development

| Component | Examples | Function | Reference |
|---|---|---|---|
| **Proteins** | TasA, TapB, BslA, SipW,CdrA, Lectins | • Adhesion<br>• Aggregation<br>• Cohesion<br>• Structural integrity<br>• Protective barrier<br>• Enzymatic activity<br>• Electron donor or acceptor | [20,27,28,38–42,46] |
| **Polysaccharides** | Pel, Psl, PIA, Alginate, Cellulose, | • Adhesion<br>• Aggregation<br>• Cohesion<br>• Protective barrier<br>• Structural integrity<br>• Water retention<br>• Binding of enzymes | [33–36,47,48] |
| **eDNA** | | • Adhesion<br>• Aggregation<br>• Cohesion<br>• Structural integrity<br>• Structural stability<br>• Intercellular connector | [20,27,44,45,49–52] |

## 3. Nanoparticle transport phenomena into the biofilm

Interactions between NPs and biofilms (Figure 2) can be described by three essentially sequential mechanisms [53]: transport of NPs to the biofilm-fluid interface; attachment to the biofilm surface (outer region) and migration within the biofilm.

It is important to note that biofilms are dynamic entities, with their structure and material/chemical properties continuously mediated by biochemical and physicochemical factors which are in turn influenced by the local environmental conditions [54]. This interplay adds to the complexity NP transport into biofilms. In general, the relative self-diffusion coefficients decrease exponentially with the radius

of the NP [55]. However their transport is also affected by the EPS matrix viscosity, cell density, liquid flow, external mass transfer resistance, physicochemical interactions of the NPs with EPS components and the characteristics of the water spaces (pores) within the EPS matrix [56].

It is essential to understand how NPs interact with and behave in biofilms in order to rationally develop improved functional nanomaterials. To-date, most published research in this field has focussed on the antimicrobial efficacy of drug delivery nanocarriers, whilst only few studies have examined the fundamentals of NP transport phenomena in biofilms.

