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Review article

Microbial resistance to nanotechnologies: An important but understudied consideration using antimicrobial nanotechnologies in orthopaedic implants

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ABSTRACT

Microbial resistance to current antibiotics therapies is a major cause of implant failure and adverse clinical outcomes in orthopaedic surgery. Recent developments in advanced antimicrobial nanotechnologies provide numerous opportunities to effective remove resistant bacteria and prevent resistance from occurring through unique mechanisms. With tunable physicochemical properties, nanomaterials can be designed to be bactericidal, antifouling, immunomodulating, and capable of delivering antibacterial compounds to the infection region with spatiotemporal accuracy. Despite its substantial advancement, an important, but under-explored area, is potential microbial resistance to nanomaterials and how this can impact the clinical use of antimicrobial nanotechnologies. This review aims to provide a better understanding of nanomaterial-associated microbial resistance to accelerate bench-to-bedside translations of emerging nanotechnologies for effective control of implant associated infections.

1. Introduction

Bacterial infection is one of the deadliest causes of death. Amongst all, osteomyelitis is an age-associated infection with mortality risk scaling progressively with age. A retrospective analysis of 10 615 patients with the ages ≥ 65 found that patients (between 65 and 74) with chronic osteomyelitis had a death rate of ~24% and this figure is nearly double for those aged >85 [1]. Osteomyelitis is closely correlated to implant associated infections (IAIs) which develop primarily by nosocomial pathogens invasion to the implant area during orthopaedic implantation surgery [2,3]. Traditional treatment of IAIs comprises of a long-term (~3 months) administration of either intravenous, oral or mixed antibiotics [4]. Despite numerous clinical successes, over-use of and over-reliance on antibiotics have led to antibiotic resistance which significantly limits the efficacy of antibiotics and subjects patients with IAIs to complications such as severe inflammation, amputation, and even death [3,5,6]. According to a report by Willyard, resistant bacteria is estimated to cause >700 000 deaths per year globally and this figure is expected to further increase due to the sluggish development of new antibiotics or strategies to combat bacterial resistance [7].

As the size of the aging population continues to expand, the demand for orthopaedic implants is expected to increase exponentially. In the U. S., there are over 7.5 million implantation procedures of various sorts (e. g., dental, joint, fracture fixation devices, spinal) carried out annually (Fig. 1A). This figure is projected to substantially increase by 2030, as the demand for primary total hip and total knee arthroplasties in the U.S.

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has been continually increasing [8]. By then, the global market size of orthopaedic implants is estimated to reach US$ 79.5 billion, almost 2 folds of the market value in 2019 [9]. Alongside a surge in the demand for orthopaedic implants is a heightened risk of IAIs. Infection rate in general ranges from 2 to 5% for primary implantation surgery and this can double after revision procedures [10,11]. Yet, the infection rate for different types of prostheses may vary significantly (Fig. 1B). IAIs is not only a health issue but also a huge financial problem to low-income families. The estimated cost borne by hospitalization post revision surgery can be as high as US$24 -73k in Europe and approximately US$ 100k in the US [12–14]. This number can be considerably greater when taking into account the after-hospital medications and care for patients with IAIs [15]. Details of the healthcare costs for various orthopaedic implants within the US are summarized in Fig. 1C. As IAIs carries many adverse socio-economic consequences on healthcare expenditure, work productivity, and life longevity [16,17], increasing efforts have been spent in the last three decades to construct effective infection control measures (Fig. 1D). There is thus an urgent need for a practical anti-IAI therapy.

IAIs primarily results from perioperative bacterial inoculation onto the implant surface, although in rare cases it can be caused by hematogenous seedings of pathogens [18]. The main causative bacteria in IAIs is *Staphylococcus (S.) aureus*, with others including *S. epidermidis*, coagulase negative *staphylococci* (CNS), *enterococci*, and *Pseudomonas (P.) aeruginosa* [19–21]. These pathogens are proficient at adapting to everchanging environmental conditions such as light [22], pH [23], temperature [22,23], nutrient status [22,24], and exposure to foreign substances (e.g., antibiotics [25], nanomaterials [26], host tissue [27], prosthetics [28], etc.). They can alter genotype as well as phenotype to re-establish homeostasis in response to environmental challenges, laying a solid foundation for antimicrobial resistance acquisition [29,30]. Among these bacteria, *S. aureus* warrants the greatest concern due to its inherent virulence and capacity to adapt to harsh environmental conditions [31]. The conversion of wild-type *S. aureus* to methicillin-resistant *S. aureus* (MRSA) is not a rare phenomenon occurring in patients with prolonged use (~11 weeks) of antibiotics, and as a result ~ 8.5% of such patients had experienced treatment failure post prosthetic bone/joint surgery [32]. Mechanistic investigations have

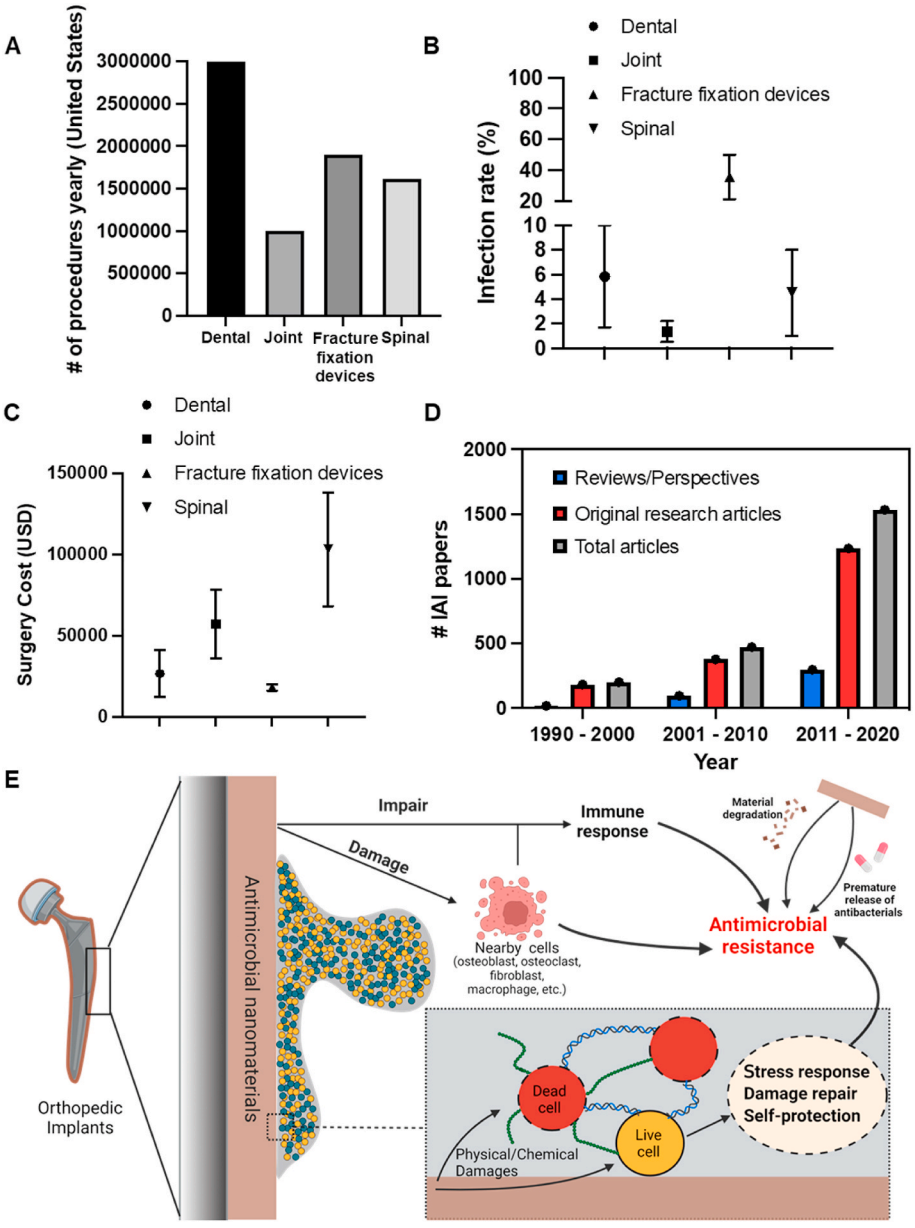


Fig. 1. Statistics of IAI and their socio-economic costs. **(A)** Estimated number of implant procedures per year in the United States: dental implant = 3 million [70], joint implant = 1 million [71], fracture fixation devices = 1.9 million [72], spinal implant = 1.62 million [73]. **(B)** Estimated IAI rates of various implant procedures: dental implant = 1.7–10% [74,75], joint implant = 0.5–2.2% [76], fracture fixation devices = 21–50% [77–79], spinal implants = 1–8% [80]. **(C)** Estimated surgery costs for various implants (USD): dental implant = 12–41k [81], joint implant = 36k – 78k [82], fracture fixation devices = 16k – 20k [83], spinal implant = 68k – 138k [84,85]. **(D)** Number of publications relevant to IAIs from 1990 to 2020. Research interest in IAI has been increasing exponentially every decade since 1990, signifying increasing interest in the field. Original research articles include full papers and communications while reviews/perspectives include perspectives, book chapters, and reviews. Results are obtained from ScienceDirect database with advanced search query "implant associated infection" sorted by year. **(E)** A general summary of the potential pathways in which current antimicrobial nanotechnology used in orthopaedic implants can lead to antimicrobial resistance. Nanomaterials with different compositions, antibacterial mechanisms, and synthesis pathways may predispose the patients to a higher risk of antimicrobial resistance by a series of materials associated detriments, such as impaired immune function, toxicity to the nearby healthy cells, and bacterial adaptation through mechanisms mainly involving stress response, damage repair, and protection. In addition, premature release of antibacterial compounds and early degradation of nanomaterials also contribute to microbial resistance. Created with Biorender.com.

found a strong correlation of staphylococcal resistance to a synergistic action of pathogenic stress response, damage repair, and self-protection [33–36]. Another pathway to acquire antimicrobial resistance is by forming a sessile bacterial community [37]. Biofilm development is the result of the competition between bacteria and other molecules/living cells to colonize the implant surface [38]. Once formed, living bacteria will be encapsulated by the external protective layer which activates a series of protection mechanisms and hampers the utility of aseptic treatments [39].

There have been substantial interests in the use of advanced nanotechnologies to combat antimicrobial resistance. With tunable physicochemical properties, such as size, morphology, topography, and surface chemistry, nanomaterials are able to (i) elicit innate bacteria-killing effects, (ii) allow for a precise control of antimicrobials release, (iii) endow implants with fouling-proof property, and (iv) modulate host immune responses [40,41]. Nanomaterials can be bactericidal through physical or chemical pathways. Drawing inspiration from special topographies studied in nature, various biomimetic materials have been developed. Mimicking cicada and dragonfly wings, nanopillars, nanowires and nanoblades have been designed to exert intrinsic bacterial killing effects [42,43]. Alternatively, desirable bactericidal performance can result from increased surface charge potential of a nanomaterial [44]. Instead of physical damage, some nanomaterials are known to be antibacterial by its capability to induce reactive oxygen/nitrogen species (ROS/RNS) intracellularly. Stress-evoking nanoparticles, such as iron oxide [45], zinc oxide [46], silver [47], gold [48], silicon dioxide (SiO_2) [49], and hydroxyapatite (HA) nanoparticles [50] have been extensively researched for antibacterial applications. On the other hand, the biocompatibility and physiochemical stability of nanomaterials have made them compatible for delivery of natural and synthetic antibacterial compounds. With assistance of nanomaterials, antibiotics can be concentrated and protected from degradation [51]. The first example using nanomaterials (i.e., nanoliposome) for antibiotics delivery was reported by Onyeji et al., in 1994 [52]. Since then, successful attempts have further extended the materials depository to metal nanoparticles [53], HA nanoparticles [54], nano polymers (e.g., chitosan [55], PLGA (polylactic-co-glycolic acid) [56], PCL (polycaprolactone) [57]), and many other advanced formulations [58]. The nano delivery systems can be further modified to impart bacteria-targeting and controlled release properties with special ligands or functional molecules responsive to external stimuli, such as light, magnetic field, temperature, pH, and ultrasonic power [59,60]. Apart from materials with active bacteria-killing effects, antibacterial properties can be achieved with nanomaterials that prevent planktonic bacterial adhesion onto the implants or stimulate the host immune responses with suitable immune cues [41,61]. These two approaches present a distinctive strategy against antimicrobial resistance that can function as a stand-alone or with active bacteria-killing machinery to improve therapeutic efficacy.

