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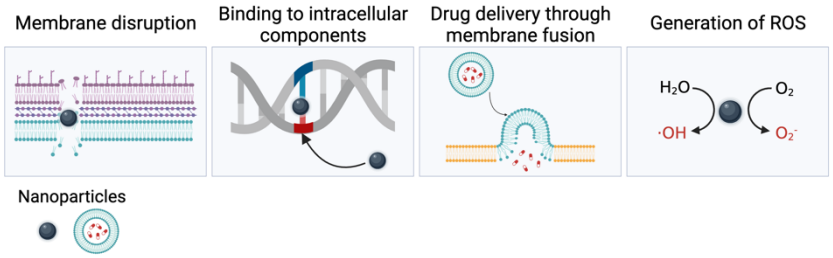
Overview of nanotherapeutics for bacterial infections

Antibiotic resistance is one of the biggest threats to global health, food security, and development, affecting people regardless of age, gender, and origin. Despite being a natural process, the misuse of antibiotics has speeded up the development of the phenotypic and genotypic drug resistance mechanisms through which bacteria can guarantee their survival. These mechanisms include limitation of drug uptake, drug target modification, drug inactivation, and active drug efflux. As a consequence, conventional antibiotics are becoming less effective and a growing number of bacterial infections, including the ones causing pneumonia, tuberculosis, gonorrhoea, and salmonellosis, are becoming harder to treat [1,2].

On the lookout for non-conventional approaches to surpass and mitigate the effects of antibiotic resistance, nanomaterial-based systems render new opportunities in the therapeutics field as drug carriers or, even, as inherent antimicrobial agents. Nanoparticles (NPs) have tuneable size, shape, and surface chemistry, and a high surface-to-volume ratio that confers them unique physicochemical properties (*e.g.*, electrical, magnetic, optical, etc). Hence, nanomaterials represent an opportunity to create

novel and cutting-edge therapeutic strategies to combat both planktonic bacteria and biofilms as they can circumvent existing resistance mechanisms by employing an array of bactericidal pathways. For example, positively charged NPs can engage in electrostatic interactions with the negatively charged groups on bacterial surfaces, resulting in damage to the membrane and leakage of cytoplasmic contents. Nanomaterials can also bind to various intracellular components like ribosomes, proteins, and DNA, disrupting their normal functions. Additionally, nanomaterials possessing catalytic properties can boost the production of reactive oxygen species (ROS) like hydroxyl radicals and superoxides, leading to oxidative stress within the bacterial cells. Furthermore, nanomaterials can serve as carriers for therapeutic agents, some of which readily penetrate bacterial cells through membrane fusion, thus facilitating the delivery of their cargo (**Fig. 6.1**) [3–5].

Figure 6.1. Antimicrobial mechanisms of nanoparticles. Created with



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6.1 INORGANIC NANOMATERIALS

Inorganic NPs encompass a wide range of materials, including transition metals (*e.g.*, gold (Au), silver (Ag), copper (Cu)), metal oxides (*e.g.*, iron oxides (FeO, Fe_2O_3, Fe_3O_4), magnesium oxide (MgO), titanium dioxide (TiO_2), aluminium oxide (Al_2O_3), zinc oxide (ZnO)), metalloids (*e.g.*, silica (SiO_2)), carbon (e.g., carbon nanotubes), and more. Their antimicrobial effect relies on certain characteristics such as size, shape, ζ-potential, presence of ligands, pH, roughness, stability, crystal structure, and chemical composition [6].

Inorganic NPs composed of transition metals are capable of forming strong coordination bonds with non-bonding orbitals characteristic of

heteroatoms (*i.e.*, N, O, and S) abundant in biomolecules (*e.g.*, proteins and nucleic acids), effectively interfering with their biological function.

Positively charged metal-based NPs can also establish strong electrostatic interactions with the negatively charged bacterial cell wall, regardless of its composition (*i.e.* Gram-positive or Gram-negative bacteria), resulting in its disruption and increased permeability [7]. In addition, NPs can also release free metal ions into the extracellular space, which can enter the cell and interfere with metabolic pathways [3,7].

Another antimicrobial mechanism utilised by this class of NPs is the production ROS, such as free radicals and peroxides, leading to the oxidative stress of bacterial cells. Indeed, ROS production is a natural product of cellular respiration and low levels of ROS (which act as redox signalling messengers) are needed for the normal physiological functioning of cells [8]. In addition, bacteria have a plethora of antioxidant enzymes (*e.g.*, glutathione (GSH), superoxide dismutase, and catalase) whose role is to protect biological macromolecules from oxidative stress [9]. Nevertheless, excess ROS production caused by metal NPs can break this balance and eventually kill the bacteria by damaging the cellular membrane, DNA, and mitochondria. In fact, this is the main antimicrobial mechanism triggered by various metal-based NPs, such as Au, Cu, ZnO, and TiO_2. Therefore, this class of NPs can be adopted for antimicrobial purposes with broad-spectrum activity. One major shortcoming is that this lack of specificity in their mechanisms of action also means that they can target pathogenic bacteria and mammalian cells alike [12].

In particular, silver NPs (AgNPs) have a strong antimicrobial activity due to the high affinity of Ag^+ ions to amines, phosphates, and thiols, functional groups widely found in proteins and nucleic acids [10]. In fact, AgNPs are the most commercialised engineered nanomaterials, accounting for over 50% of global nanomaterial consumer products, being extensively used in personal care products, textiles, food packaging, and healthcare products, due to their recognised antibacterial, antifungal, and antiviral properties [11,12]. As such, AgNPs have been extensively studied and used alone or in combination with other nanomaterials, in the form of nanocomposites or hybrid materials [13–20].

Metallic copper NPs offer an affordable alternative to silver-based nanomaterials; however, they have major limitations as the requirement

for higher dosage concentrations and instability due to the rapid oxidation of metallic copper to cupric oxide (CuO) and cuprous oxide (Cu_2O) upon exposure to air. In fact, the formation of an oxide layer at the surface of copper NPs is inevitable due to the greater thermodynamical stability of the oxide phases [21]. In addition, the release of high concentrations of Cu^{2+} (and its reduced form Cu^{+}) is toxic to bacterial and mammalian cells alike, due to the generation of excessive ROS [10,22,23]. Despite these limitations, the antimicrobial properties of copper have been recognised for centuries, with various forms of copper compounds employed by ancient civilisations for a range of purposes. For instance, the ancient Egyptians used metals like silver and copper to sterilise drinking water and treat wounds. Romans documented numerous medicinal applications of copper for different ailments. The Aztecs utilised copper to alleviate sore throats, while in Persia and India, copper was employed in the treatment of eye infections and venereal ulcers. In the past decades, Cu-based nanomaterials with low or non-cytotoxic profiles have been successfully developed against both Gram-positive and Gram-negative bacteria, such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enterica,* and *Staphylococcus epidermis*, amongst many other pathogenic microorganisms [24].

The greater stability of copper oxide phases has driven research towards the development of copper oxide nanomaterials, as an alternative to metallic copper. Several studies reported the antibacterial activity of copper oxides against Gram-positive and Gram-negative bacteria, such as *Lactobacillus brevis, Pseudomonas aeruginosa, Escherichia coli*, and especially against methicillin-resistant *Staphylococcus aureus* (*MRSA*) [25–29]. In addition, copper oxide nanoparticles, especially Cu_2O, have been reported to show lower host cell cytotoxicity, good environmental acceptability, and remarkable broad-spectrum antibacterial activity. However, the major limitation of copper oxide NPs when compared to metallic copper is the lack of sufficient stability due to their strong tendency to aggregate [30].