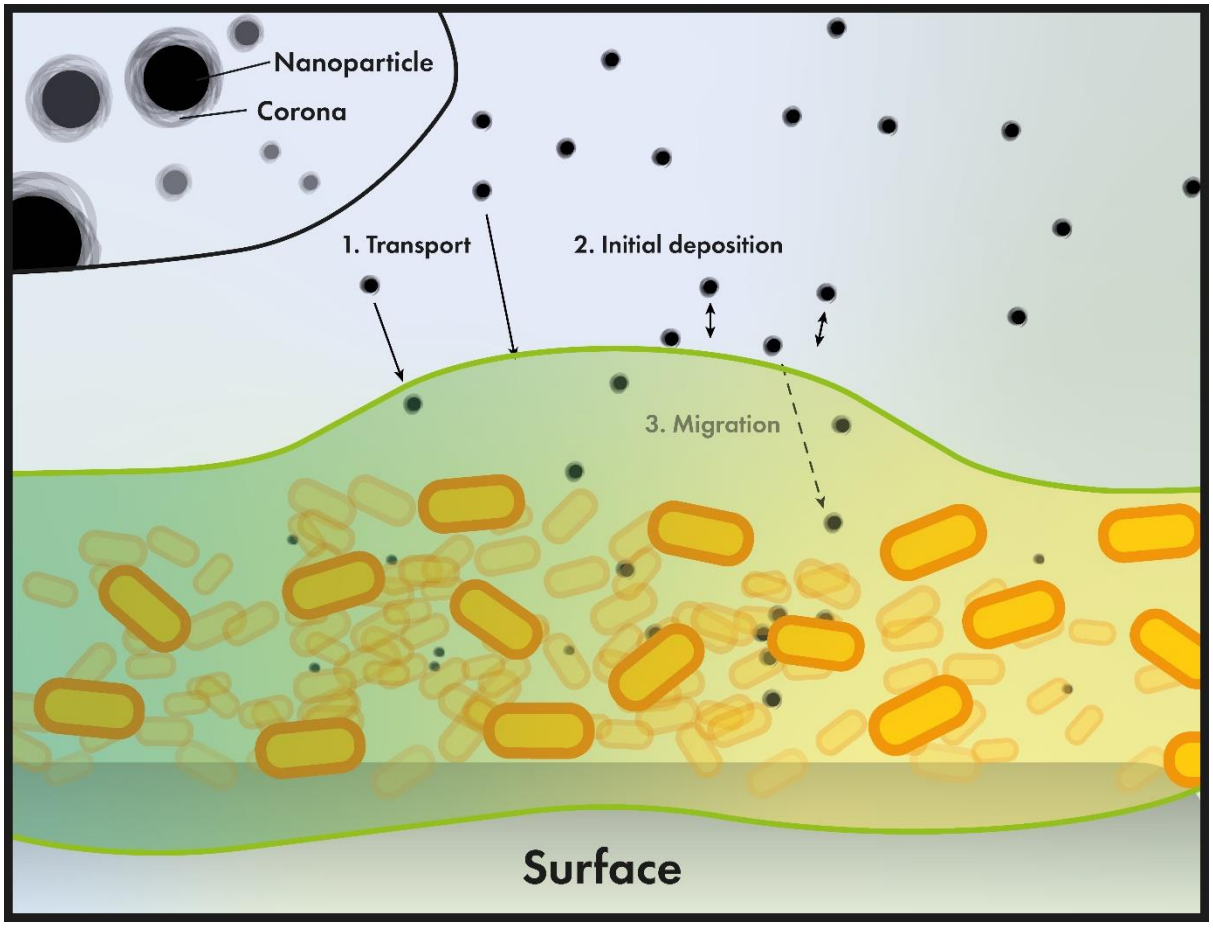


**Figure 2.** Representation of the stages (transport, initial deposition and migration) involving NPs transport phenomena within bacterial biofilms. As NPs are incorporated in the matrix, sorption (and sometimes reactive) processes result in their surface becoming covered in biofilm matrix constituents, a corona structure. This process changes NPs' properties (composition, size, charge, surface functionality). NPs interact differently with distinct biomolecules and these interactions are influenced by the environment where NP-biomolecules are inserted, such as the complex EPS matrix. A myriad of different interactions affects the transport of NPs in the matrix, due to the complex physicochemical composition of the EPS.

To eradicate unwanted bacterial biofilms, it seems rational that functional NPs should penetrate the entire matrix. The initial step of penetration in the EPS matrix is mostly controlled by the size of the NPs [55], whereas the interactions with EPS components are governed by NP's surface properties (charge and functional groups). When NPs come in contact with an environment containing organic molecules, a corona-like coating is formed on the NP surface, and the nature of this corona influences the NP-biofilm interactions [53]. In the matrix, different biomolecules, such as proteins, polysaccharides, nucleic acids, lipids and metabolites can adsorb on the NP surface [57].

## 4. Nanoparticle-biofilm interactions

The physicochemical characteristics of the NPs (size, shape, surface charge, hydrophobicity and functional groups) determine their interaction with biofilm components, both in the EPS matrix and on the bacterial surface [58–60]. NPs only remain pristine in any biological environment for very short timescales [53,61]. On first coming in contact with a biofilm, NPs inevitably interact with a complex mixture of **macromolecules** that alters their surface properties, and the different properties are ascribed to the so-called "**biomolecular corona**" [62,63], often referred to as a "protein corona" [64]. The mechanisms of protein corona formation are not yet fully elucidated, but several studies have shown that its composition and evolution are correlated to both the NP properties (size, shape, surface charge and curvature, functionalisation) and biological characteristic of the medium (concentration, topology, ratio of physiological media to NPs concentration) [64]. While the formation of the protein corona and its effect on the NPs interactions are extensively reported in the biomedical context (e.g. blood plasma), a comprehensive investigation of the NP-protein corona in biofilms is lacking [64]. In this context, it must be pointed out that also another kind of corona can be formed on NPs released in the environment, namely a Natural Organic Matter (NOM) corona [53,64,65]. The complexity of the biofilm together with the complex nature of the NP corona, further adds to the difficulty in generalising the nature of biofilm-NP interactions.