While many reviews have exhaustively summarized the advantages of nanomaterials for infection control and their potential to prevent antimicrobial resistance [40,62–65], the increased investment and lacklustre clinical translation of antimicrobial nanotechnologies suggest a need to further examine unwanted side effects for a safer and better nanomaterial design. In the context of IAIs, one of the safety concerns for nanomaterials is acute nanotoxicity to nearby cells (e.g., osteoblast, osteoclast, macrophage) [66–68], which has gained increasing recognition in recent antimicrobial research (Fig. 1E). In addition, existing antimicrobial nanotechnology systems often leave behind surviving pathogens, yet the effects of nanomaterials on these remnant pathogens remain largely unexplored. Given that bacteria can constantly adapt to a changing environment by reprograming their genetic footprints and metabolic activities [22,23,36,39], it is likely that the survivor cells would slowly acquire antimicrobial resistance through long-term exposure to the nano antimicrobials, a flipside that is often cloaked by their apparent bactericidal effects. The design principles, fabrication techniques and manufacturing defects of nanomaterials may produce detrimental off-targets to nearby tissues and the immune system while promoting chronic and persistent infections. Adding to all these is bone integration with implants, an important factor that may lead to microbial resistance if left undealt with when devising new antibacterial formulation. Typically, the use of nanomaterial-based bone graft (e.g., HA bioceramics and nanobioglasses) can significantly increase osteogenesis, but satisfactory synergistic antibacterial effects and replacement of autologous bone graft, the gold standard-of-care, have not been achieved yet [69]. Like bone transplants, achievement of desirable *in vivo* osteoinductive and antimicrobial effects by nanoengineered antibacterial materials is another hindrance for further clinical applications.

To tackle antimicrobial resistance with advanced nanomaterials, a comprehensive consideration on their designs and mechanisms for constructing a "implant of the future" is urgently needed. Here, following a review of the current mechanistic understanding of the development of resistant strains and biofilms, we present a unique analysis of existing antimicrobial nanotechnologies focusing on their potential risks in antimicrobial resistance. Current antibacterial nanotechnologies depending on the design principles are categorized into biochemical functionalization or physical surface changes. We also provide considerations to guide future explorations of antimicrobial nanotechnology.

2. Development of antimicrobial resistance: mechanisms

S. aureus is the most common causative agent of IAI. The prevalence of *S. aureus* infections can be partially explained by its commensal lifestyle that allows it to exist in human body asymptomatically as part of the normal microbiota, and its capacity to secrete virulent factors when it becomes pathogenic, making it difficult for the host immune system to cope with [86,87]. The overuse of antibiotics has made *S. aureus* infections increasingly difficult to treat due to emergence of resistant phenotypes [88]. In extreme cases where antibiotics cannot achieve an optimal result, surgical intervention is required to treat infected implant site [89,90]. In general, antimicrobial resistance can be categorized into three groups: (i) development of genetically mutated variants, (ii) formation of biofilms, and (iii) intracellular pathogens. In the following sections, we will summarize the mechanisms attributable to antimicrobial resistance, with a specific focus on *S. aureus*.

2.1. Genetically mutated resistant strains

From a historical perspective, there are two main types of *S. aureus* mutations. Since the introduction of penicillin by Alexander Fleming in the 1940s, nosocomial infections by *S. aureus* were once effectively controlled. However, resistant variants developed rapidly in this staphylococcal species and was reported shortly after discovery of the antibiotic [91]. At the molecular level, studies have shown that staphylococcal resistance to penicillin is mediated by β-lactamase encoded gene termed *blaZ* (Fig. 2A) [31,92]. Prolonged exposure to penicillin activates self-cleavage of *BlaR1* and *BlaI*, two upstream markers of *blaZ*, which in turn switches on the functional synthesis of β-lactamase by *blaZ*, resulting in a rapid enzymatic clearance of penicillin. As suppression of the β-lactam rings mediated bacteria cell wall synthesis underlies the fundamental design of penicillin and many other β-lactam antibiotics, the overexpression of β-lactamase mediated gene (i.e., *blaZ*) in penicillin-resistant *S. aureus* has rendered most of the β-lactam antibiotics ineffective.

Methicillin, a semi-synthetic penicillinase-resistant antibiotic was introduced to deal with penicillin-resistant *S. aureus*. Although resistance towards methicillin developed at a slower pace, the mutation of penicillin-binding proteins (PBPs) has led to a new *S. aureus,* i.e., MRSA, which resists to almost the entire class of antibiotics [31]. It is now known that such mutation is governed by *mecA* gene (the gene responsible for methicillin resistance). *MecA* belongs to a genomic island called staphylococcal cassette chromosome (SCC) and is responsible for

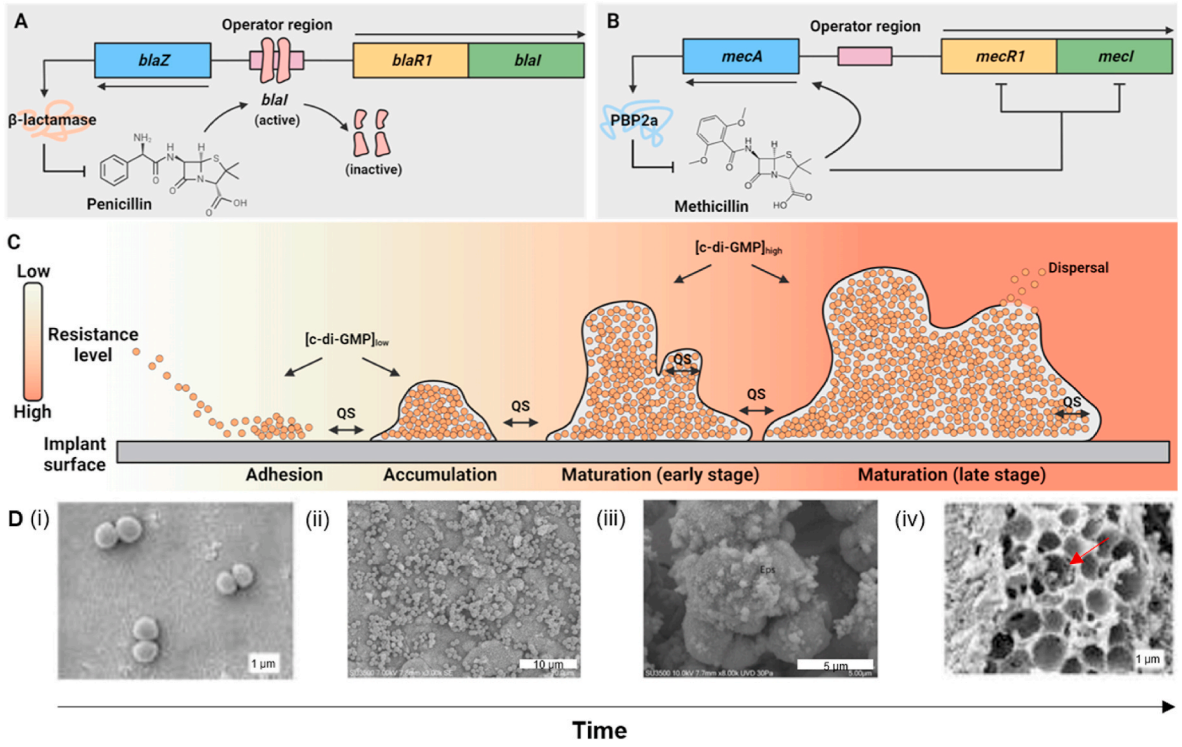


Fig. 2. The developmental process of antimicrobial resistance and key mechanisms. **(A)** The key genetic changes leading to resistance to penicillin. Overexposure to penicillin can inactivate the functionality of *blaR1* and *blaI*, which in turn increases the production of β-lactamase through overexpression of *blaZ*. **(B)** The key genetic changes leading to resistance to methicillin. Like penicillin, the extended use of methicillin can result in mutation of *mecA*, which produces a protein called PBP2a that is known to effectively inhibit almost the entire class of antibiotics. **(C)** Biofilm formation on the implant surface. Colour gradient (green to red) is correlated to the level of antimicrobial resistance. As the development of biofilm progresses, bacteria become more resistant to not only antibiotics, but also the host immune responses. Activation of quorum sensing (QS) and c-di-GMP is important for successful biofilm formation. At the late stage, some bacteria can be released from the mature biofilm and dispersed to other part of the host body, causing disease transmission and intra-host spread of the bacterial infection. Created with Biorender.com **(D)** Representative SEM images that correspond to the major stages of biofilm formation. Initial attachment **(i)** is followed by rapid proliferation and aggregation **(ii)**. Secretion of EPS and formation of the biofilm occurs **(iii)**. Dispersal of the bacteria inferred from the empty cavities observed, indicated by the red arrow **(iv)**. Reproduced with permission from MDPI and John Wiley and Sons [99,100].[99,100]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

synthesis of a PBPs mutant, namely PBP2a [93]. Unlike other homologues, PBP2a is exceptionally inhibitory to all β-lactam antibiotics including those designed to cope with β-lactamase antagonization. At the transcript level, it is believed that activation of *mecA* is controlled by *mecI* and *mecR1*, reminiscent of the regulation of *blaZ* by *blaR1* and *blaI*. Upon exposure to β-lactam antibiotics, *mecRI* will inactivate *mecI* and meanwhile switch on *mecA* for PBP2a production (Fig. 2B) [31].

MRSA was first identified in 1961 and antimicrobial resistance has since progressed substantially [94]. It should be noted that the term "MRSA" has been extended to describe many recently found resistant staphylococcal strains (e.g., oxacillin-resistant *S. aureus*) [95]. Vancomycin is the first-line and virtually the only effective antibiotic therapy for severe MRSA infections [96]. Unfortunately, the heavy reliance on vancomycin to counter MRSA has gradually led to resistance to this very last defender [97]. In spite of the fact that vancomycin-resistant *S. aureus* (VRSA) has only been reported sporadically and the relevant mechanistic actions remain poorly understood [98], the gradual increment of minimum inhibitory concentrations (MICs) frequently seen in MRSA after prolonged vancomycin treatment is a clear sign of the incoming third-phase mutation [94]. Taking into account the stagnant movement of new antibiotics discovery coupled with the rapid progression of bacterial evolution, a clinically-relevant strategy against antimicrobial resistance is in urgent need.

2.2. Biofilm

In addition to genetic mutants, *S. aureus* is able to reside on the surface of implants and form a sessile bacterial community called biofilm. Biofilm is composed of aggregated bacteria encased by a self-secreted protective matrix, namely extracellular polymeric substance (EPS) [101]. EPS can provide a robust barrier to prevent penetration of exogenous toxicants and stimulate a series of intra-/inter-cellular cyto-protective actions [102,103]. Biofilm formation is generally divided into three distinct stages: adhesion, accumulation, and maturation (Fig. 2C) [18]. The establishment process is rather swift (few hours) and usually accompanied by a chain of functional biological activities, such as cell-cell communication, metabolic changes, gene exchange, and spatial rearrangement [104–106].

Upon insertion of an orthopaedic implant into a patient body, pathogens in close proximity - residing on or within the host - start to converge towards the implant site. There is a competition between native cells such as osteoblasts, and pathogens for interaction with the implant surface which is commonly called "race for the surface" [107]. Rapid integration between the orthopaedic implant and the host is crucial for preventing bacteria surface colonization. When bacteria adhesion occur before integration, host defence would have difficulty preventing colonization [108]. The insertion of a foreign body and tissue damage from implantation activates an inflammatory response involving the coagulation cascade and innate immunity [109]. Neutrophils are amongst the first immune cell type that are activated to clear cell debris and pathogens. However, there are implant specific events that can lead to additional activation of the neutrophils. The surface morphology of the implant has been shown to affect production of reactive oxygen species [110]. Furthermore, neutrophils express β2