Conversely to AgNPs and CuNPs, AuNPs are much more inert and biocompatible, yet have significantly lower antibacterial activity. Despite this drawback, AuNPs can be easily functionalised with antibiotics, cationic moieties, or even enzymes, to increase their antibacterial effect [31]. For example, a hybrid system composed of ampicillin conjugated to lysozyme-capped gold nanoclusters was developed to treat

MRSA infections, increasing ampicillin antibacterial effect against this strain and other non-resistant bacterial strains through different mechanisms which include: (i) increasing ampicillin concentration at the target site; (ii) allowing for multivalent presentation and increased permeation of ampicillin; (iii) lysozyme-mediated cell wall lysis; (iv) bacterial efflux pump dysfunction; and (v) Au ion mediated destabilisation [32].

In a different example, amoxicillin and ofloxacin antibiotics were individually loaded in Au-silica core-shell mesoporous NPs and tested against *Pseudomonas aeruginosa*. The NPs allowed a 2-fold (for ofloxacin) and 20-fold (for amoxicillin) decrease in the quantity of antibiotics needed to inhibit bacterial growth. [33].

Metal oxide NPs, such as ZnO, TiO_2, and Al_2O_3 NPs are another group of nanomaterials with potent antibacterial activity. Amongst these, ZnO NPs have entered the spotlight due to their low toxicity and greatest biocompatibility [12,27–29]. In addition, ZnO NPs also render high antibacterial effectiveness at low concentrations (0.16-5.00 mmol/L) against a wide range of strains and at a relatively low cost [34]. For example, gelatin-coated ZnO NPs not only showed antibacterial activity but also inhibition of biofilm formation against *Pseudomonas aeruginosa* and *Enterococcus faecalis* at a concentration of 50 μg/mL, though higher activity was observed for the Gram-negative bacteria over the Gram-positive [35]. The green synthesis of ZnO NPs yields coated nanoparticles with enhanced antimicrobial activities. For example, ZnO NPs prepared in *Azariachta india* aqueous leave extract have a surface layer composed of terpenoids, flavonoids, phenolic acid, and proteins, that increases the concentration of H_2O_2 at the surface. Therefore, they present increased ROS activity and consequently better antimicrobial activity than bare ZnO produced using synthetic methods. Indeed, these nanoparticles presented antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabillis*, and *Bacillus subtilis* at micromolar concentrations [36].

6.2 POLYMERIC NANOMATERIALS

Polymeric nanomaterials can be composed of natural or synthetic polymers and generally present good biocompatibility, stability, the ability to encapsulate both hydrophilic and hydrophobic drugs, and the ability to be assembled into a varied array of three-dimensional nanostructures, such as micelles, vesicles, dendrimers, nanofibers, and nanogels.

6.2.1 *Natural polymers*

Natural polymers are materials that occur in nature and can be extracted from a variety of plant, animal, and microbial sources. They are biocompatible, biodegradable, and eco-friendly [37,38]. Indeed, most of these polymers are characterised by having a heteroatom backbone (*e.g.*, –C–O–, –C–N–, C–S–, among many others), which facilitates hydrolysis and bond cleavage. This kind of chemistry not only endows natural polymeric nanomaterials with control over drug release, but it also facilitates their excretion from the body [39]. Natural polymers fall into three major classes: polysaccharides (*e.g.*, chitosan, cellulose, starch, hyaluronan, dextran), proteins (*e.g.*, collagen, fibrin, elastin, soy protein), and bacterial polyesters (*e.g.*, poly-3-hydroxybutarate (P3HB), poly-4-hydroxybutarate (P4HB), poly-3-hydrovalerate (PHV) and copolymer poly-3-hydroxybutarate-*co*-poly3-hydrovalerate (PHBV)) [38]. Due to their physicochemical properties, such as solubility, hygroscopicity, viscosity, and easy derivatisation, polysaccharides are widely used in the production of nanomaterials for therapeutics and drug delivery purposes [40].

Polysaccharides are widely found across different biological organisms, spanning from the simplest prokaryotic cell to complex organisms like plants and animals. These natural polymers consist of monosaccharide units linked by glycosidic bonds. The most typical examples in biomedicine are chitosan, cellulose, hyaluronic acid, and dextran. However, the diversity of chemical structures that these polymers provide (different stereoisomerism, branching degree, degree of N-acetylation, degree of polymerisation, O-glycosidic bond type, etc.) and their ionic nature (cellulose and dextran are an exception for the latter feature), confer a broad spectrum of biological properties and activities (**Fig. 6.2**) [41].

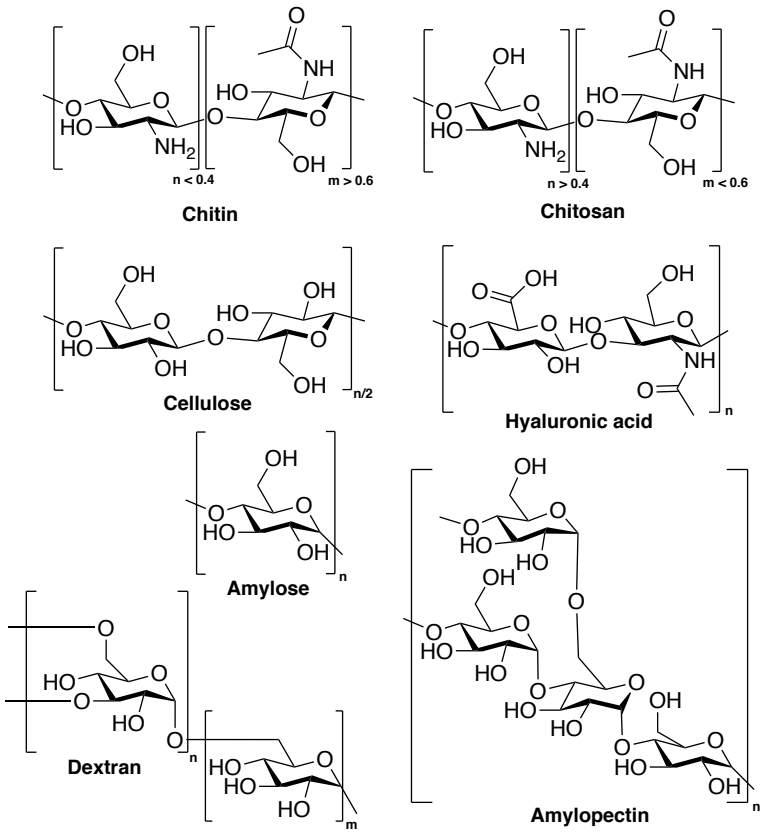

Figure 6.2. Chemical structures of some polysaccharides used in the synthesis of nanomaterials. *n* and *m* indicate the degree of polymerisation. Created with ChemDraw 21.0.0.28.

Chitin is the primary component of the crustaceans' exoskeleton, and it is also the second most abundant polysaccharide across living organisms. However, its insolubility in water and other common solvents limits its use in the field of nanomedicine. Chitosan, a chitin derivative obtained from partial *N*-deacetylation, is more commonly used since its solubility in acidic conditions is increased not only due to the protonation of the basic amino groups but also due to the decrease in polymer's molecular weight (from 1000-2500 kDa in chitin to 100-500 kDa in chitosan) [41]. In

line with this, chitosan has good biocompatibility and biodegradability, and, due to its greater positive net charge in mildly acidic conditions, it provides a greater antibacterial effect than chitin. The positively charged amines can electrostatically interact with negatively charged phospholipids, proteins, and carbohydrates present on the surface of the bacterial cell, and inhibit bacterial growth [42].