## 5. Physicochemical characteristics (or properties) influencing the NPs-biofilm interaction

The interaction between (engineered) NPs and bacterial biofilms are modulated by the physicochemical properties of both the particles and biofilm [66]. These properties and their interactions determine the degree of particle uptake, the specificity of interaction with both the biofilm matrix and bacterial cells, and in some cases, the toxicity mechanism. The exact role, extent and mechanism of each interaction is not yet fully understood because they take place in a dynamic physiological context whereby a cascade of collateral phenomena can be triggered from a single specific interaction. For instance, it has been established that the simple adsorption of NPs on the cell surface can lead to physical damage of the cellular membrane as well as production of **reactive oxygen species** (ROS); the latter can, in turn, negatively affect the cell metabolism (e.g. through inhibition of mitochondria activity, protein production, DNA synthesis, etc.) [67]. Although there is ample evidence from the scientific literature that bacteria in the "biofilm phenotype" are more resistant to antimicrobial agents than their planktonic counterpart [24,68], and that this increased **resistance** is largely caused by the EPS matrix protecting the cells, the research directed to the study of the antimicrobial action of NPs often has not taken into account the interaction between the NP and the matrix, but has instead focused almost exclusively on the NP-cell interaction [61,69]. Furthermore, the likely occurrence of (protein) corona adsorption around the NPs in the EPS matrix can modulate the interaction of the particles with the biofilm; in spite of this, the corona formation for NPs inside the biofilm matrix has not yet been discussed.

Despite the complexity of bacterial biofilms [31], some general observations can be described about the main physicochemical factors that dictate the NP-biofilm interaction. The most important interactions are electrostatic, **hydrophobic** and **steric**. These are mainly physical interactions, although also chemical and biological interactions can take place between the EPS matrix and NPs [70]. In general, **electrostatic interactions** are of paramount importance in the regulation of biofilm formation, in the first step of adhesion to surfaces and following cohesion of the EPS matrix [20,71]. Furthermore, hydrophobic interactions play a major role in the context of biofilm formation and subsequent regulation [31,72]. Steric interactions are important particularly for the colloidal stabilisation of the NPs: steric stabilisation can prevent NP aggregation, even in media with high salinity or **ionic strength**, and this is very important in determining the interaction with the biofilm EPS [70]. It is important

to note that the overall interaction is often determined by a combination of forces, and in the complex biofilm matrix the establishment of the extent of contribution of each factor is non-trivial.