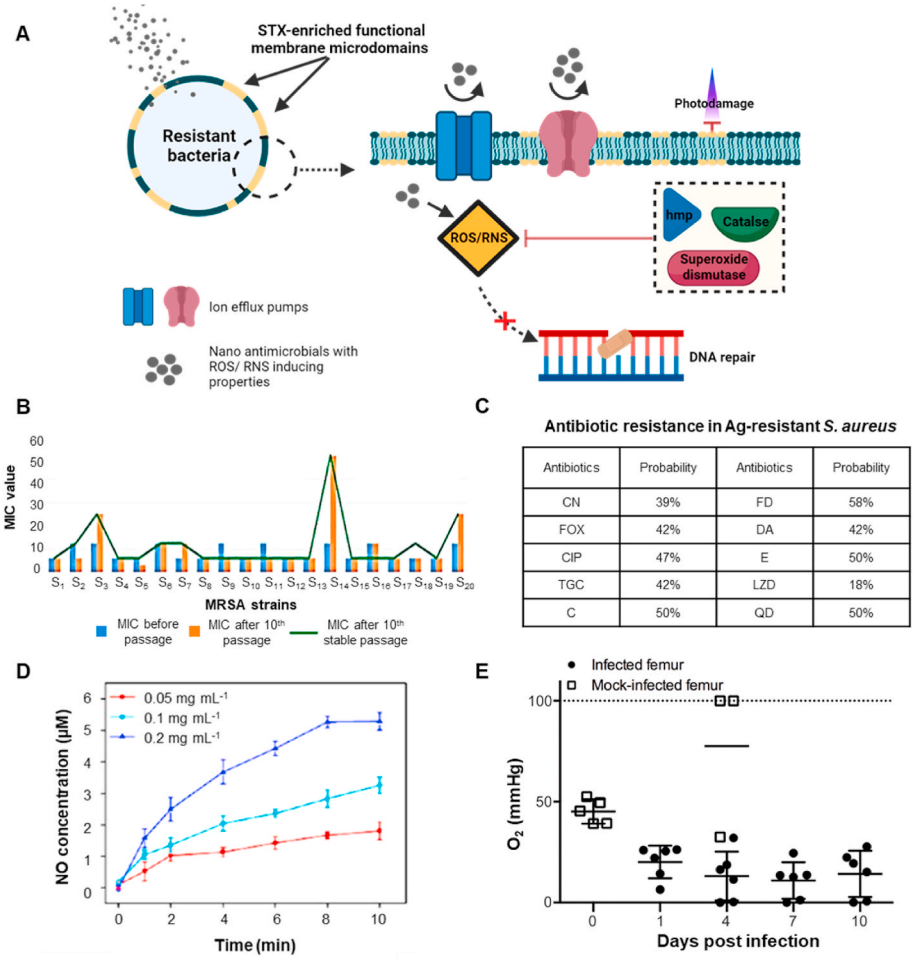


Fig. 3. Antimicrobial nanotechnologies with oxidative stress- and heat-evoking properties can prime the patients receiving orthopaedic implants for antimicrobial resistance **(A)** A schematic depicts how resistant bacteria (e.g., MRSA). resist to oxidative stress induced damages. **(B)** MIC of 10 nm Ag nanoparticles against 20 strains of *S. aureus* before passage, after 10th passage conditioned by a sub-lethal dose of the particles, and after 10th stable passage in nanoparticle-free condition following the 10 passages of conditioning experiments. Reproduced with permission from John Wiley and Sons [190]. **(C)** A computed table shows the probability of occurrence of multidrug resistance in Ag-resistant *S. aureus* [191]. n = 38. Chronic exposure to silver can allow *S. aureus* to cross-adapt to a broad range of antibiotics. The probability of resistance to a certain antibiotic was calculated by the number of resistance events over the total number of strains. Abbreviations: CN, gentamicin; FOX, cefoxitin; CIP, ciprofloxacin; TGC, tigecycline; C, chloramphenicol; FD, fusidic acid; DA, clindamycin; E, erythromycin; LZD, linezolid; QD, quinupristin/dalfopristin. Reproduced with permission from Dove Medical Press [191]. **(D)** A NO release profile as a function of time. The burst release of NO and lack of sustainability can limit its long-term therapeutic efficacy against bacterial infections Reproduced with permission from American Chemical Society [195]. **(E)** Oxygen level in murine femur tissues infected by *S. aureus* as a function of time. *S. aureus* osteomyelitis triggers reduced oxygen availability in infected murine femurs, which can limit the therapeutic efficacy of oxygen-dependent PTT. Reproduced with permission from PLoS [211].

integrins that are able to bind additional sites provided by the host serum proteins such as fibrinogen and complement factors that are coated around the implant for neutrophil activation [111]. A prolonged activity can result in metabolic exhaustion and depletion of oxidative resources of neutrophils which is detrimental to their ability to kill bacteria [112]. This provides bacteria an opportunity to evade the immune system and attach themselves onto the implant surface.

Bacteria adhesion can be further broken down into two phases. The initial attraction of the bacteria to the implant surface involves non-specific interaction forces through a combination of van der Waals, gravitational forces, surface electrostatic charges and hydrophobic interactions [105,113]. The subsequent phase happens through specific binding of the bacteria surface to the host extracellular matrix (ECM) proteins coating the implant. The ECM proteins layer that forms around the implant surface consists of collagen, albumin, fibronectin, fibrinogen and laminin [108]. Although this layer helps with host tissue integration, it is ironically also conducive to bacteria attachment [114]. Bacterial and cell adhere to implants via different pathways. Unlike bone cells such as osteoblast which employ a variety of integrins to bind to these ECM proteins [115], *S. aureus* adheres to implant substrates via the cell wall-anchored microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) as well as the secretable expanded repertoire adhesive molecules (SERAMs) [116]. With the assistance of these anchoring proteins, staphylococcal pathogens can bind to some of these ECM proteins via specific ligand-receptor interactions. This binding process then becomes irreversible when the attractive forces between the bacteria and the proteins are greater than their repulsive electrostatic forces [117,118]. Once bacteria have successfully evaded the immune system and attaches itself, biofilm formation occurs, making it even harder for the immune system to detect and eradicate bacteria.

Quorum sensing (QS) is a pivotal process for biofilm formation following the aggregation of pathogens onto the implant. In general, QS regulates gene expression in response to change in cell density by a library of autoinducers [119]. The signalling pathway by which QS in gram -positive and -negative bacteria is regulated appears to be different. For gram-positive bacteria like *S. aureus*, they use two-component adaptive response proteins for detection of autoinducers via a phosphorylation/dephosphorylation cascade [120]. The autoinducers are some self-secreted peptides through a dedicated ATP-binding cassette (ABC) transporter. Gram-negative bacteria, in contrast, respond to increased cell density via LuxR-type mediated signalling pathway [120]. They used a different set of autoinducers called homoserine lactone signalling molecule (HSL) to regulate the downstream genetic modifications in response to the changing cell density. Recent studies have shown that cell-cell communication driven by QS has an inextricable connection to antimicrobial resistance [121]. Many biological events such as virulence factor production, stress adaptation, and metabolism happens through this mechanism [119]. Another key player for biofilm formation is cyclic diguanosine monophosphate (c-di-GMP). c-di-GMP acts as a biological switch of pathogenic life pattern, that is, to “swim” or to “stick”, by modulating expression of a plethora of adhesins and activity of flagella [122,123]. In addition, it also assists with the production of biofilm associated exopolysaccharides. Downstream of these key players are genes belonging to the family of outer membrane porin (*omp*). It has been revealed that *ompC*, *ompF*, and *ompT* play an important role in biofilm formation via regulation of the pathogenic structural proteins [124]. Nonetheless, QS is a double-edged sword as hyperactive QS can otherwise lead to

disassembly of surface-bound biofilm and trigger interconversion of resistant bacteria to drug-susceptible wild type [125]. When EPS is fully shaped, biofilm enters its maturation phase where the bacterial residents in the biofilm becomes extremely tolerant to antimicrobials [126]. Even if antibiotic treatment did eradicate the bacteria population, within the biofilm exists a small subpopulation of cells that are completely tolerant to antibiotics which can be referred to as persister cells. This subpopulation of cells remains in a dormant state and as the antibiotic level drops, they resuscitate and start to repopulate the biofilm causing relapse [127]. This can cause chronic inflammation around the implants. As bacteria continues to proliferate and biofilm matures, resources eventually become limited, and waste accumulates. These unfavourable environmental or stress cues can trigger bacteria dispersal, allowing the bacteria to disseminate to get nutrients, or be tolerant to cues, both furthering the infection in the body which can further exacerbate the orthopaedic infections [128].

2.3. Intracellular pathogens

Another potential source of microbial resistance is pathogens which reside within the host cells. Several intracellular bacteria have been identified, such as *Mycobacterium* (*M.*) *tuberculosis*, *Salmonella* (*S.*) *enterica*, *Chlamydia trachomatis*, *Listeria monocytogenes and S. aureus.* [129] Host cells provide a protective niche for these intracellular pathogens, making them harder to reach by antimicrobials relative to their extracellular counterparts [130]. In the case of *S. aureus*, an insidious variety of this strain called small-colony variants (SCVs) has been implicated to give rise to recrudescent infections in osteomyelitis and implant associated infections [131]. This subtype differ from their parental strains with less membranous pigments, reduced growth rate, and downregulated production of exotoxins [131]. They arise spontaneously during the course of antibiotic therapy and can enter host cells via a pathway mediated by fibronectin and host-cell integrins [131]. Post-internalization genetic modifications may be an important route by which intracellular persistence occur. A microarray analysis on human lung epithelial A549 cells infected by *S. aureus* shed light on the global transcriptional changes after bacterial internalization [132]. Specifically, considerable increase of virulence and detoxification genes was observed while genes associated with metabolic activities, nutrient transport, and cell wall synthesis were terminated. Reduced levels of α-toxin production in the pathogen were also identified. These findings provide the earliest evidence to the potential risks of resistant infections caused by intracellular bacteria, yet preclinical and clinical proof is largely lacking and further exploration is necessary to fully uncover how intracellular bacteria influence orthopedic infections. It should be noted that the abovementioned resistance mechanisms are specific to *S. aureus* and may partially apply to other gram-positive bacteria. Due to presence of lipopolysaccharides (LPS)- based outer membrane, the major pathways in which gram-negative strains resist to antimicrobials are nevertheless drastically different. Readers are recommended to refer to other available documentations for more information on their drug resistance mechanism [133,134].

3. Microbial resistance to nanotechnologies

Current concepts of developing antimicrobial nanotechnologies for orthopaedic implants are based on the unique physicochemical properties of nanomaterials, such as size, shape, surface chemistries, and topography, which enable them to be highly biocompatible with low foreign body reactions and capable of preventing bacterial infections even in the presence of antibiotic resistance. In general, the antimicrobial properties of orthopaedic implants can be achieved by biochemical surface functionalization or physical surface changes. However, as bacteria can adapt and evolve, they can gain resistance against antimicrobial nanotechnologies, especially when there is incomplete removal of bacteria or material associated damage to peripheral tissues at implant sites. In this regard, the risks of resistant bacteria infections posed by the applied nanomaterials should be avoided. In the following section, we will evaluate the existing antimicrobial nanotechnologies with respect to their potential pathways leading to antimicrobial resistance. We also refer readers to more comprehensive reviews that focus more on the antimicrobial mechanisms and toxicological aspects of various nanotechnologies [135–137].

3.1. Biological and chemical functionalization

3.1.1. Antimicrobials delivery nano systems

Traditional antibiotic treatment via intravenous injection or oral administration often results in premature drug degradation and off-target absorption by non-pathogenic microorganisms [138,139]. The lower-than-expected dosage of antibiotics in the infected area is considered a major cause of antibiotics resistance [140,141]. To circumvent this problem, nanomaterials with a fine-tuned porous or core-shell structure are employed to encapsulate and deliver antibiotics. In contrast to the conventional strategies, large payload of drugs can be concentrated into the nanocarriers and subsequently released to the infection sites for bacteria removal. Successful delivery of vancomycin, gentamicin, and ampicillin and many other water-soluble antibiotics by mesoporous silica nanoparticle (MSN) has been reported [142]. Alternatively, hydrophilic antibiotics can be encapsulated by liposomes, a natural mimic of bacterial phospholipid bilayers, which can fuse with bacterial cell wall and allow deep penetration of antibacterial drugs to remove biofilm-mode pathogens [143]. When it comes to encapsulation of poorly water-soluble drugs, polymeric micellar structures are more suitable as they possess a hydrophobic core as drug reservoir. Carvacrol-laden poly(oxanorborneneimide)-based micellar nanocarriers, X–NCs named by the author, was reported to eradicate >80% of *S. aureus* and achieve a nearly three-fold log reduction (99.5%) in its biofilm with no discernible toxicity to the co-cultured fibroblast cells [144]. To further enhance the bacteria inhibitory efficiency, positively-charged poly(ethylene glycol)-block-poly(ε-caprolactone) (PEG-b-PCL) was devised to increase electrostatic penetration of antibiotics into acidic biofilms without being prematurely washed out. Effective killing of resistant bacteria and biofilms can also be achieved with other nano encapsulation platforms, such as carbon nanotubes, metal nanoparticles, HA nanoparticles, polysaccharides nanogels, etc., which comes with their own strengths and limitations.

Although nanomaterials offer a more efficacious mode of infection control due to a more localized mode of antibiotics administration, bacteria can continue to exploit technological gaps to acquire antimicrobial resistance. Given the structural similarity in the plasma membrane of bacteria and human cells, the drug-laden nanocarriers can be recognized and internalized by both pathogens and cells that are in proximity to the infection hotspots [145–147]. This in part contributes to a high degree of clinical irrelevance between *in vivo* preclinical and clinical data and those obtained by *in vitro* experiments, as few *in vitro* antibacterial efficiency assessments were carried out in the co-presence of bacteria and neighbouring host cells. It can be foreseen that the direct use of *in vitro* optimal dosage will lead to an unsatisfactory result *in vivo,* and the surviving bacteria cells are likely to rapidly develop resistance during extended use of nano delivery systems.