Chitosan can be used as an adjuvant or delivery carrier, protecting the carried molecules from enzymatic degradation [37]. For example, poly(lactic-*co*-glycolic acid) (PLGA)-chitosan composite NPs enhanced the antibacterial and antibiofilm performance of ciprofloxacin against *Enterococcus faecalis* [43]. On the other hand, composites of chitin/chitosan with metallic and metal oxide NPs (*e.g.*, Chitin/Ni, Chitin/Ag, Chitosan/Ag, Chitin/Cu, Chitosan/ZnO, and Chitosan/TiO_2) showed antimicrobial activity against both Gram-positive and Gram-negative bacteria [44–51].

Hyaluronic acid, naturally found in the extracellular matrix, is a hydrophilic linear polysaccharide composed of alternately linked D-glucuronic and *N*-acetyl-D-glucosamine units *via* β-1,3 and β-1,4 glycosidic bonds. Opposite to chitosan, hyaluronic acid is a negatively charged polysaccharide that undergoes rapid enzymatic degradation [52]. Bacteria often have the ability to adhere to wounds and plastic materials, but further spreading that enables penetration into deeper tissues requires the use of enzymes as invasion factors. Many pathogenic Gram-positive bacteria (*e.g.*, *Staphylococcus aureus*) produce hyaluronidase, an enzyme capable of degrading the hyaluronic acid present in the extracellular matrix and greatly enhancing bacterial infiltration. Baier and co-workers showed that hyaluronic acid-based nanocapsules containing the antimicrobial agent polyhexanide could be efficiently cleaved in the presence of bacterial hyaluronidase and efficiently killed the pathogenic bacteria [53]. When a sustained cargo release is desired, the conjugation of synthetic polymers, such as polyethylene glycol (PEG), to hyaluronic acid slows down and controls its degradation [54,55]. This approach, however, has been more extensively studied in the delivery of anticancer drugs since hyaluronic acid targets CD44 receptors, which are overexpressed in cancer cells [56–60].

Starch is a non-toxic complex polysaccharide formed by two different sugars: amylose and amylopectin. The former is a linear polymer of α-(1,4)-linked D-glucose units, while the latter is a highly branched polymer

composed of short chains of α-(1,4)-linked D-glucose with branches formed by α-(1,6) linkages at the branch positions [41]. Due to its insolubility, starch is generally used in combination with other polysaccharides, synthetic polymers, or metal ions, and the use of such composites shows great promise for biocide purposes in the food packaging industry due to abundant sourcing and low price [61–64]. In the biomedical field, the starch biopolymer is often used in the form of hydrogels decorated with inorganic NPs [57–61]. For instance, the incorporation of CuO NPs in carboxymethylated starch hydrogels yielded antibacterial activity against both Gram-positive and Gram-negative bacteria. In particular, hydrogels containing 2 wt.% CuO NPs also displayed low cytotoxicity and good biocompatibility [65]. Similarly, starch-based hydrogels reinforced with silica-coated copper NPs presented antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* for at least four cycles of use [66]. The possibility of using these hydrogel composites dermally paves the way for the development of wound dressing applications.

Cellulose is the most abundant polysaccharide and the primary constituent of plant cell walls, where it is mainly extracted from. Its linear polysaccharide structure is formed by β-(1,4)-linked D-glucose units, giving it a flat ribbon-like conformation. This β-glycosidic linkage enables the formation of inter- and intra-chain hydrogen bonds leading to a network that provides extra stability. At the nanoscale, cellulose finds numerous applications in drug delivery due to its unique physical properties (*e.g.*, good mechanical strength and stiffness), large specific surface area, low cytotoxicity, and renewability [63–65]. This configuration of cellulose coined the term nanocellulose and it can be found across the four main natural sources (*i.e.*, bacteria, plants, algae, and animals) in the form of fibrils or crystals [69].

Dextran is mostly obtained from *Leuconostoc mesenteroides* and its basic structure consists of main chains formed by (1,6)-α-D-glucose with various ratios of linkages and branches. Contrary to cellulose and starch, dextran is highly water soluble, which facilitates its use in the development of novel nanotherapeutics that can be taken orally or even by injection. Indeed, dextran sulfate sodium NPs have shown inherent antibacterial effects at minimum inhibitory concentrations (MICs) below 250 μg/mL against both Gram-positive and Gram-negative bacteria,

including *Escherichia coli*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Proteus vulgaris*, due to their ability to passively diffuse through the bacterial cell pores. The antimicrobial effect of the particles was almost equivalent to the standard antibiotic ciprofloxacin [70]. However, the main drawback of dextran is that larger molecular weights of 60 kDa are poorly excreted from the kidney and remain in the blood before being completely metabolised, so its clinical use should be restricted under this cut-off value [41]. In another example, amphiphilic dextran-decanoate NPs loaded with ciprofloxacin presented antibacterial activity against the oral bacteria *Enterococcus faecalis* at a MIC of 0.7 μg/mL, showing potential for the treatment and prevention of root canal infections [71].

Polyhydroxyalkanoates (PHA) are natural polyesters produced through enzyme hydrolysis by certain types of bacteria and other organisms as means of energy and carbon source during periods of detrimental conditions, such as lack of macro elements, trace elements, or oxygen, for their thriving [72,73]. From a chemical point of view, PHAs are constituted of *R*-3-hydroxy and *R*-4-hydroxy fatty acids with different chain lengths, spanning from three to fourteen carbon atoms [13,74] (**Fig. 6.3**). The degradation of these compounds produces the corresponding *R*-hydroxyalkanoic acids (such as 3-hydroxybutyric, 3-hydroxyvaleric and 4-hydroxybutyric acids) that are recognised as degradation products and are conveniently removed from the body. In fact, 3-hydroxybutyric that is naturally found in the human bloodstream [75]. It is noteworthy to mention that some PHAs show antimicrobial activity and their potency seems to be correlated with the chain length of the monomeric fatty acid. Although the mechanism still remains unclear, it has been attributed to their detergent-like properties which alter the permeability of the bacterial cell membrane [76–78]. These biogenic polymers outperform many others in the field of nanomedicine due to the lack of toxicity, biocompatibility, biodegradability, as well as high drug-loading capacity [72].

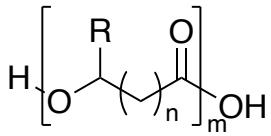

Figure 6.3. Schematic representation of polyhydroxyalkanoates (PHAs), where *R* represents an alkyl group, *n* the chain length of the repeating unit (up to $n = 8$), and *m* the degree of polymerisation. Created with ChemDraw 21.0.0.28.

Amongst PHAs, poly-3-hydroxybutyrate (PHB) has several advantages including biocompatibility and the ability to degrade *in vivo* and *in vitro* without the generation of harmful by-products, as it happens with the acidic degradation products of synthetic polymers, such as PLGA, that could lead to an inflammatory response [72]. Using electrospinning techniques, PHB can be tailored to form nanofibrous scaffolds as carriers for antibiotic drugs. For example, PHB nanofiber mats surface-loaded with kanamycin sulphate showed antimicrobial activity against *Staphylococcus aureus* by a sustained release of over 95% of the antibiotic drug within 8h [79]. Similarly, different porous morphologies of PHB electrospun meshes have been assessed for the release of levofloxacin against *Micrococcus luteus, Serratia marcescens*, and *Escherichia coli.* In nanofiber mats loaded with levofloxacin, a slower release was observed with 30.4% to 32.5% of the incorporated antibiotic being released during 24 hours and displaying bacterial killing efficacy against Gram-negative and Gram-positive bacteria [15]. Hence, sustained drug release with enhanced antimicrobial activity can be achieved for different fibrous nanostructures of the PHB polymer.