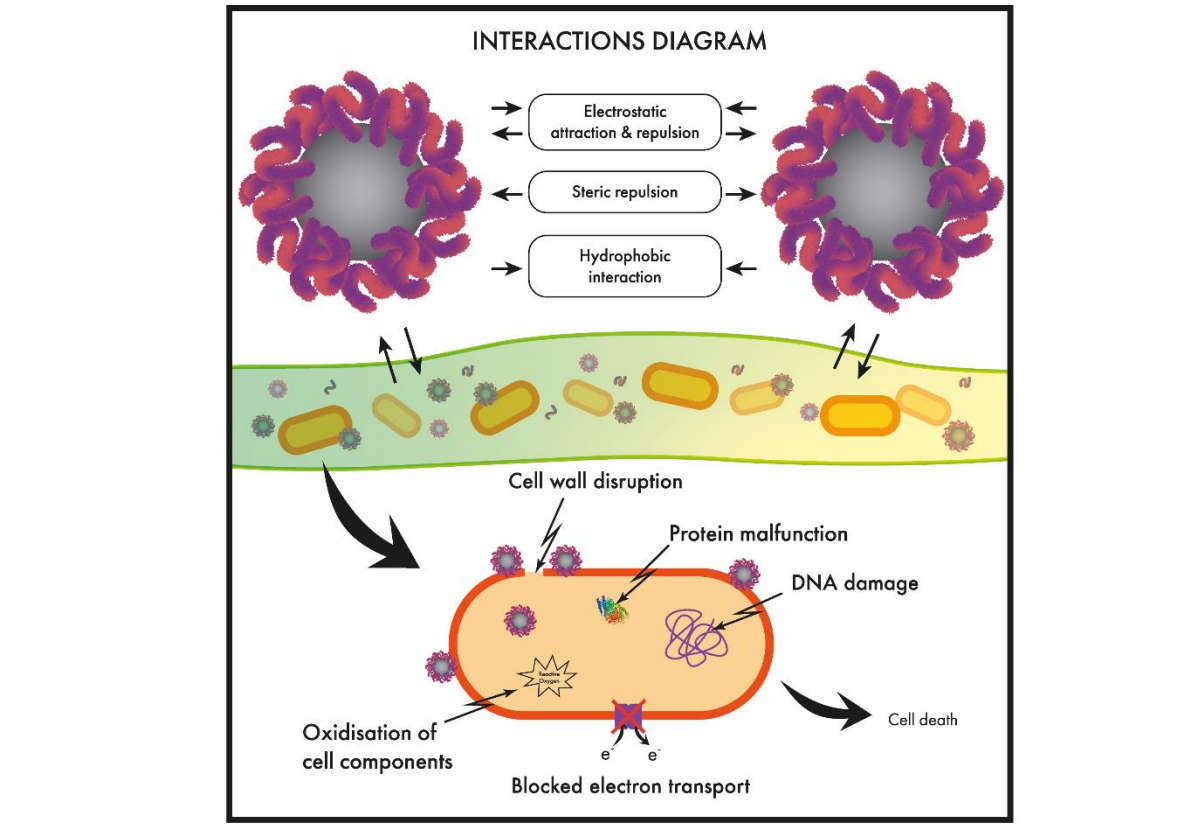


**Figure 3.** Graphical diagram describing the different physicochemical interactions between NP and bacterial biofilms.

In general positively charged NPs are more likely to interact with the EPS substances (polysaccharide skeleton, proteins, humic and uronic acids, and DNA) having, on the whole, a negative charge [73], and also with the generally negatively charged bacterial cell wall [74]. Rotello and co-workers studied the different interactions of an *E. coli* biofilm with, respectively, neutral, anionic and cationic quantum dots (QDs) (d = 7.5-24 nm)[75] reporting that, after 1 hour of incubation in 72-hour biofilms, both neutral and anionic QDs were not able to penetrate within the biofilm, whilst cationic ones were able to penetrate and diffuse within the matrix. Furthermore, they also reported a different localisation for hydrophilic cationic QDs and hydrophobic cationic ones. The hydrophilic particles were not co-localised with the bacteria, indicating interaction with only the EPS, whereas the hydrophobic ones were co-localised with the bacteria. These data suggest that a synergistic action of both surface charge and functional

groups, not only the net charge, might determine the specific interactions with the biofilm components.

With regards to hydrophobic interactions, Mitzel *et al*. [76] analysed the interaction of carboxylate- and sulphate-modified polystyrene latex NPs (d = 20 nm) with two *P. aeruginosa* engineered strains, one with hydrophilic cell walls and the other hydrophobic. It was shown that the interaction was much stronger between NPs and hydrophobic bacterial cells within the biofilm, rather than the hydrophilic ones. The effect of ionic strength on the NP retention was investigated, showing that the retention of the hydrophobic particles was not influenced by it; and it was hypothesised that this greater stability against increase in ionic strength could be due to hydrophobic EPS components acting as stabilisers.