To circumvent the off-target effects, recent studies have focused on improving the bacteria recognition capacity of nanocarriers by surface conjugation of bacteria-targeting molecules. Marina et al. reported a levofloxacin-loaded MSN that was able to selectively remove Gram-negative bacteria via surface decoration of lectin concanavalin A (Con A), a glycoprotein that can be recognized by a wide range of microbes but did not exhibit cytotoxicity to mammalian cells [148]. Similarly, gram-positive *S. aureus* can be targeted via a cyclic 9-amino-acid peptide CARGGLKSC (CARG) and anti *S. aureus* was manifested by a CARG-loaded porous silicon nanoparticles [149]. In spite of the increased specificity towards bacteria, the capacity of the nanomaterials

to retain antibiotics till the point of release is still in question. Thus far, a burst release of the loaded antibiotics (~10–60%, <5 h) is often observed in reported nano delivery systems [148–151], and the existence of bacteria-targeting molecules on the nanoparticles can further accelerate the drug release [148]. Considering the time frame of an orthopaedic implant surgery, which usually lasts for 1–2 h [152], it is likely that some drugs would be inadvertently released in the process of surgical operation without any meaningful clinical outcomes. The premature loss of antibiotics from the nanomaterials can lead to lower-than-expected dose of antibiotic reaching the target infection region, and this sub-toxic dosage not only cannot kill bacteria sufficiently but further prime the bacteria to become increasingly tolerant to antibiotics.

To address this, external stimuli-responsive agents have been incorporated into the nano delivery systems to improve the release kinetics of antibiotics, enabling controlled and sustained release upon triggered by environmental changes, such as light, pH, temperature, magnetic field, or ultrasonic power [153]. With these highly localized, precise, and controlled nano delivery modalities, bacterial resistance progression can be largely impeded; yet the uncertainty of their long-term stability in the physiological environment and ADME (absorption, distribution, metabolism and excretion) profile *in vivo* requires future investigation in order to fully verify their practicality for hospital use [154]. Importantly, majority of these drug delivery systems only kill 80–90% of bacteria, which is far below the clinically relevant antibacterial efficacy (2–3 log reduction) [155]. Additionally, whether there is sufficient penetration to access deep tissue via this delivery method remains to be observed. Even if all the technical barriers are perfectly resolved in laboratory, most hospitals lack the relevant infrastructure for these smart systems to operate. In its biological essence, antibiotics induced antimicrobial resistance is governed by several central genetic mediators, e.g., *mecA*, which can activate a cascade of downstream antimicrobial resistance-promoting functional activities [156]. Although proven effective against a broad spectrum of pathogens and biofilms in preclinical trials, without fundamental novel differences in their bacteria-killing mechanisms from intravenous or orally administered antibiotics, nano delivery strategies are likely only to slow down microbial resistance to antibiotics in the real physiological context.

To avert antibiotic induced microbial resistance, smart nanomaterials formulations have been proposed to elicit bactericidal effects without recourse to any antibiotics. One example of such "antibiotics-free" strategies is to use nanomaterials to deliver antimicrobial peptides (AMPs). AMPs are primarily situated in the phagolysosomes of phagocytic cells, yet its existence in the extracellular environment can also be found in the event of infections. The bactericidal effect of AMP is achieved via synergistic actions comprised of extrinsic (e.g., membrane depolarization) and intrinsic pathways (e.g., inhibition of protein and DNA synthesis) [157,158]. Attributing to its multi-faceted killing mechanism, AMP is less likely to lead to antimicrobial resistance. As a promising alternative of antibiotics, there have been over 200 assortments of natural/synthetic AMPs identified over the past two decades. Delivery of AMPs for infection control has been achieved with the assistance of inorganic (e.g., silicon nanoparticles, nanoclays) or polymeric (e.g., PGA, PLGA, chitosan) nanomaterials, which has been comprehensively reviewed elsewhere [159].

Despite its potent resistant bacteria- and biofilm-removing property, the use of AMPs may give rise to antimicrobial resistance for several reasons. On one hand, many AMPs are known to cause haemolysis, as exemplified by Tachyplesin-1 [160,161]. Haemolysis refers to an abnormally fast destruction of erythrocytes and can generate excessive heme in the blood streams. The overconcentrated free heme in turn compromises immune systems by impairing phagocytosis of macrophages and neutrophils [162], elevating the likelihood of patients undergoing implantation surgery to suffer from bacterial infection and microbial resistance. On the other hand, the potent cytotoxicity inflicted by AMPs is not limited to bacteria. A mild toxicity to several mammalian cells has been recorded [163,164], and one example is the notorious nephrotoxicity of colistin, which has largely restricted its clinical applications [165]. In fact, bacteria like *S. aureus* have evolved several mechanisms as countermeasures of AMPs, although a complete abrogation of their lethal damages seems to be quite impossible [166]. There is a huge fitness cost for bacteria to gain resistance to AMPs. For instance, some bacteria may attempt to evolve enzymatic reactions to degrade AMPs, yet due to lack of unique epitopes in many AMPs that could be selectively recognized by bacterial proteases, collateral damages to bacterial functional proteins can be hardly avoided. While resistance to AMPs present negative impacts on bacterial survival, it may at the same time impair the ability of our innate immune systems to clear bacterial infections. Therapeutic use of a synthetic AMP named pexiganan was reported to induce cross-resistance in *S. aureus* to human neutrophil defensin 1, a pivotal part of host immune responses [167]. Such unintended consequence might be an additional consideration when deploying AMP-based aseptic strategies. Although many have reported that AMPs is relatively harmless to bone cells and no observable adverse effects on bone growth and local immune functions *in vitro* [168–170], the clinical relevance of these *in vitro* findings remains to be seen. As the cytotoxicity to bone cells and nearby immune cells is a critical contributor to orthopaedic implant associated microbial resistance, future studies are necessary to fully elucidate the toxicological profile of AMPs especially in a more physiologically relevant condition. As an alternative approach, some AMPs can be grafted onto nanoparticles to exert antimicrobial functionality. Different material compositions and strategies to enable AMP-conjugated nano antimicrobials can be found in other review papers [171,172]. Despite enhanced stability and antibacterial performance by surface decoration relative to encapsulation by nanocarriers [173], the potent cytotoxicity of AMPs remains a significant risk factor yet to be resolved, which might lead to antimicrobial resistance.

3.1.2. Antibacterial nanomaterials

Apart from nano delivery systems, some nanomaterials carry an innate bacteria-killing property that can be directly used for infection removal. Amongst all, metal nanoparticles have shown a great promise to combat MRSA and resistant biofilms related infections. Primary research attention has been given to surface coating silver and gold nanoparticles onto implants to prevent bacterial infection, in part due to their excellent chemical inertness and corrosion resistance, in addition to their potent bactericidal effects [174–176]. Silver and gold nanoparticles can be directly conjugated onto medical implants via salinisation or embedded into a nanocomposite film (e.g., hydroxyapatite) preceding the final deposition onto the implant surface [177–179]. The bactericidal effectiveness of silver and gold nanoparticles has been manifested in a broad spectrum of pathogens, including *S. aureus*, *P. aeruginosa*, *E. coli*, and resistant mutants (e.g., MRSA), although the exact mechanisms remain elusive [180]. A generally accepted theory for the antibacterial mechanism is centred on the ROS-inducing properties of these nanomaterials. Like many other nanoparticles, free radicals (e.g., $OH\bullet$, $O_2\bullet^-$) can be generated when the dissolved Ag^+ and Au^+ interact with the oxygen containing molecules present on the plasma membrane or in the cytoplasm via Fenton chemistries [181–184]. Excessive ROS produced by the metal nanoparticles can lead to a cascade of downstream growth-inhibitory actions, such as membrane polarization, organelle damage, metabolic dysfunction, DNA replication anomaly, and eventually cell death. The complexity of the ROS-mediated oxidative damages is believed to explain the superior non-selective bactericidal property of the nanoparticles and complicate the developmental process of antimicrobial resistance. While it is generally agreed that ionization of the particles and its downstream oxidative stress induction are the major killing action. Yet, this perception is under debate as physicochemical properties, in particular surface chemistries of nanoparticles play an important role in mediating the bacterial responses. It has been revealed that cationic polymer

coated silver nanoparticles caused damages to bacteria via a drastically different pathway compared to the pristine form [185]. It is also noteworthy to mention that expression of genes with relation to regenerative purposes has also been achieved with nanomaterials such carbon and silver [186,187]. Despite extremely low probability, acquisition of pathogenic resistance against these nanomaterials is not impossible.

There have been accumulating evidence pointing to the capability of bacteria, e.g., *staphylococci*, to evolve various protection, detoxification, and repair mechanisms in order to resist to the oxidative stress (ROS/RNS) induced damages [35]. (Fig. 3A) Staphyloxanthin (STX), a membrane-bound carotenoid pigment, is often present in the *S. aureus* colonies isolated from human patients. STX gives the bacteria a yellowish colour and is an effective detoxifying agent, preventing damages from exogenous singlet oxygen (O_2) and photo-induced oxidation [188]. Studies have also identified the capability of resistant staphylococcal strains to reprogram the ion efflux pumps, allowing a more efficient excretion of internalized metal nanoparticles out of the bacterial cytoplasm [189]. In addition, activation of detoxifying enzymes, such as superoxide dismutase, catalase, and haemoglobin-like protein (*hmp*) and DNA repair machinery (e.g., excision repair, recombinational repair) further abrogate the toxic effects of metal nanoparticles [35]. In this regard, it is possible that through this multidimensional self-protective mechanism, *S. aureus* may gradually evolve to become increasingly resilient to the metal nanoparticles induced oxidative damages. In fact, *in vitro* resistance to metal nanoparticles, such as magnesium, silver and gold, has been reported in *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and many other types of bacteria [180]. Specific to *S. aureus*, a recent study from Elbehiry et al. reported a strong particle-dependent resistance in 198 strains after a long-term exposure to sub-toxic doses of silver and gold particles (10–20 nm); and the likelihood of resistance occurrence was shown to be negatively correlated to the colloidal stability of the nanoparticles (Fig. 3B) [190].

Although bacterial resistance to metal nanomaterials *in vivo* is poorly understood, there is a recent report showing that prolonged exposure to silver-based wound dressing led to an increased silver resistance (4-fold increase in MIC) in 4 of the 38 clinical staphylococcal isolates (Fig. 3C) [191]. Furthermore, the chronic exposure to silver ions was found to desensitize the bacteria to a broad range of antibiotics, pointing to a presumably more worrisome consequence of metal-resistant bacterial infections. The exact mechanism as to how metal-resistant bacteria cross-adapt to antibiotics is not clear at the moment, but a better understanding of this cross-resistance action will be useful for devising countermeasures and furthering the utility of both metal antimicrobials and antibiotics. Specifically, genetic profiling of changes in metal/antibiotic-resistant strains at the transcript level to decouple the shared molecular actions can be an interesting proposition. In addition to the direct effects on bacteria is the innate nanotoxicity of the particles to neighbouring bone cells [67,192,193], which might further ease the bacteria to adhere to the implants resulting in biofilm-mode microbial resistance. Taken together, special considerations on the nano safety for orthopaedic implants, particularly on to how to mitigate the risks of antimicrobial resistance associated with the applied metallic nanomaterials are required.

Apart from metal nanoparticles, some scientists have aimed to develop antimicrobial nanotechnologies to deal with resistant bacteria infections by harnessing the inherent bacteria killing property of nitric oxide (NO). A four-fold log reduction in planktonic-mode MRSA has been manifested by S-nitrosoglutathione (GSNO) conjugated PLGA nanoparticles [194]. Using L-arginine as a NO precursor, over 99% of *S. aureus* biofilm can be eliminated by the NO-releasing indocyanine green-modified mesoporous polydopamine (MPDA) [195]. (Fig. 3D) Yet, current design of NO-releasing nano formulations often suffer from transient release of NO ($t_{1/2} < 12$ h), limiting its long-term bacteria--killing efficiency. In addition to the poor durability is its working principle, i.e., execution by oxidative stress induction, that approximates to that of the metal nanoparticles. In *S. aureus*, resistant variants have been identified to produce a higher level of flavohemoprotein *hmp*, which was found to enhance its tolerance to exogenous high NO concentrations [196]. Although bacterial resistance to NO has not been reported thus far, it is expected that bacteria with the complex set of protection mechanisms against oxidative stress damages may develop resistance to the NO-based antimicrobial nanotechnologies through a similar pathway.