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) polymer has been proven as an effective material for bone tissue engineering due to its biocompatibility and slow degradation rate. Nanocomposites formulated with PHBV, nanodiamond, and nanohydroxyapatite loaded with vancomycin have been developed for bone fixation devices with antibacterial activity. When tested for the treatment and prophylaxis of bone infection, these nanocomposites guaranteed a sustained *in vitro* release of the antibiotic over 22 days [80].

The combination of PHA nanomaterials with inorganic NPs not only brought about an alternative to antibiotics for the treatment of infections but has also shown enhanced antimicrobial activity in some cases. For example, PHBV nanofibers had negligible activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* bacteria, but when loaded with 5-13 nm AgNPs showed antibacterial activity against both strains [16]. In addition, PHBV polymer extracted from a mixed bacterial culture has been demonstrated to be a good stabilising agent for the *in situ* preparation of AgNPs, since the antimicrobial activity of the NPs is dependent on their size, size distribution, and aggregation propensity. The AgNP-PHBV

materials presented strong antibacterial activity against *Salmonella enterica* at low concentration (0.1-1ppm) [80].

6.2.2 *Synthetic polymers*

As opposed to natural polymers, which are found in plant, animal, and microorganism sources, chemically synthesized polymers are non-naturally occurring polymers obtained by different synthetic methods. In the field of medical materials, polylactic acid (PLA), PLGA, polyurethane (PU), poly(methyl methacrylate) (PMMA), polyvinylpyrrolidone (PVP), silicone rubber, and polyvinyl alcohol (PVA) polymers, are the most used due to their biological compatibility (**Fig. 6.4**) [37].

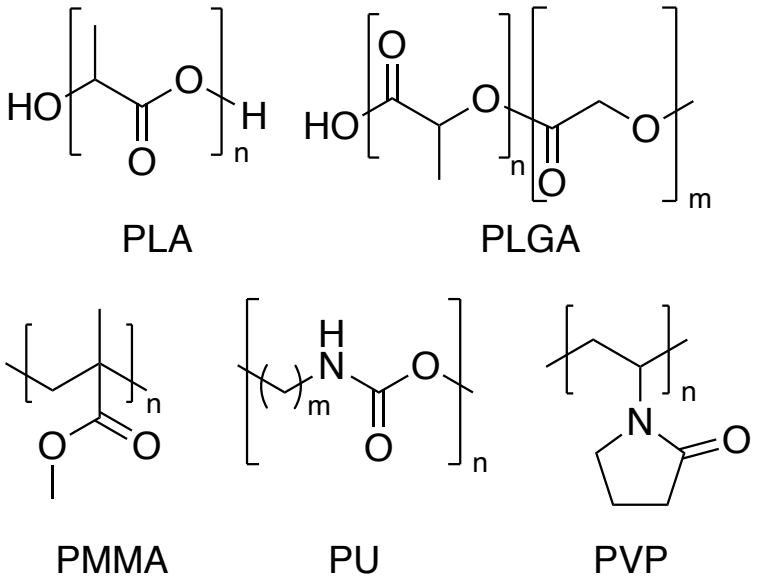

Figure 6.4. Chemical structures of the synthetic polymers most used in nanomedicine. PLA: polylactic acid. PLGA: poly(lactic-co-glycolic acid). PMMA: poly(methyl methacrylate). PU: polyurethane. PVP: polyvinylpyrrolidone. *n* and *m* indicate the degree of polymerisation. Created with ChemDraw 21.0.0.28.

PLA NPs can be synthesised by a wide range of processes that produce solid colloidal particles ranging from nanometre to micrometre in size. For example, double emulsion and salting out methods are suitable to produce micro-sized PLA particles, whereas nanoprecipitation and dialysis enable the formation of PLA NPs. Such methods, among many others, allow the encapsulation of bioactive molecules, such as genes, proteins, vaccines, and small molecule drugs [81]. PLA, PGLA, and their copolymers are recognised by the U.S. Food and Drug Administration (FDA) as non-toxic, biocompatible, and completely biodegradable. When used as carriers, due to their high structural integrity, the slow degradation of PLA and PLGA NPs enables the controlled release of drugs. For example, PLA NPs loaded with rifampicin and functionalised with a cationic peptide (poly-L-lysine) have shown to interact strongly with *Staphylococcus aureus*, under planktonic and biofilm modes of growth [18]. On the other hand, PLGA NPs loaded with 1,3-bis[3,5-bis(trifluoromethyl)phenyl]urea have recently been demonstrated to be a potent antimicrobial system against the cariogenic bacterial strain *Streptococcus mutans*, responsible for dental caries. The drug-loaded PLGA NPs not only significantly inhibited the growth and lactic acid production of planktonic *Streptococcus mutans*, but also *Streptococcus mutans* biofilms, while presenting low cytotoxicity [82].

The exceptional mechanical properties of PU materials (*e.g.*, elasticity, strength, and resilience) enable their use in the biomedical field, such as in the production of artificial organs, catheter interventions, and polymeric drug capsules. PU nanomaterials do not present inherent antimicrobial activity, yet such properties can be rendered by their functionalisation with quaternary ammonium salts or with metal-based NPs [83–85]. For example, antimicrobial PU catheters doped with AgNPs have shown good antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, eradicating up to 80% of the bacterial population when challenged in a killing test [86]. Notwithstanding, although PU materials are non-teratogenic, non-allergenic, and present low cytotoxicity, they cannot be naturally degraded, which implies environmental pollution [30]. To overcome this drawback, biodegradable PU materials, such as starch and cellulose derivatives of PU have been developed. In fact, urethane-functionalised starch is stated to improve the properties of native starch such as pore size, volume, and binding

sites [37,87]. Desai and co-workers developed cross-linked starch polyurethanes as nanocarriers for anti-tuberculosis drugs and showed that streptomycin-loaded and isoniazid-loaded nanocrystals were 42 and 7 times more active against *Mycobacterium tuberculosis* H37Rv strain than the respective free drugs, respectively. A pH-dependent drug-release study showed that the antibiotic-loaded starch-derived nanopolyurethanes have a sustained release profile for isoniazid, rifampicin, and pyrazinamide, whereas burst release was observed for streptomycin, suggesting that the drug is present in the nanosphere's outer layer [87]. Several other hybrid starch-based PU nanocomposites in combination with inorganic NPs have been designed and tested satisfactorily against Gram-positive and Gram-negative bacteria [88,89].

PVP is a water-soluble polymer made from *N*-vinylpyrrolidone monomer. Approved by the FDA, PVP attracts considerable attention because of its excellent chemical and physical properties. In this sense, PVP has been widely studied as a stabiliser of AgNPs since it has an impact on the control of the reduction rate of the Ag ions and the aggregation process of Ag atoms during nanoparticle synthesis. Hence, hybrid nanomaterials based on PVP and AgNPs not only present antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but they also have shown antifungal activity against *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata* and *Aspergillus brasiliensis* [90–92].

Synthetic polymers open the possibility of mimicking the three-dimensionality of naturally occurring nanostructures, such as micelles and vesicles, or even creating novel artificial nanostructured scaffolds, such as dendrimers (**Fig. 6.5**).