Manipulation of the electrostatic forces occurring in the context of NPs-biofilm interactions is another interesting topic. In a very recent paper, Harper *et al.* [77] reported that the screening of the electrostatic forces within the EPS can be exploited to enhance the diffusion of nanomaterials within the biofilm. The authors used alpha tocopherol phosphate (α-TP) liposomes (d = 700 nm) as antimicrobial nanomaterial, against oral biofilm *Streptococcus oralis.* The α-TP liposomes have a negatively charged surface, and the data presented show that they are administrated to the biofilm using a phosphate buffer (negative electrolyte) as they are not able to penetrate inside the matrix; instead if Tris buffer (positive electrolyte) is used the liposomes can diffuse inside the biofilm, with no modification of their negative surface charge. This is evidence that the positive electrolytes of the buffer are able to screen the electrostatic repulsion that occurs between the negatively charged liposome and the EPS components (negatively charged on the whole). The consequent minimisation of the electrostatic repulsion allows the α-TP liposomes to diffuse in the matrix and reach the target cells.

However, steric stabilisation of the NPs with ligands such as negatively charged poly(vinylpyrrolidone) (PVP) can also contribute to the overall interaction through electrostatic phenomena. For instance, Mitzel *et al*. [78] reported a very low adsorption of PVP-capped silver NPs (AgNPs, d = 78-134 nm) onto *P. aeruginosa* biofilms. This was attributed to repulsive electrostatic forces between the negatively charged chains of PVP and the EPS polysaccharides of the biofilm. Steric interactions in the biofilms

can also be a determining factor in the availability and consequent toxicity of NPs. Adeleye *et al*. studied the stability of various copper-based NPs (d = 40-50 nm) in an aqueous environment in the presence or absence of EPS, extracted from *Isochrysis galbana* marine phytoplankton [79]. It was shown that the EPS interacts with the copper nanomaterials by sterically stabilising them, so that less aggregation occurs in comparison with the same nanomaterials in a solely buffer solution.

The above discussed NP-biofilm interactions clearly show the complex nature of the interaction itself; an additional complication is given by the fact that the nanoparticles and their properties can affect the bioentities they are in contact with, but they can also be "transformed" as a consequence of the interaction [80], undergoing chemical, physical or biological transformations that are rarely easy to predict. All these potential interactions are relevant to NP based anti-biofilm technologies.

In the following section, several examples of NP-based anti-biofilm strategies will be presented and discussed.

## 6. Nanoparticles as tools for biofilm control and eradication

Bacterial cells embedded in a biofilm are inherently more resistant to host immune responses and antibacterial chemotherapy compared to planktonic cells [24]. Proposed mechanisms for this include; reduced penetration of antimicrobials into the biofilm; inactivation of antimicrobial agents by EPS components and the altered metabolic state of bacterial cells within the biofilm [31,81]. The EPS physicochemical complexity, its variability and its component interactions make the treatment of biofilm infections a significant challenge for therapeutics [28]. New biofilm therapies, such as functional NPs, will need to focus on the whole microenvironment in order to be successful [82] and be resistant to adverse reactions [24]. An additional challenge for purely antimicrobial approaches relates to the fact that biofilm EPS components remain in place even after microbial inactivation/death. The remaining EPS matrix may facilitate subsequent colonisation by other microorganisms [28], with significant consequences, for example, the *in vivo* dispersal of biofilm bacteria in the absence of antibiotic therapy, it was reported to cause lethal septicemia in a mouse wound model [83]. Prospective biofilm dispersal agents for clinical application consequently will require careful safety evaluation and should be administrated alongside antibiotics to prevent recolonisation [28].