Using nanoparticles as a power source to generate local heat in the infection sites has emerged as a promising aseptic regimen to combat resistant bacteria infections. The fundamentals and various formulations of this hyperthermia-based strategy, also known as photothermal therapy (PTT), has been exhaustively reviewed [197–200]. Briefly, an elevated temperature (>50 °C) in the localized region can be produced by materials under near-infrared radiation (NIR), resulting in occurrence of a rapid necrotic cell death, a distinct execution mode that is believed to circumvent the particle-oxidative stress associated antimicrobial resistance. Extensive studies have demonstrated the capability of a variety of nanomaterials, including but not limited to polymeric nanoparticles, carbon-based nanoparticles, gold nanoparticles, and metal sulphide nanoparticles (e.g., CuS, MoS_2) to convert light to heat for antibacterial applications [40]. The PTT bactericidal properties are influenced by many material structural parameters, such as shape, size, surface chemistry, and crystal pattern, of which surface chemistry is the key player. For example, the antibacterial effectiveness of nano gold-based PTT scales with the level of surface hydrophobicity [201]. Surface charge potential is also an important factor. By decorating a positively-charged ligand, i.e., polyaniline, onto the materials surface, the modified chitosan gels was able to generate a concentrated heat to the infection hotspots, resulting in enhanced thermal lysis of bacteria [202]. Yet, nanomaterials enabled PTT is not without its limitations. In orthopaedic implant, excessive heat generated by the nano-based PTT can be cytotoxic to neighbouring healthy tissues [203,204], which may retard bone growth and regeneration and is a risk factor underlying the biofilm-mode resistance. To resolve this problem, current methodologies are split into two directions. One way is to contain the heat energy within the infection area to minimize damage towards surrounding healthy tissues. To this end, smart materials that can auto-transform into a hydrogel upon trigger by bacteria have been devised to generate bacteria-targeting heat [202]. Alternatively, low-temperature PTT has recently attracted increasing research interest. By reducing the functional temperature to below 48 °C, along with the synergistic effects of other antibacterial agents such as metal nanoparticles, NO, antibiotics, and other bactericidal modalities [205–207], satisfactory therapeutic outcome can be retained while the potential threat to the nearby tissues can be maximally alleviated [208]. Of note is that at temperature >48 °C, even a short-term treatment (4–6 min) can lead to a significant damage to cellular proteins and DNA [208,209], thus low-temperature PTT in comparison to the high-temperature modality is preferred as it preserves the protective functions of the host immune system against microbial. Regardless of current laboratory success in applying PTT to combat resistant bacterial infections, clinical use of PTT often suffers from insufficient penetration into deep tissue to remove bacteria therefrom [210]. Furthermore, the elevated level of antioxidants, such as glutathione, in the infection sites coupled with a hypoxic environment can largely limit the efficacy of oxygen-dependent PTT (Fig. 3E) [210]. Given insufficient removal of bacteria plays a key role in microbial resistance acquisition, future efforts are required to further improve the bacteria killing effectiveness of PTT in a clinically relevant context.

Surface grafting bacteriophages, viruses of pathogenic bacteria, onto nanomaterials is another strategy to combat antimicrobial resistance. As bacteriophages target only bacteria, they do not cause any damages towards eukaryotic cells [212], which makes them safer and less likely to impair bone cells and the local immune activities relative to the antibacterial agents mentioned earlier. There have been several research groups reporting the use of phage-grafted nanomaterials for infection

control. One example is the polyvalent phage PEL1-functionalized iron oxide nanoparticles, which was able to eradicate >99% of *E. coli* and *P. aeruginosa* and >88% of their related biofilm [213]. Interestingly, using a synthetic phage, a comparable bactericidal efficiency (>90% for *S. aureus*, *P. aeruginosa*) was achieved post treatment of the phage-mimicking silver-doped gold nanospheres [214]. Despite the reported benefits provided by bacteriophages, emergence of phage associated resistance has been documented; and the primary mechanism to drive the resistance development is believed to be random genetic mutations during the long-term exposure to the phages [215]. Nonetheless, whether such mutation-driven resistance carries positive or negative impact is still in debate. Some mutations in the pathogenic membrane receptors have been shown to sensitize the phage-resistant bacteria to antibiotic treatments or reduce their virulence [216,217]. Improved understanding on the complex phage-stimulated bacteria evolutionary dynamics may present an interesting therapeutic target for reversal of microbial resistance to antibiotics.

3.1.3. Anti-biofouling nanomaterials

In stark contrast to active bactericidal nanotechnologies, anti-biofouling nanotechnology provides a distinctive route to combat biofilm-mode resistance. Due to treatment difficulty once a mature biofilm is established, prevention of bacteria adhesion is considered the best possible strategy as bacterial colonization would not be possible if adhesion is unable to take place. When complete prevention of adhesion is unsuccessful, interference of the attachment process will also delay the initial stage of bacterial infection. Antifouling strategies was first introduced to prevent microorganism's adherence onto marine products. Metal nanopaints (e.g., Ag, Zn, Cu, As) is the first-generation fouling-proof technology that has been widely applied in the marine industry; yet its potent toxicity can pose a huge health risk to human body, rendering it unfit for long term biomedical implant use. The aim to seek for a safer and more biocompatible alternative stimulated recent development of nanoengineered fouling-proof materials. In general, anti-biofouling properties can be achieved by increasing the hydrophilicity or hydrophobicity of materials.

Polyethylene glycol (PEG) is considered the gold standard strategy to prevent surface contamination by a wide range of protein molecules and microbes. However, its intrinsic structural instability in the presence of oxygen and enzymes presents a critical hindrance for its broad application in the clinical context. Alternatives have been discovered in the recent years, including zwitterionic polymers, poly(glycerol), poly (vinylpyrrolidone), polysaccharides, poly(2-oxazoline), polypeptoids, etc. [218] The design principle and applications of these antifouling materials have been reviewed in detail elsewhere [219]. In general, it is believed that these hydrophilic materials (contact angle <90°) prevent fouling via formation of an extensive hydration layer and steric repulsive effects. Despite an excellent antifouling property, addition of nanofillers (e.g., Ag, SiO_2, ZnO, CNT, graphene) is often required to provide sufficient mechanical strength to ensure structural integrity and further improve the antifouling effectiveness of the composite material [220]. Even when no material deformation is reported, the antifouling efficiency of these materials appears to be unsatisfactory in many cases, which is often <85% and is only sustainable within a short period of time (<7 days) [221–224]. Therefore, antibacterial compounds (e.g., antibiotics, AMPs) are frequently incorporated into the antifouling formulations to achieve better therapeutic outcomes. One example is bi-layered antifouling polymeric nanofilm (26 nm in thickness), in which the top layer was designed to deliver gentamicin in a controlled manner and the base layer was designed to exhibit fouling-proof property [225]. According to the authors, this binary antifouling nano system can prevent bacteria adhesion up to 2 weeks, >2 times longer than the effective time range of existing antifouling strategies. While the combination of antifouling and antibacterial agents allows the composite materials to exert dual mechanisms to kill bacteria as well as remove bacteria adhesion, the superior adaptability of bacteria to antibiotics as well as the well-known cytotoxicity of AMPs might dampen the long-term utility of such combination antibacterial therapy.

In addition to hydrophilic nano-reinforced materials, prevention of biofouling can also be achieved by endowing the implant with a superhydrophobic surface. While hydrophobic surface (contact angle >90°) is susceptible to bacteria contamination, superhydrophobic materials (contact angle >150°) are an exception due to the extremely low surface free energy and innate self-cleaning property. The superhydrophobic characteristic of the Ti implant can be achieved by femtosecond laser ablation [226]. The laser imprinted surface presents a 10–20 μm sized convex structures, which were decorated by nano undulations (diameter ~ 200 nm). With this unique nano-engineered topography, the Ti implant was reported to remove ~100% of *P. aeruginosa* and ~92% *S. aureus* adhesion up to 18 h. Despite the simplicity of direct modification of the Ti implant surface, the long-term efficiency of biofilm inhibition *in vivo* is doubtful as Ti implant is prone to surface contamination by host serum proteins and pattern loss due to corrosion and dissolution under physiological conditions [227,228]. Adding to this is the notorious hypersensitivity and chronic inflammation caused by the dissolved Ti ions [229], which may impair the host immune function and lead to antimicrobial resistance. Although systematic analysis of the impact of Ti implant toxicity on resistant bacterial infection is lacking, allergy and inflammation triggered by titanium has been identified as a cause of bone loss and eventual implant failure. In this regard, constructing a superhydrophobic coating on the medical implant can prevent unwanted exposure of the implant and provide a better antifouling route. Superior fouling repulsion properties have been witnessed in biocompatible polymers with nanoengineered geometry. One example is the lotus-like hierarchical polycarbonate (PC)/polypropylene (PP) films [230]. These films possess a micro-dome pattern with a number of pillar-like nanostructures (size ~200 nm) erected on the fringe. According to the authors, these films were able to retain super hydrophobicity up to a year and incorporating them into medical devices have shown positive results against bacteria adherence [231]. Following the traditional Cassie-Baxter design regime, other superhydrophobic coatings, such as polydimethylsiloxane (PDMS), styrene-*b*-(ethylene-*co*-butylene)-*b*-styrene (SEBS), SiO_2-modified poly (vinylidene fluoride) (PVDF) have been developed [232–235].

Nevertheless, the lack of durability and bio-inertness of these superhydrophobic formulations may lay the basis for resistant bacteria infections. Antifouling coatings are only effective against bacteria adhesion when they are tightly bound to the implant surface without losing their structural integrity. The introduction of nanoengineered patterns onto superhydrophobic materials is critical for antifouling property, but ironically results in mechanical and chemical frailty [236]. In addition, the structural stability of coatings is also dependent on materials composition, and increased durability has only been reported in superhydrophobic materials produced by fluorine-based precursors [237]. Materials with insufficient resistance to the wear abrasion by physiological fluid or bodily movement and enzymatic reactions are prone to detachment or decomposition over time, which eventually loses its protective function against bacterial adhesion and result in biofilm-mode resistance. One potential solution is by concealing the coating materials with a lubricant that is immiscible with the fluids in the bone microenvironment. Proposed by Epstein et al., the exceptional antifouling performance of a lubricated superhydrophobic coating, termed slippery liquid-infused porous surfaces (SLIPS), was demonstrated in a broad range of pathogens including *S. aureus* and *P. aeruginosa* over a 7-day experimental period [238]. To fully understand the clinical value of this antifouling nano strategy for orthopaedic surgery, special attention shall be put to the long-term stability and fouling-proof performance as well as the toxicological profile of the materials to human body [239,240].

Apart from the material design principle, the risks of antimicrobial resistance can be correlated to subtle "defects" resulting from the materials manufacturing process. It should be noted that in some

technologies like electrophoretic deposition, it is difficult to control the material quality and antifouling coating is often produced with cracks. In the presence of cracks, the structural integrity of the antifouling coating is compromised prior to use, allowing bacteria to adhere and subsequently colonize the implant starting at the "crack area". Also, the uniformity of the coating is of vital importance to confer a stable and sustainable fouling-repellent performance; and this becomes a problem when using spray coating and ion-beam deposition. The use of toxic chemical precursors (e.g., fluorocarbon) and solvents (e.g., chloroform, toluene) in the material synthesis process should be avoided and be used

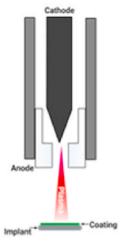
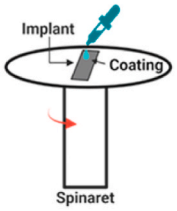
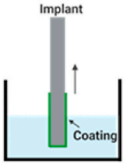
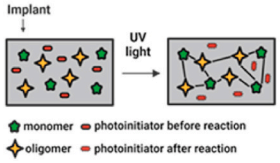
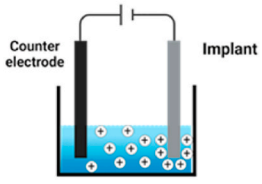
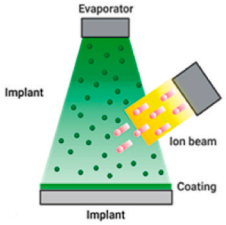
	Advantages	Disadvantages	Ref
Spray coating 	• High deposition rates • Low cost • Able to obtain a thin film down to 30μm in thickness	• Energy-inefficient due to use of high temperature • Rapid colling causes amorphous coatings	242
Spin coating 	• Simple equipment • Low volume of liquid required • Able to obtain a film with <1μm in thickness	• Not suitable for non-flat surface • Mostly for liquid with low viscosity	243, 244
Dip coating 	• Simple equipment • Ease of operation • Able to obtain a film with < 1μm in thickness	• Large volume of liquid required • High cost of precursor • Mostly for liquid with low viscosity	245
Photopolymerization 	• Time and energy efficient • Solvent-free • Precisely control the initiation of the polymerization reaction • High grafting efficiency • Flexibility of reaction temperature	• Oxygen-free atmosphere required	246, 247
Electrophoretic deposition 	• Rapid deposition • Uniformity of the coatings is ensured • Suitable for complex coating materials	• High temperature required • Difficult to obtain a crack-free coating on the substrate	248
Ion-beam deposition 	• Uniform coating • High adhesive strength • Enhanced control of microstructural properties of the coatings	• Expensive • Produce amorphous coatings	249

Fig. 4. Various fabrication strategies for creation of antifouling coating onto orthopaedic implants along with its advantages and disadvantages [242]. spray [243, 244] spin [245] dip coating [246,247] photopolymerization [248] electrophoretic [249] ion beam.

with care and caution as presence of these toxicants, if any, in the designed coating materials can incur systemic toxicity to human body [239,241]. The immune system is a known target of fluorocarbons [212], and those with immune function impaired by these toxic fluorocarbon-based coating materials may be more prone to bacterial infections and resistance. Readers can refer to Fig. 4 for a summary of various fabrication techniques for antifouling coating.