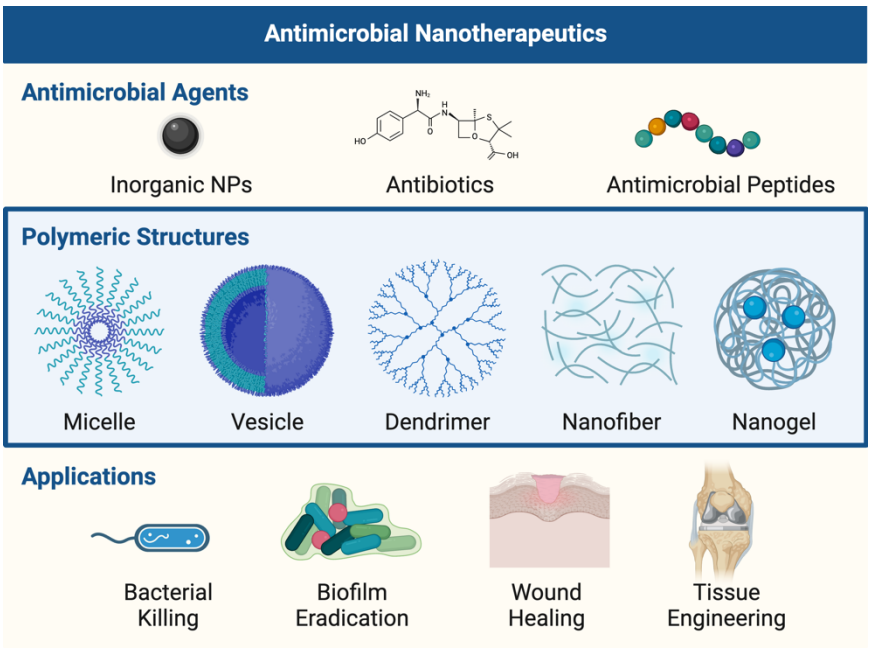

Figure 6.5. Polymeric nanomaterials can take the form of different 3D structures, such as micelles, vesicles, dendrimers, nanofibers and nanogels. As such, they can incorporate a variety of antimicrobial agents, including metallic and metal oxide NPs, antibiotics, and antimicrobial peptides, finding applications in the eradication of planktonic and biofilm-forming bacteria, wound healing, and tissue engineering. Created with BioRender.com.

Polymeric micelles and vesicles are supramolecular structures that are easily obtained through the self-assembly of amphiphilic block copolymers into a monolayer, in the case of micelles, or a bilayer, in the case of vesicles [93]. Polymeric micelles are formed by a hydrophobic core and a hydrophilic external layer solvated by water. Apart from the most known spherical shape in which micelles can assemble, other geometries such as cylinders and nanoplates can be adopted depending on the structure of the individual amphiphile and its packing configuration when assembled [93]. Independently of the shape, the hydrophobic core facilitates the encapsulation of hydrophobic drugs (*e.g.*, poor-water soluble antibiotics, antimicrobial peptides, and AgNPs), whereas the

hydrophilic surface may be functionalised with ligands by both covalent and non-covalent interactions [94–96]. For example, in the field of photodynamic therapy, lipase-sensitive methoxy poly(ethylene glycol)-*block*-poly(ε-caprolactone) (mPEG-PCL) micelles have been developed to encapsulate and deliver the water-insoluble photosensitizer hyprocrellin A (HA) drug to treat *MRSA* infections. These micelles showed better anti-*MRSA* activity in periodontal infections in mice than the HA drug by itself. In addition, light irradiation of the HA-loaded mPEG-PCL micelles enhanced its antibacterial effect by triggering ROS production [97].

Polymeric vesicles or polymersomes are closed hollow nanostructures with a bilayer membrane composed of a hydrophobic portion separating two hydrophilic blocks that face the aqueous core or the outer aqueous phase (resembling a cell membrane). The molecular composition and length of the hydrophilic and hydrophobic blocks determine the vesicle properties, such as size, stability, rigidity, and biodegradability. As for micelles, polymersomes may adopt spherical or non-spherical structures [98] (**Fig. 6.6**).

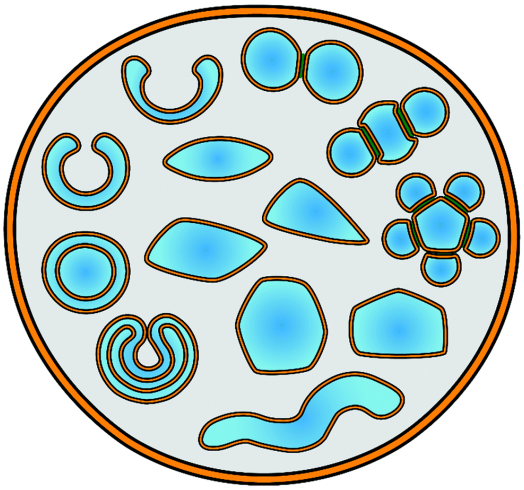

Figure 6.6. Schematic representation of non-spherical polymersomes engulfed within a larger spherical polymersome. The hydrophobic and hydrophilic domains are depicted in yellow and black, respectively. Water is represented in blue. The water compartment of the larger outer spherical polymersome is omitted for clarity. Reprinted with permission from [98].

As an additional benefit compared to micelles, polymersomes are capable of encapsulating both hydrophilic drugs in the lumen and hydrophobic drugs in the membrane. For example, poly (2-(methacryloyloxy) ethyl-phosphorylcholine)-*co*-poly(2-diisopropylamino)ethyl methacrylate) (PMPC-PDPA) vesicles have been successfully loaded with a large variety of antimicrobial agents, including proteins (*e.g.,* lysostaphin), small peptides (*e.g.,* vancomycin), glycols (*e.g.,* gentamicin), poorly water-soluble organic molecules (*e.g.,* rifampicin), and functionalised pyridines (*e.g.,* isoniazid), to treat intracellular infections caused by *Staphylococcus aureus*, *Mycobacterium bovis*-attenuated Bacillus Calmette–Guérin (BCG), *Mycobacterium marinum*, and *Mycobacterium tuberculosis* [99].

Dendrimers are nano-sized, monodisperse, and highly branched macromolecules with a central core surrounded by radially symmetric peripheral groups [100]. The repeated growth reactions during synthesis lead to a higher and tuneable degree of branching (*i.e.*, higher surface polyvalency), which enables them to engage in multivalent interactions [100]. Dendrimers have the ability to control and engineer "critical nanoscale design parameters", overcoming limitations associated with other polymeric nanocarriers, such as high polydispersity, poorly controlled shape and/or surface chemistry, and inability to make systematic size modifications below the "critical micelle concentrations" (i.e., 30-40 nm) [101]. The unique structure of dendrimers not only provides special opportunities for host-guest chemistry but also renders them promising scaffolds for drug loading within their nanocavities [94]. Dendrimers can be prepared by two different synthetic pathways: convergent and divergent. In convergent synthesis, dendrons, the branched repeating units, are first synthesized individually (up to the desired degree of branching) and then linked to a multifunctional core molecule, which will be at the centre of the dendrimer. Conversely, in divergent synthesis, the dendrimer grows outwards from the multifunctional core by the subsequential addition of new repeating units. [102]. Many antimicrobial agents including antimicrobial peptides, silver and metal-oxide NPs, and commercial antibiotics have been successfully loaded within dendrimers [94]. For example, poly(amidoamine) (PAMAM) dendrimers loaded with sulfamethoxazole enhanced the aqueous solubility of the drug by a factor of 40, in addition to increasing its antibacterial activity against *Escherichia coli* by a factor of

4 and 8 in DMSO and 0.01M NaOH solutions, respectively [103]. On the other hand, platensimycin (PTM)-loaded PLGA and PAMAM dendrimers showed an enhanced antimicrobial activity against *MSRA*, as well as improved pharmacokinetics and reduced cytotoxicity when compared to the free antibiotic [104] (**Fig. 6.7**).