Two broad strategies to counteract unwanted biofilms are the prevention or minimization of initial adhesion (passive strategy) and antimicrobial approaches (active strategy) [84]. Developments in antifouling surfaces are reviewed elsewhere [85]. Another important area is the development of antimicrobial coatings for wound dressings and implants [86]. Wound dressings functionalised with antimicrobial moieties have been shown to prevent microbial colonisation [87]. The most common example is the use of AgNPs which often demonstrate an improved inhibitory activity of microbial colonisation, adhesion and biofilm growth.

Nanotechnology has opened up the possibility for the design of sophisticated drug delivery systems. The potential exits to use nanocarriers to penetrate the biofilm and, for example, can be designed to protect the active ingredient from enzymatic inactivation or binding to the biofilm matrix or other components surrounding the biofilm infection site [88]. The encapsulation of antibiotics in organic NPs can provide increased antimicrobial potency compared to free antibiotics [89,90]. The immobilisation of antimicrobial and antibiofilm agents in nanomaterials provides an alternative path to overcome payload degradation, poor delivery of water-insoluble molecules, deficient drug uptake, excessive drug efflux and resistance development [91].

Lipid and polymer NPs are of major interest due to their biocompatibility, versatility, potential as platforms for targeted/triggered release, and ability to incorporate lipophilic as well as hydrophilic drugs. Lipid NPs or liposomes can fuse with phospholipid membranes and deliver the antibiotic directly to the cells therefore maximising therapeutic benefit while reducing unwanted side effects [88]. Several liposomal formulations for the treatment of biofilm infections are under development, but to the best of our knowledge currently no such products are on the market. Arikace™ (Transave, Inc.) a liposomal formulation containing the antibiotic amikacin is in Phase-III clinical trials [92] and Fluidsomes™ containing tobramycin is on Phase-II trial both for the treatment of cystic fibrosis-associated respiratory infections [88,93].

Drug delivery NPs with targeting ligands have potential for promoting enhanced proximity between the nanocarrier and individual bacterial cell within the matrix. Non-specific targeting relies on charge-based interactions and hydrogen bonding of the nanocarrier with the biofilm; specific targeting is based on targeting ligands that

selectively bind to a target molecule inside the biofilm. For instance, PEG-PAE micelles (d = 100 nm) conjugated with Triclosan were able to penetrate biofilms and target bacterial cell surfaces [94]. Once in the low-pH environment the degradation of the ester-linkage with PAE by bacterial lipases leads to the release of Triclosan. This targeted delivery of Triclosan was shown to enhance the antimicrobial potency against MDR *Staphylococcus aureus*, *Escherichia coli* and streptococcal biofilms compared to free antimicrobial controls. Similarly, polymer NPs based on a triblock copolymer PLGA-PLH-PEG (d = 196 nm) loaded with vancomycin selectively bound to bacteria cells in acidic conditions owing to their pH-sensitive surface charge switching [95]. The nanocarrier showed pH-responsive antibacterial efficiency. Such an approach seems promising for systemically administered drug carrier's development to target and treat Gram-positive, Gram-negative, or polymicrobial infections.

Several types of inorganic NPs have been shown to exhibit antimicrobial properties. The antibacterial activity of gold [96] and AgNPs [97–100] has been extensively reported. Although several studies have been conducted on the inhibitory effect of AgNPs on bacterial biofilms, the interactions between bacterial biofilms and AgNPs is not fully understood. Extensive sloughing of the biofilm bacteria into suspension was associated with NP-bacterial interactions but with very little change in bacterial viability. AgNP aggregates were detected in the EPS matrix suggesting a mechanism for the lack of efficacy of biofilm eradication. Although the reasons for the effect of NP (d = 5 - 150 nm) on biofilm detachment were not apparent, the data suggested that exposure time played an important role with significantly less biomass detaching after exposure over 8-24 h periods compared to initial exposure [101]. Another study compared AgNP (d = 15 – 21 nm) treatment between biofilm and planktonic cells where it was shown that biofilms were about four times more resistant to AgNP inhibition than planktonic cells [102]. The effect was partly attributed to NP aggregation, due to changes in ionic strength and interactions with complexing agents from the EPS, together with retarded silver particle/ion diffusion within the biofilm matrix. AgNP (d = 5 - 106 nm) interactions with wastewater biofilms in their natural environment has been also investigated [103]. Biofilm bacteria treated as isolated pure culture were much more sensitive to AgNPs, compared with mixtures of bacteria in the biofilm. EPS was believed to provide physical protection for bacteria under AgNP

treatment. When loosely bound EPS from bacterial biofilms was removed, they were more sensitive to AgNPs.