3.1.4. Immunomodulatory nanomaterials

In recent years, there has been rising interest to harness the body's natural ability to combat infection and heal itself. Our innate immune response is a natural host defence to remove exogenous pathogens. Despite the robustness of our immune system under healthy conditions, it is often compromised in the case of biofilm-mode IAIs due to the exceptional capability of biofilm-residing bacteria to hijack the immune system [41]. We direct readers to reviews that have discussed about the role of the immune cells during an orthopaedic implantation [41,65,250]. The importance of restoring host immune response has prompted scientists to devise smart nano strategies with immune modulation properties to combat antimicrobial resistance. Biofilm-related chronic infection is highly correlated to delayed wound closure and stagnant bone regeneration. These two vital activities have recently found to be regulated by anti-inflammatory M2 macrophages [251,252]. Because of this, special attention has been given to nanotechnologies that can promote differentiation of macrophages (M0/M1) into M2 subset. Macrophage phenotype is mediated by its genetic activity of

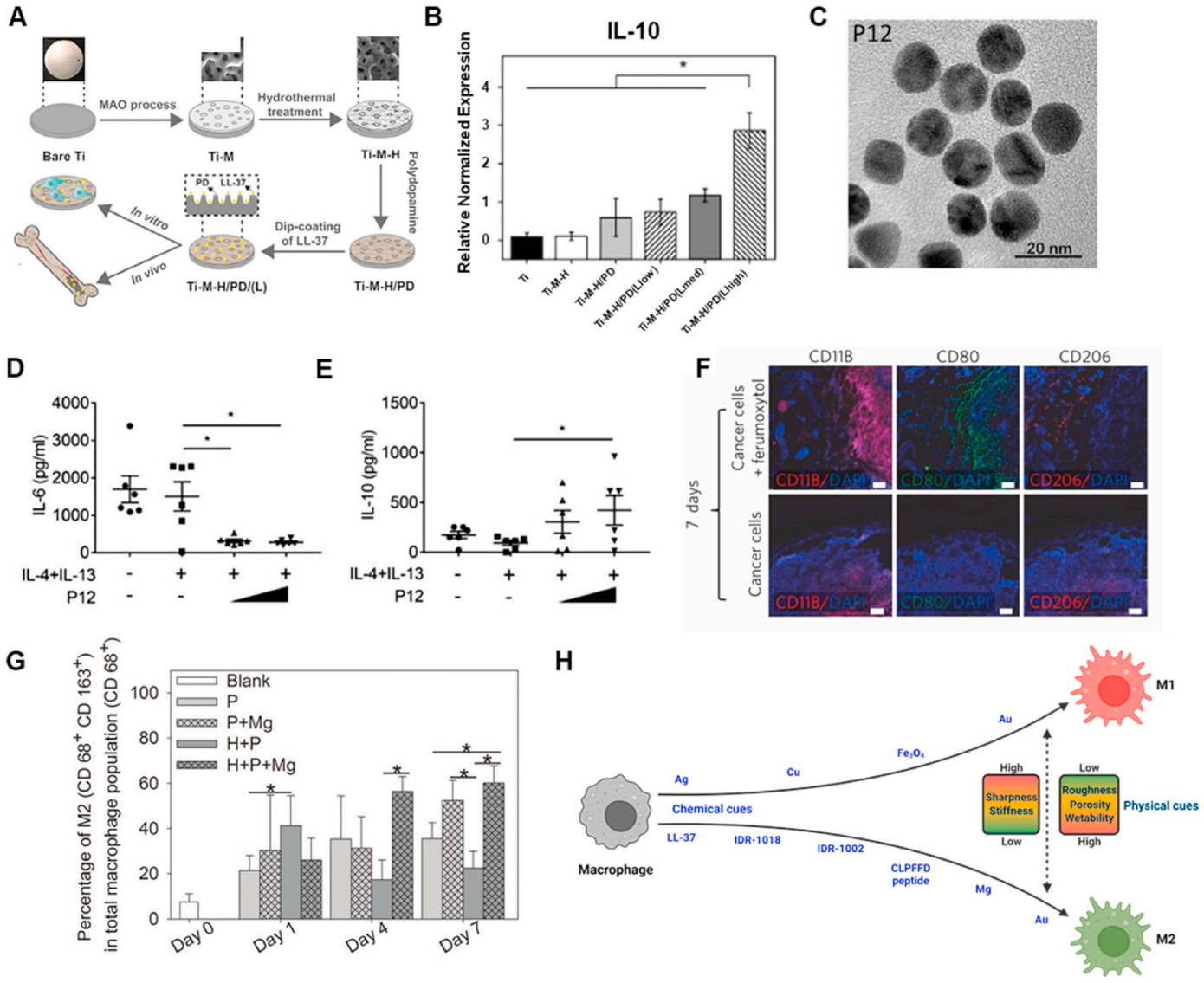


Fig. 5. Nanomaterials can control bacterial infections via modulating the immune function of macrophages. **(A)** Schematic illustration of LL-37 functionalized Ti implant [256]. **(B)** LL-37 at high concentrations significantly increased genetic expression of IL-10, a potent anti-inflammatory cytokine implicating the ability of LL-37 to induce M2 macrophage polarization. Reproduced with permission from Elsevier [256]. **(C)** A representative TEM image of synthetic host defence peptide nano mimic (P12) nanoparticles. Expression of IL-6 **(D)** and IL-10 **(E)** after co-treatment by IL-4, IL13, and P12 nanoparticles. Reproduced with permission from Springer Nature [261]. **(F)** Representative immunofluorescence staining images for CD11b (red, M1 marker), CD206 (red, M2 marker) and CD80 (green, M1 marker) of MMTV-PyMT tumour sections with or without 7-day exposure to iron oxide nanoparticles, i.e., ferumxytol. Significantly increased level of M1 markers in the iron oxide nanoparticles-treated samples suggested the presence of a large quantity of M1 macrophages in the tumour section relative to the untreated samples. Reproduced with permission from Springer Nature [268]. **(G)** Increase of M2 macrophages in the total macrophage population after exposure to Mg substrates. Reproduced with permission from the Royal Society of Chemistry [273]. **(H)** An illustration summarizes different physical and chemical cues affecting the macrophage polarization fate. Created with Biorender.com.(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammation-related cytokines. It is well known that overexpression of IL-4 and IL-10 can polarize macrophage to its M2 subtype, although the exact mechanism as to how these interleukin molecules steer macrophage molecular responses is poorly understood. Recent scientific findings shed light on the mechanistic action of IL-10 and pointed to the importance of rapamycin (mTOR) complex 1 (mTORC1) pathway [253]. The cytokine production of macrophage can be modulated by chemical and physical signals. LL-37, a member of cathelicidins family under the category of host defence peptides, has been shown to favour the differentiation of macrophages toward the wound-healing subtype (M2) [254,255]. A LL-37 coated Ti implant has been fabricated with the assistance of nano dopamine thin film (Fig. 5A) [256]. The coating with a high dose of LL-37 significantly elevated levels of anti-inflammatory IL-10, suggesting its capability to induce M2 macrophage polarization (Fig. 5B) [256]. Other host defence peptides, such as innate defence regulators (e.g., IDR-1002, IDR-1018) and defensins (e.g., human neutrophil peptides) are also conducive to M2 polarization [257–260]. Interestingly, a synthetic host defence peptides nano mimic (P12) composed of hexapeptides with a predetermined amino acid sequence (i.e., CLPFFD) as the surface décor and gold nanospheres (~13 nm) as the substrate displayed a potent M2 polarization effect *in vitro* (Fig. 5C–E) [261]. The use of host defence peptides and their derivatives in theory should not induce any microbial resistance, owing to the complicated and non-specific killing mechanisms [41]. Yet, there have been rising scepticism on the practicality of some host defence peptides, e.g., LL-37 and human neutrophil peptides, as they tend to be undesirably cytotoxic to eukaryotic cells (e.g., osteoblasts) and thus may put patients at risk of post-implantation complications like delayed wound healing and biofilm-related infections [262–264]. Conversely, IDR-1018 is able to accelerate cell proliferation, alongside its exceptional immune-protective and anti-biofilm functions [265]. Taking into account its absence of cytotoxicity and excellence in immune modulation, IDR-1018 may be a promising research target for developing future antimicrobial nanotechnologies to combat resistant bacteria and biofilm related infections for orthopaedic implants.

Interestingly, some metal nanoparticles can modulate host immune responses at non-inhibitory doses. In contrast to the well-studied antibacterial mechanism, mechanistic understanding of the immunomodulating property of metal nanoparticles remains poorly elucidated [266,267]. It is often observed that the immunomodulatory effects are material dependent. For example, some metal nanoparticles, such as iron oxide, silver, copper are prone to induce M1-typed polarization (Fig. 5F) [266,268–270]. Carbon nanomaterials are also able to induce inflammatory responses dependant on dose and time. They have also been suggested to promote M1/M2 polarization of macropahges [270,271]. On the contrary, magnesium is able to significantly reduce inflammatory response by reduction of inflammatory cytokines production and promotion of M2 phenotype (Fig. 5G) [267,272,273]. Even metal solvation is considered the upstream event of the immune modulatory activities, the differential outcome driven by metallic ion composition points to a ion-specific modulatory actions. Adding to the complexity of this, metal-led immune modulation such as those seen in gold nanoparticles can be influenced by different surface charge potential [274,275]. As a balanced inflammatory environment is of vital importance for a timely tissue repair and bone wound closure, the versatility of metal nanoparticles in modulating immune response and inflammation status can be further harnessed to intelligently control the implant-residing microenvironment for a better surgical result with reduced chance of IAIs and antimicrobial resistance. Notwithstanding these encouraging findings, it should be mentioned that at non-inhibitory levels, the metal ions related ROS generation might prime the bacteria to develop resistant phenotypes, as supported by some previous laboratory findings [180,190]. In addition, current available models that centre on selective induction of M1/M2 phenotype might lead to a prolonged activation of pro-/anti-inflammatory phenotype, which might cause collateral damages to the surrounding tissues in the long run and predispose the patients to biofilm-related chronic infections [41,276]. Therefore, an on-demand release of metal ions is recommended to offer timely regulation of inflammatory environment without causing damages to the surrounding cells and unwanted metal exposure to pathogens.

Alternatively, host immune response can be mediated by physical properties of the nanomaterials. Physical features, such as shape, porosity, roughness, wettability, and stiffness can substantially alter the immune responses upon immune cells in contact with the materials (Fig. 5H). In general, materials with higher roughness, a smoother shape, larger pores, higher hydrophilicity, and lower stiffness tend to direct macrophages to their anti-inflammatory subtype [250]. When harnessing the physical property of materials for a better immune modulatory outcome, special attention shall be put on the effects of these physical signals towards bacteria-implant interaction. For example, a hydrophobic implant surface, albeit conducive to early inflammatory phagocytic activities, also favours the adhesion of bacteria and subsequent biofilm formation. Likewise with materials that have high roughness which tend to promote M2-regulated bone wound healing but concurrently allows bacteria to colonize. Such trade-offs must be taken into account for designing a nano immunomodulatory material to maximize its therapeutic efficacy at the early (inflammatory) stage and meanwhile avoid development of biofilm-mode resistance at the later (would healing) stage.