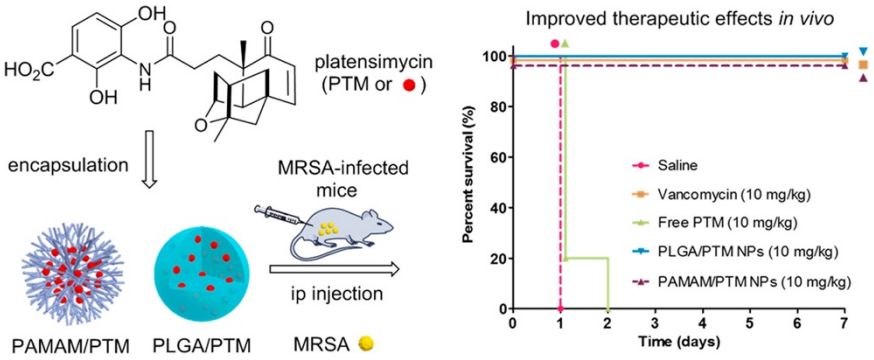

Figure 6.7. Schematic representation of PTM-loaded PLGA and PAMAM dendrimers and percentage of survival of mice infected with *MRSA* 7-days post-infection. Reprinted with permission from [104]. Copyright 2020 American Chemical Society.

Finally, dendrimers can be, *per se*, effective antimicrobial agents. As for cationic antimicrobial peptides, dendrimers with positively charged surfaces usually have strong interactions with negatively charged bacterial cell membranes [94]. For instance, a cationic dendrimer designed by capping a mannose-functionalised PAMAM dendrimer with a quaternary ammonium compound, 1-hexadecyl-azaniabicyclo[2.2.2]octane (C_{16}-DABCO), showed to inhibit the growth of *Streptococcus oralis*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Escherichia coli* at low micromolar MICs. Moreover, the activity of the dendrimer against these microorganisms is 10 times more potent than C_{16}-DABCO alone [105].

Polymeric nanofibers are one-dimensional nanostructures whose diameter is less than 100 nm. This class of nanomaterials exhibits a high surface-to-weight ratio, which makes them suitable for a wide range of applications in the medical field, such as antimicrobial materials for wound healing [94,106]. Polymeric nanofibers can be prepared through

different techniques, including self-assembly [107], template synthesis [108], thermal-induced phase separation [109], melt-blown [110], and electrospinning [111]. The latter is a superior technique for preparing nanofibers from polymer solutions, with proper control over their chemical composition and diameter, in addition to its simplicity and versatility [112]. The simplest method to incorporate antimicrobial agents into nanofibers is by the solubilisation/ suspension of the target agents in the electrospinning precursor polymer solution [94]. Different blends of antibiotics (*e.g.*, ampicillin and ciprofloxacin) can be combined with different nanofiber mats to render antimicrobial activity against both Gram-positive and Gram-negative bacteria [113,114]. However, one of the main drawbacks of these systems is the burst release of the loaded drugs, which limits their application when sustained mid- to long-term antimicrobial activity is desired. Polymers can also be combined with other nanomaterials, such as metallic NPs or carbon nanotubes, before electrospinning, opening new avenues to combat antimicrobial resistance [115].

Finally, nanogels or hydrogels are crosslinked, water-swellable, biocompatible polymeric networks that can swell or shrink upon external physical or chemical stimuli [116,117]. Their tuneable size, from nanometres to micrometres, their large surface area for multivalent bioconjugation, and their stimuli-responsive swelling and collapsing triggered by external factors (*e.g.*, pH, temperature, enzymes, or ionic strength) provide them unique advantages for on-demand delivery of antimicrobial agents [94,116]. Nanogels can be synthesized by a myriad of methods depending on the raw materials adopted, yet their synthesis strategies roughly fall into three major groups: monomer polymerisation, physical or chemical cross-linking, and template-assisted nanofabrication [118]. The obtained three-dimensional aqueous network structure of nanogels enables the incorporation of small bioactive molecules, as well as macromolecules such as DNA and proteins [119,120]. However, nanogels loaded with small molecules have seen more significant development compared to their biomacromolecule-loaded counterparts, primarily because the latter exhibit greater instability due to their large molecular weight and complex structure [120–122]. At the target site, drug release occurs through three main mechanisms: (1) gel

degradation; (2) gel expansion and drug diffusion; and (3) cleavage of drugs covalently linked to the nanogel matrix [123].

In line with this, encapsulation of conventional antibiotics into nanogels has been widely studied aiming to protect drugs from degradation and enhance their targeted delivery to infected tissues at therapeutic concentrations. [19,124]. For example, several studies have used nanogels to deliver the last resort antibiotic vancomycin in higher dosages as a tactic to overcome drug resistance [118,125–127]. Xiong and co-workers developed a bacterial-lipase sensitive polymeric triple-layered nanogel (TLN) using the amphiphilic diblock copolymer mPEG-PCL to initiate the ring-opening polymerisation of the difunctional monomer 3-oxapentane-1,5-diyl bis(ethylene phosphate) (DEGDP). In this manner, hydrophobic PCL segments collapsed and surrounded the cross-linked polyphosphoester core of DEGDP, leading to a compact and hydrophobic matrix that behaves as a molecular fence and prevents non-specific drug leakage. Therefore, only when the TLN senses lipase-secreting bacteria, will the PCL fence degrade to release loaded vancomycin [127]. The same research group reported a strategy to target macrophages infected with *MSRA* using mannosylated nanogels with a polyphosphoester crosslinked core and encapsulated vancomycin. The antibiotic is only released in the presence of bacterial phosphatase and phospholipases which efficiently degrade the nanogel core (**Fig. 6.8**) [126].

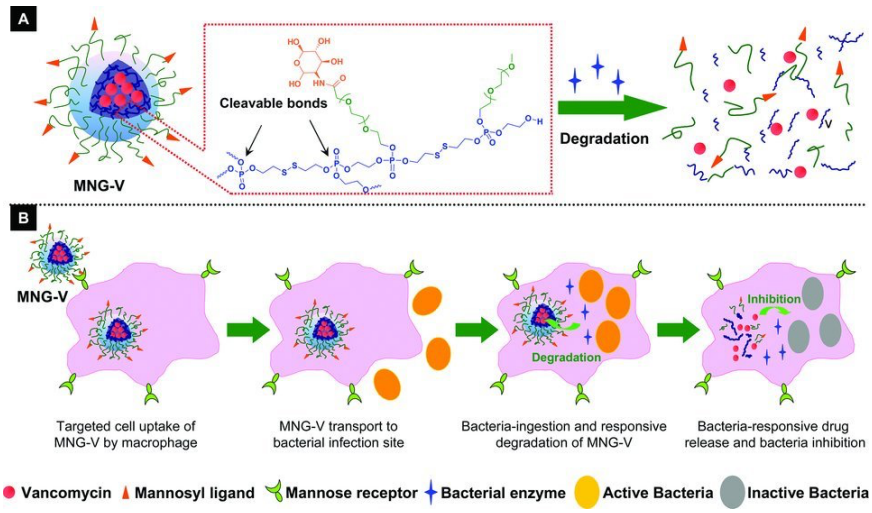

Figure 6.8. (A) Schematic representation of vancomycin-loaded mannosylated nanogels (MNG-V) and antibiotic release by the action of bacterial phosphatases and phospholipases. **(B)** Schematic illustration of nanogel-uptake by macrophages, intracellular trafficking, nanogel degradation, vancomycine release and bacterial killing. Reprinted with permission from [126].

As for antibiotics, the incorporation of AgNPs in nanogels renders their controlled and sustained release while limiting nonspecific cellular uptake and possible cytotoxicity [118]. For example, bactericidal activity against *Staphylococcus aureus* and *Escherichia coli* has been achieved by the photoresponsive release of AgNPs from chitosan and aniline nanogels [128,129] (**Fig. 6.9**).