The EPS matrix is a complex mixture, with spatial and temporal variations dependent on several factors such as microbial physiology, environmental conditions (fluid chemistry, temperature), nutrient availability and hydrodynamics. Targeting the EPS is a vital but still under-exploited approach for biofilm control allowing for the disruption of the matrix and potentially increasing the cell susceptibility to antimicrobial strategies. NPs can play a very important role as “carriers” of EPS matrix disruptors, and several approaches have been proposed [85,104,105].

A promising strategy consists of using engineered NPs as carriers for specific quorum sensing (QS) inhibitors. A significant reduction of cell-to-cell communication was observed when β-cyclodextrin-functionalised silica NPs (d = 15 and 50 nm) were added to *Vibrio Fischeri* cultures; β-cyclodextrin is a non-specific binding agent for acylhomoserine lactones (HSLs) signalling molecules [13]. Silver [106,107] and AgCl-loaded $TiO_2$ NPs (d = 6 - 7 nm) [108] have also been successfully exploited to inhibit quorum sensing in *P. aeruginosa*, *Chromobacterium violaceum*, *Serratia marcescens* and *E. coli* biofilms. Zinc NPs (ZnNPs, d = 24 nm) synthesised from *Nigella sativa* seed extract ZnNPs demonstrated broad-spectrum QS inhibition in *C. violaceum* and *P. aeruginosa*. Elastase, protease, pyocyanin, and alginate production were significantly inhibited. Sub-inhibitory concentrations of ZnNPs were able to inhibit the biofilm formation and disperse preformed mature biofilms of *C. violaceum*, *P. aeruginosa, Listeria monocytogenes* and *E. coli* [109].

Other promising approaches to disrupt the EPS include the use of enzyme-functionalised NPs or enzyme mimicry. Gold-based NP (d = 25 nm) and Silica-based nanobeads (d = 501, 638 nm) functionalised with Proteinase K were effective in disrupting the structure of *Pseudomonas fluorescens* biofilms, decreasing surface coverage and thickness [110,111]. This reusable enzyme functionalised nanobeads approach could be a cost-effective approach to disperse biofilms compared to free enzymes. A DNase-mimetic artificial enzyme composed of gold NPs with cerium (IV) complexes were immobilised onto the surface of magnetic $Fe_3O_4$ / $SiO_2$ NPs. This artificial nanoenzyme with DNase-like activity exhibited high cleavage ability towards eDNA, with improved stability and easy recovery. Substrates coated with these NPs

inhibited bacterial adhesion and biofilm formation for long periods. Additionally, by EPS degradation the NPs were efficient in the dispersion of established biofilms. Furthermore, the combined use with traditional antibiotics increased the ability to eradicate enclosed bacteria and eliminate biofilms [11].

## Concluding remarks and future perspectives

While biofilms are the prevalent form of bacteria in nature [23], their recalcitrance presents a major challenge that has generally not been adequately met by conventional antimicrobials. The self-produced biofilm matrix creates a physically and chemically complex barrier which partly shields the embedded cells from antimicrobial therapy, immune responses and environmental challenges. The spatial and temporal variability of biofilms, both in terms of chemical and microbial composition, have added a further layer of complexity that have inhibited eradication strategies.

Nanotechnology is a promising route for new antimicrobial and delivery system approaches particularly in the context of enhanced penetration and targeted delivery of antimicrobials within the biofilm. Moreover, EPS targeting strategies allow for matrix disruption, enhancing the susceptibility of the remaining biofilm to antimicrobial therapy.