3.2. Physical surface changes

3.2.1. Bionic surface topography

The use of surface micro-/nano-patterning to impart bacteria-killing property to medical implants was inspired by the antibacterial function of several naturally occurring surfaces, such as cicada and dragonfly wings [40]. A large number of studies have strived to create an antimicrobial bionic surface for orthopaedic implants by means of mechanical and chemical treatments. Extensive summary of the available technologies for surface patterning and their respective working principle has been documented elsewhere [277]. To summarize, the main focus is to produce a material surface geometry with a large aspect ratio (e.g., pillars, wires, tubes) and an optimized equilibrium of many other physical parameters including, but not limited to size, charge, wettability, rigidity, and pattern spacing of the designed nanostructures. High aspect ratio nanostructures have been loosely defined in literature but can typically be referred to structures that have an aspect ratio (ratio of the length to width) equal or greater than 10:1 [278]. Among all, laser ablation is accepted as the most cost-effective and versatile method to create patterned implant surfaces. Eghbali et al. reported the excellent *anti-E. coli* performance (>90% inhibition) of a micro-groove patterned Ti–6Al–4V implants fabricated by nano-second laser ablation [279]. The surface-enabled bactericidal effects were found to scale with the increase of groove distance. Alternatively, by employing dip-pen nanolithography and soft lithography technologies, a TiO_2 micropattern was successfully grown onto the medical-grade stainless steel 316L surface (Fig. 6A). Coupled with the light-mediated photocatalytic effect of TiO_2, this patterned implant was able to remove 96% of the resistant *S. aureus* (Fig. 6B) [280]. The large bandgap of TiO_2 (3.0–3.2 eV) however restricts further applications as the as-observed bacterial killing effects occur only under UV light radiation. By pairing TiO_2 with a molecule that has a lower bandgap and ROS inducing property, the photocatalysis-enabled antibacterial function can be manifested in the visible light range [281]. Orthopaedic implants can also be modified by anodic oxidation to produce a bactericidal surface, as supported by a recent example of nanotubular structured Ti implants showing inhibition of *S. aureus* colonization by >90% up to 5 days [282]. Other techniques, such as hydrothermal synthesis, vacuum casting, and micro molding have also claimed success in creating antibacterial implant surface patterns. Such a large depository of pattern fabrication technologies has enabled flexibility in designing unique nano-/micro-patterns onto medical implants with appreciable antibacterial

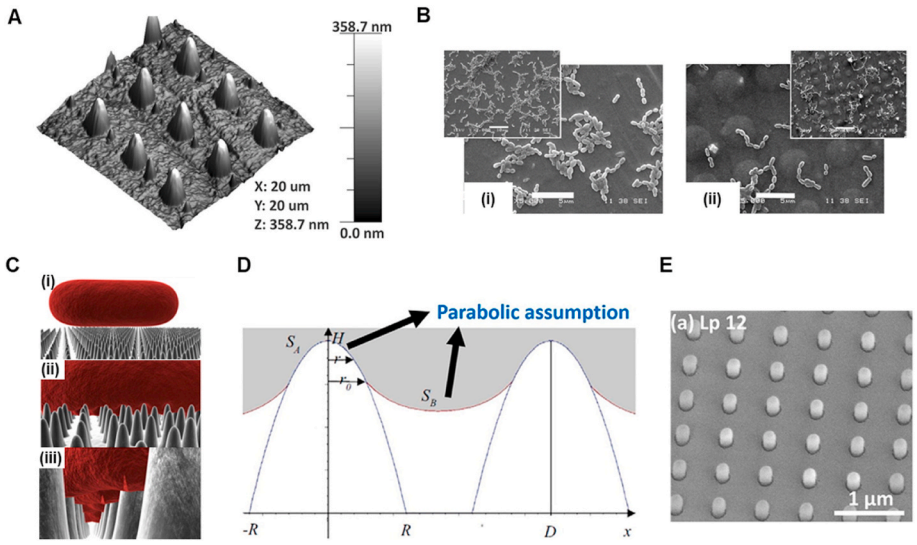


Fig. 6. High-aspect-ratio nanostructured surface in antimicrobial applications. **(A)** An atomic force microscopy (AFM) image of the TiO_2 patterned stainless steel 316 implant substrate. **(B)** SEM images showing the adhesion of *streptococcus* mutants on stainless steel 316 implant substrate **(i)** without TiO_2 micropatterns and **(ii)** TiO_2 micropatterns. Scale bar = 5 μm. Inserts correspond to the respective SEM images with lower magnification. Scale bar = 10 μm TiO_2 patterned surface possessed significantly reduced number of adhered bacteria. Reproduced with permission from Springer Nature [280]. **(C)** a possible antibacterial mechanism proposed by Pogodin et al. **(Ci)** Bacterial contact with nanostructured patterns. **(Cii)** Bacterial membrane begins to stretch when bacteria interact with the nanostructures. **(Ciii)** Bacteria membrane rupture occurs when the stretching force is overwhelming, causing cellular lysis and bacterial death. Reproduced with permission from Elsevier [283]. **(D)** Another biophysical model proposed by Xue et al. in an attempt to explain the nanostructure induced bacterial lysis [284]. H is the height of the nanopillars, 2R is the bottom width of the nanopillars, S_a denotes the contact area of the part of the bacterial membrane covering the nanostructure, S_b denotes the area of the suspended membrane, r_0 is the distance from the dividing line to the x-axis, and D is the distance between two adjacent nanopillars. In this model, the rupture event is mainly caused by the combination of gravity and van der waals. Reproduced with permission from Elsevier [284]. **(E)** A dome-like nanopatterns created by direct laser writing (DLW) with laser power (lp) of 12%. Although 3D printed surface patterns for antibacterial applications remains largely under-studied, future development of this additive manufacturing technology may be valuable for a highly reproducible and industrially scalable surface patterned implant with intrinsic bacteria-killing property. Reproduced with permission from American Chemical Society [296].

efficacy, yet special considerations are needed when strategizing such high-aspect-ratio patterned implants for clinical antibacterial applications.

Consensus on the antibacterial mechanism of these bionic surfaces has not been reached at present. Pogodin et al. proposed a biophysical model to explain the interaction between the nano-pillared cicada wings and bacteria (Fig. 6C), and it is believed that nanostructures with higher roughness, smaller spacing, larger radius and height tend to eradicate bacteria more efficiently [283]. The antibacterial mechanism is centred on the ability of the nanostructures to stretch bacterial membranes in the process of bacterial adhesion. Contrary to this proposition, Xue et al. put forward another theory that suggests sharper nanostructures with larger spacing can tear apart bacteria more effectively by gravitational and van der Waals force (Fig. 6D) [284]. This theory, according to the author, is supported by the exceptional clearing of gram-negative strains by nano-pillared wing surface. While the two theories converge to the membrane rupture killing mode, further investigations are warranted to address the contention on the effects of pattern spacing and sharpness on its antibacterial effectiveness to facilitate a better design of nano-patterned antibacterial implants. Regardless of the discrepancies in the two biophysical models, it is factual that there is a minimum requirement of height to width ratio for the nanostructures to be antibacterial while an excessively sharp structure can ironically promote bacterial growth [285]. In addition, it is often seen that gram-positive bacteria are more resistant to nanostructure induced damages, owing to their thicker peptidoglycan membrane that is unable to be stretched at ease [286]. The higher resilience of gram-positive bacteria should be taken into account when determining the optimal aspect ratio of a nanopattern. Furthermore, whether the lethal damage offered by those nanostructures is purely mechanical is still in debate. In contrast to Ti and stainless steel implants, it is often observed that high-aspect-ratio silicon implants are poorly antibacterial [287]. A better understanding of the structure-activity relationship of the applied surface topographies would be extremely useful as an instructive guideline to further the utility of these biomimetic antibacterial surfaces to combat resistant bacterial infections.

With recent studies on the bactericidal effect of carbon and graphene materials, a second class of mechano-bactericidal mechanism has been proposed [288,289]. It involves the interaction of surfaces with sharp nano-edges from graphene nanowalls or nanosheets and the bacterial membrane. When in contact, the lipophilicity of graphene reorientates the lipids from the phospholipid bilayer. This disrupts the integrity of the membrane structure allowing the insertion of the nano-edges into the membrane causing physical damage, resulting in pore formation and subsequent cell death [290]. While oxidative stress mediated by the graphene has been suggested, studies have also showed that in order for the bactericidal activity to take effect, the orientation of the edges has to be perpendicular to the surface. It has been shown that vertically aligned graphene nanosheets have a much stronger bactericidal effect when compared to randomly or horizontally aligned nanosheets [291]. These carbon nanomaterials have been shown to be effective against multi-drug resistant pathogens like MRSA [292,293]. Results have been promising, with a study by Elias and colleagues demonstrating effective antibacterial properties of graphene oxide and carbon nanofibers against multidrug-resistant bacteria. In addition, this method was combined with light-emitting diode irradiation as a novel combination method [292].

Another concern on the practicality of the bionic surfaces is that the reliance on bacterial adhesion onto the implant may ironically cause protein fouling. The antimicrobial efficiency could be significantly dampened by forming a protein complex layer on the nano geometries. In addition, bacterial adhesion is usually accompanied by accumulation of dead cells onto the nanostructured surfaces. If cellular debris cannot be cleared in a timely manner, the formation of a debris layer can cover the active functional patterns and be a source of nutrients in support of subsequent bacterial adhesion [294] Furthermore, the reliance on the interaction between pathogens and the bionic surface to exert bactericidal function also renders this technology unable to remove planktonic pathogens. The long-term structural stability of the patterned surface due to constant mechanical displacement from bodily motion is also questionable. Implants will be subjected to different kinds of wear, some with frictional contact with soft tissue while others will rub with metals or bone. All of these physical forces can accelerate the deformation/degradation of the bionic patterns, and this leads to a large degree

of uncertainty on their long-term utility when applied into clinical applications. Surface wear, corrosion, and dissolution of nanostructures in the physiological condition are likely to produce solid particulate debris and soluble ions that can be toxic to the nearby cells. As physicochemical properties (e.g., surface chemistry, size, aspect ratio, crystal structure) of the nanostructure are important parameters affecting its mechanical, chemical, and physiological stability, it is perceived that by engineering the material with careful consideration of these factors, cytotoxicity of the material can be minimized. Important to note is that some preliminary findings have identified slow dissolution of TiO_2, the main constituent in the high-aspect-ratio Ti implant, in the physiological condition. Therefore, aside from mechanical barriers, the hydrolysis of nanostructured patterns is also a risk factor leading to insufficient clearing of bacteria and antimicrobial resistance. Adding to all these is the potent Ti^+ toxicity to healthy tissues, which subjects the patients to a higher probability of chronic inflammation and immune function impairment [229]. All of these risk factors are ought to be considered for future design of bactericidal high-aspect-ratio implant surfaces in order to reduce the chance of resistant bacterial infection.

Manufacturability of the bionic surfaces is another challenge to translate the findings in laboratory into bedside applications. Important metrics that have to be considered are scalability, quality, reliability and cost. Current fabrication methods involve using either additive (bottom-up) or subtractive (top-down) processes. The quality and reproducibility of the patterns remain difficult to ensure. New nanomanufacturing procedures are needed to allow the translation of lab scale fabrication of nanostructures to industrial scale production. The high-volume production has to ensure quality and reliability with no large variation in aspect ratio which may affect the bactericidal properties of the nanostructure. To address this, Chen and his team have managed to develop a scalable platform for continuous printing of 3D nanostructures [295]. Interestingly, highly ordered dome-like nanopatterns can be fabricated using direct laser writing (DLW), an advanced 3D fabrication technology (Fig. 6E) [296]. Owing to a high programmability and structural

Table 1
SWOT analysis of different antimicrobial nanotechnologies.

	Strength	Weakness	Opportunity	Threat	Ref
Drug delivery systems	• Able to kill bacteria before attachment onto implant surface	• Unable to provide long term protection due to premature depletion of drugs	• Design of 'smart' delivery systems with release via various cues (temperature or pH) can vastly improve efficiency of strategy • Co-delivery of a cocktail of various antimicrobial molecules can serve multiple therapeutic functions.	• Premature depletion of drugs, causing emergence of antibiotic resistant strains	[136, 297]
Antimicrobial nanoparticles	• Intrinsic antimicrobial properties against antibiotic resistant bacteria strains	• Innate toxicity to host cells after long term exposure	• Immunomodulatory properties are largely unexplored and potentially can be harnessed with well controlled delivery	• Optimization is needed to prevent prolonged inflammation	[171]
	• Non-selective bactericidal property can potentially hinder development of antimicrobial resistance	• Development of resistance is not impossible	• Can be synergized with other strategies to improve antimicrobial effect	• Toxicity to humans cannot be fully replicated in animal models	
Anti-biofouling materials	• Inhibits bacterial attachment	• Eukaryotic cell attachment may be compromised	• Improvements in manufacturing process can greatly improve scalability	• Prevents bacteria colonization but will not confer permanent bactericidal effects, leading to infection once anti-biofouling property is compromised	[298]
	• No toxicity concerns	• Suboptimal bactericidal effect [some polymers like chitosan can kill bacterial to some extent]	• Other antimicrobial techniques can complement the strengths of anti-biofouling properties	• A struggle to ensure that the entire implant surface is adequately coated. • Mechanical and chemical frailty in the long term	
High aspect ratio nanostructure	• Anti-bactericidal effect using mechanical lysis and poration with reduced likelihood to promote resistance	• Accumulation of dead cells surface may decrease anti-bactericidal effects	• Strong demand for innovative anti-microbial materials	• Higher cost of production and price tags may deter use	[288]
	• Localised protection that reduces adverse systemic outcomes	• Wear and tear during surgery and continual use	• High applicability to a broad range of implants	• Low scalability in manufacturing due to specialised equipment needed	
	• Minimally affects larger eukaryotic cells and may even promote osseointegration with osteoblasts	• Limited (pre-)clinical data about efficacy	• Relative ease to comply with Good Manufacturing Practices		
Immunomodulatory nanotechnology	• Potential for sustainable and long lasting anti-microbial protection	• Possible cytotoxicity when release of immunomodulatory biomolecules is not well controlled	• Current boom in immunomodulatory materials including early phase clinical research	• Batch-to-batch variation during complex material synthesis	[250, 299]
	• Multiple synergistic mechanisms of protection including immune cell recruitment and macrophage polarization		• Programmable properties in a broad range of antimicrobial settings	• Regulatory approvals can take longer due to involvements of many chemical and biological factors	
	• Localised protection that reduces adverse systemic outcomes		• Ease of integration with existing strategies such as polymer coatings and nano-structured implant surfaces	• Potential high costs as all components must be pharmaceutical grades	

controllability, 3D printing technology may be further developed in the future to enable an industrially scalable manufacturing strategy for fabrication of advanced antibacterial nanostructured surfaces.