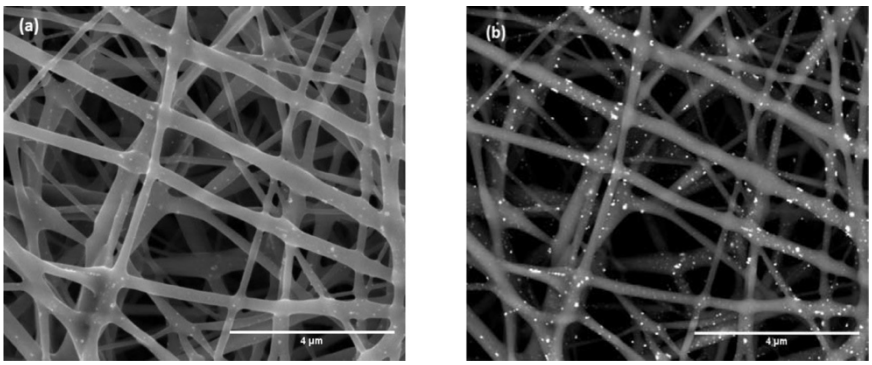

Figure 6.9. Field emission scanning electron microscopy (FE-SEM) image of PCL nanofiber mats with AgNPs-nanogels immobilised onto their surface. **(a)** secondary and **(b)** backscattering electrons show the difference between electronic density of the AgNPs-nanogels and the PCL nanofibers**.** Reprinted with permission from [129].

Many efforts have also been made in the design of environmentally friendly hydrogel nanocomposites by using biodegradable synthetic polymers, such as poly(*N*-isopropylarclamide) (PNIPAAm), or natural-occurring polymers, such as polysaccharides [130–134]. For instance, agarose-based hydrogels containing tannic acid-Fe(III) NPs presented excellent photothermal conversion capability, being able to kill 99% of bacteria within 10 min of near-infrared radiation (NIR). The bactericidal

effect was evaluated *in vitro* and *in vivo* using mice with *Staphylococcus aureus*-infected wounds [132].
Alike other nanostructures, hydrogel applications go beyond antibiotic delivery. Nanogels with intrinsic antimicrobial moieties, such as surface-functionalised with quaternary ammonium compounds or cationic guanidine groups, have been developed to physically damage the bacterial cell membrane (*i.e.*, generate pores and alter permeability) and, ultimately, cause bacterial cell death [118,135–139].

6.2.3 *Polymer-antibiotic conjugates*

As illustrated by the many examples above, the physicochemical interactions between an antibiotic and its carrier offer a means to entrap these bioactive agents in polymeric nanomaterials. However, this approach has two main drawbacks: the loading capacity, often limited to ≤10% weight, and the occurrence of burst release, in which more than 50% of the drug can be released within 24 hours [140].
On the other hand, the covalent ligation of small bioactive molecules to polymeric carriers (*i.e.*, drug-polymer conjugates) offers unique advantages, such as controlled drug release, active intracellular delivery, prolonged drug circulation in the bloodstream, reduced immunogenicity, enhanced stability, and the potential for targeted delivery through the incorporation of targeting moieties. In addition, the kinetics of drug release can be tailored based on the bonds that link the antibiotic to the polymer (*e.g.*, ester, amide, urethane), the type of formulation (*e.g.*, powder, hydrogel, coating, microsphere), and the polymer chemical composition. Beyond linker chemistry, numerous other physicochemical factors, including steric effects, polymer molecular weight, and nanocarrier composition, play a significant role in drug cleavage and release rates [140,141]. A thorough assessment and careful selection of all system design parameters should be conducted to allow a proper spatiotemporal control over the drug release.
Norfloxacin (NOR) covalently linked to dextran with one of two tetrapeptide linkers (*i.e.*, Gly-Phe-Ala-Leu-NOR or Gly-Phe-Leu-Gly-NOR) is an example of an early study, in which the bioactive agent was linked through an amide bond susceptible to lysosomal conditions. The use of cathepsin B at pH 5.5 during 7h, however, did not promote the release of free drug in any of the two systems, but of the dimers Leu-NOR

(80%) and Gly-NOR (25%) [142]. When the linker was replaced by a methoxy-terminated Gly-Phe-Gly-Gly linker, in which NOR was covalently bound to the α-carbon of Gly at the C-terminus, and mannosylated dextran polymer was used, NOR was released as a free drug – 27% release at pH 7.4, 40% release at pH 5.5, and ≈65% release at pH 5.5 in the presence of Cathepsin B, in a 24h-period [143].
In a different study, polymeric nanobiotics composed of an isoniazid (INH)-conjugated α-keto polyester and encapsulated clofazimine (CFZ) showed to efficiently reduce granuloma and bacterial burden in a zebrafish model of mycobacterial infection, outperforming the combination therapy with the free drugs at the same concentration [144]. (**Fig. 6.10**).

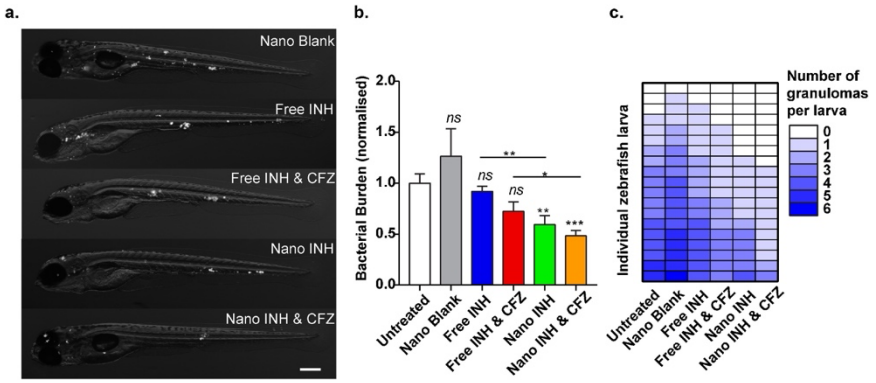

Figure 6.10. Effect of nanobiotics at 3 days post infection on zebrafish infected with fluorescently-labelled *M. marinum*. **a.** representative images (scale bar, 200 μm). **b.** quantification of bacterial load (results plotted as mean ± SEM from 2 independent experiments; n = 21). **c.** Quantification of granuloma number at 3dpi. Results are plotted as mean ± SEM from 2 independent experiments (n = 19)**.** Reprinted with permission from [144].

Drug-conjugated hybrid nanomaterials have also been developed for drug delivery applications. For instance, azithromycin (AZM)-conjugated clustered nanoparticles (AZM-DA NPs) have been developed as therapeutic agents for the treatment of *Pseudomonas aeruginosa*-induced chronic lung infection. AZM-DA NPs are prepared by the electrostatic complexation between AZM-conjugated poly(amidoamine) dendrimer

(PAMAM-AZM) and 2,3-dimethyl maleic anhydride (DA)-modified poly(ethylene glycol)-*block*-polylysine (PEG-*b*-PLys). In an acidic biofilm microenvironment (pH 6.0), AZM-DA NPs disassemble and promote the release of the secondary cationic PAMAM-AZM NPs. The small size and positive charge of PAMAM-AZM NPs enhanced the permeabilisation of both inner and outer bacterial membranes and, consequently, increased the internalisation of AZM as compared to the free AZM (**Fig. 6.11**) [145].