Although there are some examples of antimicrobial catheters, implants, wound-dressings containing AgNPs available for clinical use, sophisticated antibiofilm strategies are still underdeveloped with studies mostly focused *in vitro* and only two products currently in clinical trials; Arikace™ (Phase-III) and Fluidosomes™ (Phase-II) [81,93].

Ultimately, the development of successful strategies to combat biofilms requires a multidisciplinary approach in order to tackle the various challenges that biofilms present (see Outstanding Questions). Engineered NPs represent a very promising tool for the accomplishment of this task; however it must be stressed that, their commercial deployment in certain settings (process plants, healthcare surfaces etc) will depend not only on their effectiveness but also their acceptance from a regulatory perspective in the context of the release of nanomaterials. The assessment of nanomaterial impact on human health and the environment is currently an open debate and the future

commercial development of NP-based biofilms technologies will depend on overcoming such regulatory hurdles.

It is important to note the need for systematic *in vivo* studies to evaluate the effectiveness of these new technologies in the biomedical context. Specificity is a key factor in clinical applications, where it is necessary to distinguish between pathogenic and commensal bacteria and host tissue. Strategies based on smart release by environmental cues, such as pH, oxygen concentration, reducing potential, improve selectivity and drug delivery. The use of aptamers, antibodies and peptides also enhance specificity [28]. However, the synthetic route and cost must be considered. Another critical factor to be investigated is how the NPs are modified in the biological milieu, such as blood and how these changes will affect their function. Furthermore, a rigorous assessment of NPs' toxicity and impact on commensals is needed. Future directions should focus on the complete biofilm eradication, by addressing simultaneously the EPS matrix and the cells, enhancing the therapeutic effect, while minimizing toxicity and resistance development.

**Glossary**

**Antibiotic:** an antimicrobial substance with the ability to kill bacteria or inhibit bacterial growth.

**Antimicrobial:** is an agent that kills microorganisms or stops their growth.

**Biofilm:** aggregate of surface-adhering microorganisms that are embedded within an extracellular polymeric matrix.

**Biofilm matrix:** self-produced hydrated matrix of extracellular polymeric substances (EPS) composed of several (bio)molecules (proteins, carbohydrates, lipids and nucleic acids).

**Biofouling:** unwanted growth of microbial communities on artificial surfaces in aquatic environments.

**Biomolecular corona:** adsorption of biomolecules onto an inorganic surface, such as nanoparticles. The biomolecules form a coating wrapping around the nanoparticle changing its characteristics (size, charge, surface)

**Electrostatic interactions:** interactions between objects with an intrinsic charge, can be repulsive (same charge) or attractive (opposite charge).

**EPS:** extracellular polymeric substances (polysaccharides, proteins, lipids and extracellular DNA) produced by microorganisms during biofilm formation. It is responsible for the integrity and structure of the biofilm and can also act as a protective barrier.

**Hydrophobic interactions:** is the interaction between water and hydrophobes (low water-soluble molecules, normally with a long carbon chain), which causes non-polar species to aggregate in water.

**Ionic strength:** it is a measure of the concentration of ions in a solution and represents the strength of the electrical field in this solution.

**Macromolecules:** large molecules consisting in monomers joined by covalent bonds, such as proteins, carbohydrates, lipids and nucleic acids.

**Nanoparticles (NPs):** particles that have at least one dimension under 100 nm.

**Nanocarriers:** a nanomaterial used to transport another substance, such as a drug.

**Reactive oxygen species:** chemically reactive chemical compounds with oxygen, metabolic by-products of aerobic respiration.

**Resistance:** the ability of the microorganism to prevent the action of an antimicrobial to act against it.

**Steric interactions:** effects resulting from repulsive forces between overlapping electron clouds of molecules.

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