A SWOT (strength, weakness, opportunity, threat) analysis of different antimicrobial nanotechnologies is presented in Table 1. There is a great variety of test methods with the ability to test for antimicrobial activity of a material however with variety comes variations and tweaks in each method resulting in difficulty to do side by side comparisons between different materials. It is important to standardize methods in order to understand the behaviours of the antimicrobial material and if possible, further improve on standardized methods to test for effectiveness of antimicrobial materials that mimic its intended setting. Readers are recommended to refer to a toolkit list which summarizes commonly used characterization techniques for antibacterial nanotechnology research (**Table 2**).

4. Conclusion and future perspectives

Our society sees an ever-increasing demand for orthopaedic implants amidst an increasingly aging population. The greater need for orthopaedic implants can give rise to IAI cases and further emergence of resistant bacterial species. Even though antibiotics can treat IAIs in many cases and confer a life of better quality to patients post implantation surgery, a number of patients are still required to undergo surgical intervention, e.g., debridement, implant ex-plantation, and even amputation due to resistant bacteria infections. The slow and gradual development of resistance to vancomycin and the sluggish progress of new antibiotic discovery further exacerbate the situation. To circumvent the limitations of the conventional antibiotic administration regimens, such as lack of specificity, early drug degradation, and potent side-effects, advanced nano delivery systems have been devised to enable a controlled and precise drug delivery in the targeted region. Yet, due to overreliance on antibiotics, these drug delivery approaches can slow down the pace of antibiotic resistance at best. AMPs is considered one of the promising replacements for antibiotics, as it can remove bacterial infections via a multi-modal killing mechanism. Special considerations are however needed for further development and selection of AMPs for hospital use especially because some AMPs have documented organ toxicity and haemolysis inducing property. Another bactericidal agent that kills bacteria via a complex set of mechanistic actions is metal nanoparticles. Irrespective of the material type, metal nanoparticles generate bacteria killing effects mainly by disruption of homeostasis with significantly increased levels of oxidative stress. Similarly, the oxidative stress inducing property also accounts for the antibacterial action of those nitric oxide loaded nanomaterials. Although the complexity of the killing mechanism renders antimicrobial resistance unlikely to happen, recent mechanistic understanding implicates the pivotal role of oxidative stress in MRSA development, justifying the scepticism on the long-term utility of these stress-evoking antimicrobial nanotechnologies.

Antifouling nanotechnologies offer a different bacteria-cleansing route by removing bacteria adhesion, a precondition for biofilm formation, instead of actively killing bacteria. Nonetheless, current examples of antifouling nanoengineered materials often suffer from lack of mechanical strength, physiochemical stability, durability, therapeutic efficacy, the last of which is usually remediated by incorporation of antibiotics, nanoparticles, and AMPs into the fouling-repellent recipe. The antifouling formulations containing these antimicrobials concurrently inherits their own set of limits and thereby possess a heightened risk of antimicrobial resistance. It should be mentioned that aside from the composition of materials, the fabrication process is also an important source of the aforesaid limitations. Therefore, special considerations are needed when strategizing the manufacturing process and technique. Specifically, the uniformity and structural integrity of the designed materials are ought to be ensured and introduction of toxic chemicals and environmental pollutants shall be avoided. Another critical issue

Table 2
A list of available characterization techniques useful for antimicrobial nanotechnology research.

Antibacterial Properties	Purpose/Objectives	Assays	References
Cell adhesion	Observation of bacterial attachment on material	Microscopy Techniques (SEM, TEM, AFM, Confocal, Optical)	[300–303]
Biofilm formation	Visualization of biofilm	Fluorescence imaging (CLSM, epifluorescence microscopy)	[304,305]
Bacteria viability	Detection of bacteria viability or activity	Live/dead assay, CLSM, plate colony count, FDA assay, DAPI stain	[306–310]
Biomaterial Attributes			
Chemical composition	Determination of material composition/ Verification of uniformity in coating	NMR, FTIR, XPS	[301,311, 312]
Morphology	Investigation of morphology of the material	Microscopy Techniques (SEM, TEM, AFM, Confocal, Optical)	[303,306, 313,314]
Biodegradability	Analysis of material degradation *in vitro* or *in vivo*	Fluorescence imaging, SEM	[312,315]
Cytotoxicity	Characterization of toxicity of materials	Live/dead assay, CCK-8	[316,317]
Loading capacity	Measurement of loading capacity of material as molecular reservoir	BCA assay, fluorescamine assay	[316,318]
Release kinetics	Assessment of release profile of loaded biomolecules from material/ Ensure controlled release of loaded biomolecules over 30 days	Fluorescence imaging, ELISA, UV-VIS	[312,319, 320]
Bactericidal effect	Evaluation of bactericidal effect of materials/Aim for 2–3 log fold reduction of bacteria for clinical relevance	ISO 22196, agar zone of inhibition, spray inoculation assay, immersion inoculation assay, touch transference inoculation assay	[321,322]
Immunomodulation	Determination of immune responses elicited by material/ The polarization and immune cell profile is balanced and not skewed.	ELISA, macrophage phagocytosis assay, immunofluorescent staining of immune cells	[312,319]
***In vivo* assessment of biomaterials**			
Immune cell recruitment	Recruitment of immune cells to site of implantation	Fluorescence imaging and tissue histology	[323,324]
Immunogenicity	Assessment of side effects and immunomodulation	Immunofluorescent staining of immune proteins	[324]

Abbreviations: AFM: Atomic force microscopy; BCA: Bicinchoninic acid; CCK-8: Cell counting kit-8; CLSM: Confocal laser scanning microscope; DAPI: 4′,6-diamidino-2-phenylindole; ELISA: Enzyme-linked immunosorbent assay; FDA: Fluorescein diacetate; FTIR: Fourier-transform infrared spectroscopy; ISO: International Organization for Standardization; NMR: Nuclear magnetic resonance; SEM: Scanning electron microscopy; TEM: Transmission election microscopy; UV–Vis: Ultraviolet–visible spectroscopy; XPS: X-ray photoelectron spectroscopy.

that is non-negligible and often arises from antifouling nanotechnologies is the non-discriminative prevention of both pathogens and host cells from adhesion onto the implant. Compromised tissue cell attachment to the implant can eventually lead to bacterial colonization and implant failure. An ideal antifouling coating should deter bacterial adhesion while favouring host cell integration. One possible research direction is to engineer the structural properties of coating materials to impart them with selectivity to bacteria. This has been tentatively proved by a recent work of Pham et al., where the authors showed that black silicone implant coating can selectively remove pathogens while allow propagation of COS-7 fibroblast cells onto the substratum [325]. Alternatively, high cell compatibility and anti-bacterial adhesion may be simultaneously achieved by incorporating antifouling and host cell adhesion-promoting coating [326].

The intrinsic bactericidal properties of bionic implant surface have also received a significant research interest, probably owing to its simplicity with minimal use of chemicals, which is presumed to substantially ease the FDA approval process. Notwithstanding its advantages, the heavy reliance on the direct contact with bacterial cells to inhibit their viability can ironically become a weakness leading to protein contamination and a lower-than-expected therapeutic efficacy. The bactericidal effect of the nanostructures is only able to take effect on the bacteria that adhere and make direct contact with the surface. Planktonic bacteria will therefore be able to avoid getting killed. The direct exposure of tissues to Ti implant can cause Ti^{+} related cytotoxicity and chronic inflammation, which is often associated with the weakened ability of bone cells to "race for the surface" and thus paves the way for bacterial adhesion onto the implant. Another point of consideration would be the mechanical stability of the nanotechnology, and this is not only applicable to bionic implant surfaces but also other strategies (e.g., antifouling) whose bacteria-removing effects are premised on the structural integrity and tight connection to the implant surface. Specific to bionic surfaces, the designed patterns have to withstand the mechanical forces produced by bodily movement and avoid early loss of function due to hydrolysis in the physiological condition. To improve the mechanical stability, a flexible pattern that can withstand those wear and tear forces may be an interesting research direction. It is interesting to note that a flexible vertically aligned carbon nanotubes (VACNTs) can further improve the antibacterial efficiency via structural deflection upon contact with bacteria [306]. This novel design principle may be valuable for orthopaedic implants. The manufacturability of the bionic surfaces is another barrier impeding the translational progress, but this problem may be resolved by harnessing the benefits of 3D printing technology. Future explorations are warranted to address these bottlenecks to fully unlock the potential of these biomimetic surfaces.

Another key aspect of infection control that has been often disregarded in prior research is our immune system. Although effective, the existence of biofilm-mode infection can evade the immune system and abrogate its function. It is now well known that EPS can significantly reduce chemotactic migration of neutrophils and macrophages toward the infection region, masking the embedded bacteria from immune recognition and phagocytosis [327]. Nanomaterials with immunomodulatory functions is therefore an alternative strategy by restoring the host immune response. The central focus is to use nanomaterials to enable a smart manoeuvre of macrophage polarization status, i.e., from M1 to M2 or vice versa. Since a balanced inflammation status in the bone microenvironment is essential for osteogenesis and wound closure [41], future research should aim to devise a smart nano "switch" that can direct an on-point polarization to either inflammatory or wound-repairing subset. As implant associated immune response involves a complex interplay of many types of immune cells, e.g., monocytes, neutrophils, T/B cells, it may be valuable if future developments consider crosstalk within the entire set of immune networks. For instance, given that regulatory T cells are an important stimulator of other immune cells' wound healing activity [250], it will be useful if we can devise a nanomaterial that can recruit regulatory T cells to accelerate implant wound closure. Additionally, given osteogenesis is mediated by the local immune responses, an antibacterial immunotherapy that can also promote bone cells integration with implant may have important clinical values. Several recently identified immunomodulators, such as IDR-1018 have shown a great potential for clinical use due to its exceptional immune-protective, anti-biofilm, and biocompatible properties. These immunomodulatory agents are promising targets in future research.

However, none of these antimicrobial nanotechnologies can perfectly ward off resistant bacterial infections and pathogenic resistance progression at a stand-alone mode. From the perspective of bacterial resistance evolutionary dynamics, it is more effective to prevent antimicrobial resistance by integrating known antimicrobial therapies to produce a strategy that has a set of multi-dimensional killing mechanisms. One example is light-mediated metal nanoparticle based photothermal therapy which combines heat and metal ion-enabled oxidative stress to limit bacteria from developing resistance. Likewise, nano antimicrobial implant coating with both active bacteria-cleansing and immunomodulatory properties is promising to restrict the resistance emergence. Apart from combination therapy, recent development of bacteriophage engineering and vaccines, albeit in their infancy and effective against only a limited range of strains, can enable scientists to devise a antimicrobial nanotechnology that is tailored to a specific need [40]. It needs to be mentioned that regardless of the huge progress seen in recent years, few examples have been adopted into clinical applications. One major problem is believed to be the sub-par reduction in bacteria for all these lab-based antibacterial nanotechnologies. A log-fold change of 2–3 is usually the threshold of efficacy of antimicrobial materials, pathogenic inhibition *in vivo* remains a cumbersome issue yet to be resolved for most of the past research [155]. The incomplete clearance of implant-residing bacteria is likely to generate persister cells and predispose the patients to antimicrobial resistance associated dire consequences. From a material perspective, to achieve an optimal therapeutic outcome, we should ensure that the designed material formulation is exceptional at eradicating pathogens in clinically relevant conditions and meanwhile avoid potential long-term material induced side-effects. Timing of the therapy and the release and retention of the nanoparticles will greatly affect their toxicological profile. Although acute nanotoxicity has been widely accepted into the current experimental paradigm for evaluation of a new nano antimicrobial, little is known about its long-term effects. With a better understanding of the correlation of materials of different design principles and material fabrication techniques to the bacterial evolutionary dynamics, it is recommended that future explorations shall shift the focus to attain the clinically relevant antibacterial efficacy and examine the long-term impacts of nanomaterials on patients' health, rather than conceive new antimicrobial recipes with mere conceptual fascination. Special focus shall be placed on assessment of the ability of emerging antimicrobial nanotechnologies to remove biofilm, as it is the major cause of resistant bacterial infection. The nature of quorum sensing functioning bidirectionally may be further harnessed to control biofilm-mode infections in different disease states [125]. As bone integration is an important facet for successful wound closure and void of implant related infection, future antibacterial nanomaterials should strive to achieve both bacterial-killing and osteoinductive. After all, considering that antimicrobial resistance does not develop in just a day or two, only time can eventually tell us whether the advanced antimicrobial nanotechnologies would undergo the same fate as antibiotics whereby bacteria would be trained to become increasingly formidable and hard to treat.

Declaration of competing interest

There is no conflict of interest.

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