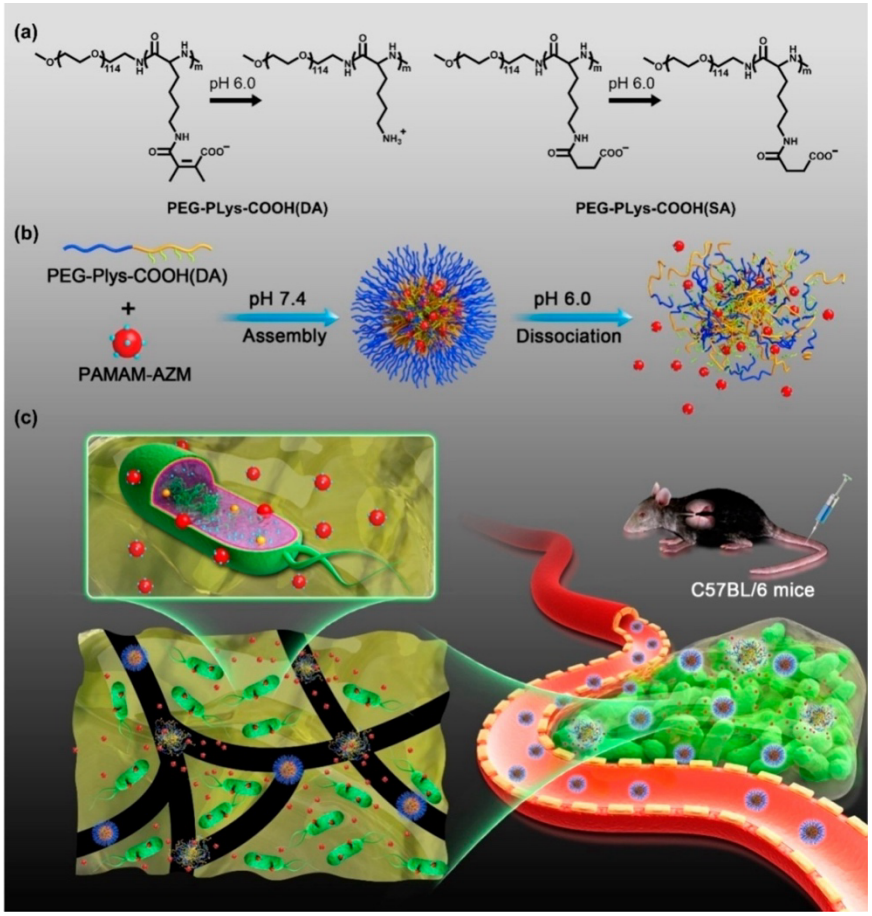

Figure 6.11. (a) Change of chemical structure of 2,3-dimethyl maleic anhydride (DA) and succinic acid (SA) (negative control) modified poly(ethylene glycol)-block-polylysine (PEG-b-PLys) in acidic pH; **(b)** Illustration of the self-assembly of azithromycin-conjugated clustered (AZM-DA) NPs at pH 7.4 and release of

secondary AZM-conjugated poly(amidoamine) dendrimer (PAMAM-AZM) NPs in an acidic biofilm microenvironment; **(c)** Illustration of the accumulation of AZM-DA NPs in biofilms and subsequent release of PAMAM-AZM NPs for enhanced biofilm penetration, permeabilisation of the bacterial membrane, and increased AZM internalization**.** Reprinted with permission from [145]. Copyright 2020 American Chemical Society.

6.3 LIPOSOMES AND LIPID-BASED NANOPARTICLES

Lipids are a wide group of small biomolecules that can be either hydrophobic or amphiphilic and play a vital role in physiological and pathophysiological events of living systems [146,147]. As for amphiphilic block copolymers, amphiphilic lipids, such as phospholipids, spontaneously self-assemble into ordered lyotropic liquid-crystalline phases (*e.g.*, monolayers, lipid bilayers, micelles, liposomes, and tubules) in the presence of water [148]. Liposomes are spherical hollow nanostructures composed by at least one phospholipid bilayer with an inner aqueous compartment. Like polymersomes, liposomes can deliver both hydrophilic and hydrophobic drugs. Liposomes building blocks are charged or neutral lipids that can be natural, such as L-*α*-phosphatidylcholine (PC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), or synthetic, such as 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [149]. With recognised biocompatibility and biodegradability, liposomes have been approved for clinical use for almost 30 years now [150].

In a study by Sande *et al.*, the anti-staphylococcal activity of vancomycin-loaded liposomes has been investigated through two different liposome formulations: (1) dicethylphosphate (DCP) liposomes, composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), DCP, and cholesterol; and (2) dimyristoylphoshatidylglycerol (DMPG) liposomes, composed of DSPC, DMPG and cholesterol. The DCP and DMPG liposomal vancomycin formulations showed a two-fold to four-fold decrease in MICs compared to the free drug, when tested against the hospital associated-*MRSA* strain NRS-35 and the community-associated *MRSA* strain LAC. The DPC-based liposome formulation was the most efficacious, showing bacterial killing efficacy against a seven *MRSA*

strains. The authors suggested that the increased bacterial killing efficacy of the liposomes might be related to the fusion of liposomal components with the bacterial cell wall enabling an increased amount of antibiotic to reach the cytoplasm [151].

Mycobacterium abscessus nontuberculous infections have risen considerable concern since this human pathogen, responsible for a wide spectrum of soft tissue infections, is naturally resistant to many common antibiotics [152]. Rifampicin is a broad-spectrum antibiotic and a first line drug used for the treatment of drug-susceptible Tuberculosis, with proven antibacterial activity against intracaseum bacilli [156]. However, rifampicin is not suitable for the clinical management of *Mycobacterium abscessus* lung disease, since this bacterium possesses monooxygenase enzymes capable of inactivating this antibiotic [154,155]. However, liposomal formulations composed by hydrogenated phosphatidylcholine (HSPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol (DPPG) loaded with rifampicin significantly increased drug intramacrophage bioavailability and reduced intracellular mycobacterial viability, when compared to the free drug [156]. In a recent review article, Gosh and De present a list of FDA-approved liposome-based antibiotic delivery systems [157].

Other types of lipid-based antimicrobial delivery systems, such as lipidic micelles [158], vitamin-lipid NPs [159], solid lipid NPs (SLNs) [160], nanostructured lipid carriers (NLCs) [161], and lipid-coated hybrid (LCH) NPs [162], have been developed for the eradication of wide number of bacterial infections, including the ones caused by *Helicobacter pylori*, *MSRA*, *Echerichia coli*, *Pseudomonas aeruginosa*, amongst others.

6.4 CONCLUDING REMARKS

The rapid emergence of antibiotic-resistant bacteria poses one of the greatest threats to public health as conventional therapies and commercial antibiotics are dropping their effectiveness. In the race for the discovery of new strategies to prevent a scenario in which commonplace infections prove fatal, nanomaterials stand in the limelight due to their unique physicochemical properties that can be seized to overcome common resistance mechanisms.

Nanoparticle-driven drug delivery emerges as a beacon of hope, shielding antibiotics from enzymatic degradation, enhancing their targeted delivery to afflicted sites in therapeutically potent concentrations, and minimising undesired side effects. Drugs can either be entrapped or chemically conjugated to NPs, with the latter offering a myriad of possibilities in orchestrating spatiotemporal controlled release of the therapeutic payload. Meanwhile, nanomaterials can also display intrinsic antimicrobial properties, either by direct disruption of bacterial cell membranes (*e.g.*, nanoparticles functionalised with cationic groups) or by instigating the generation of ROS (*e.g.*, metallic nanoparticles).

The clinical implementation of nanotherapeutics still faces considerable challenges, mainly related with their complex chemistry and polydispersity, which poses difficulties related to cost-effectiveness, scale-up, and Chemistry, Manufacturing, and Controls (CMC) management. Still, the development of computational approaches allowing a better understanding of nano-bio interactions and predictive biodistribution, pharmacokinetics, and toxicology, along with a harmonised international regulatory framework, is expected to facilitate clinical translation in the near future.

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