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Abstract

Microbial colonization on biomaterials is the main cause of failure of a successful implantation. In fact, local infections can eventually evolve in severe sepsis that might finally end up in a multi-organ failure and death of the patient. Besides, infection has become one of the toughest problems in the medical world, as microorganisms become more resistant to known drugs. Scientific research has been focussing on exploring new strategies to combat this life-threatening problem. In this review, information was collected about currently used polymeric biomaterials in the medical field and the main bacterial infections associated with their implantation. Furthermore, drug-free strategies to overcome this complication are explored, and the existing methodology required for assessment of the antibacterial activity is also described.

Introduction

The emergence of antibiotic-resistant infections is a continuous threat to public health. Despite the efforts made by the World Health Organization, antimicrobial resistance (AMR) is still a real problem that threatens the successful prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi.[1,2] The impact of AMR on patients in medical institutions is a serious challenging problem that leads to increased morbidity and mortality rates. In fact, currently in the European Union, AMR infections cause approximately 25,000 deaths per year. Globally, the mortality rate is 700,000 people, but it has been estimated that deaths attributable to AMR infections will probably rise from the current estimate to ten million lives annually by 2050.[3]

Moreover, AMR induces dramatic changes in the healthcare system through augmentation of costs associated with prolonged hospital stays, and implementation of safety, hygiene and environmental protective habits. In hospital surveillance programs the most commonly tracked AMR microbes are bacterial species (*e.g. Staphylococcus aureus*, *Enterococcus* spp., *Clostridium difficile*, *Escherichia coli* and *Klebsiella pneumoniae*).[4] Bacterial infections are particularly problematic because several bacterial strains can easily and rapidly mutate their genes obtaining an increasing resistance to a wide-spectrum of currently used antibacterial drugs.

Nowadays, medical practice is dependent on a large number of instruments, devices and implants. Biomaterials used to produce medical devices (*e.g.* pacemakers, biosensors, artificial hearts, blood tubes) and implants (*e.g.* sutures, bone plates, joint replacements, ligaments, vascular grafts, heart valves, intraocular lenses, dental implants) are widely applied to improve the quality of life of patients for the replacement or regeneration of traumatized/degenerated tissues/organs, assistance in healing, or improvement of tissue functions and/or correction of abnormalities. Unfortunately, these materials often present an optimal surface for bacterial adhesion leading to the biofilm formation. Biofilm structures are characterized by a complex community interaction that provides microbes with a high tolerance to antibiotics and immune cells.[5] Therefore, resistant bacterial strains continue to emerge and cause extreme infections to humans. Despite the presence of advanced sterilization procedures, it is still complicated to eradicate bacteria and maintain sterility of materials for biomedical applications, without frequent use of disinfectants.[6] The incidence of implant-related infections is constantly increasing mainly due to the growing number of orthopaedic replacements in the aging population and its longer residency time inside the patient (continuous risk for infection during their implanted lifetime).[7] A notable example of the growing request is the number of total hip replacements in the USA, that in a period of only 17 years increased two-fold, and the number of total knee arthroplasties increased almost five-fold.[8] Thus, there is an urgent need to develop new strategies to solve this challenging situation.

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The use of potent antibacterial materials that are effective against a broad range of pathogenic bacteria could help to mitigate and/or eradicate these infections. In this context, drug-free polymeric materials with intrinsic antibacterial properties have gained interest from both academic and industrial point of view. Antibacterial polymers that are biocompatible and provided with suitable physicochemical properties can be used in bioactive medical devices for diverse biomedical applications. Moreover, the use of the intrinsic antibacterial capacity of drug-free antibacterial polymers is a great strategy to mitigate the AMR infections in the society.

In this review, we collected information about common polymeric biomaterials used in the medical field and the main bacterial infections associated with their implantation.

Furthermore, possible drug-free strategies to overcome bacterial infections were also proposed, and the methods for biological characterization of biomaterials in respect to different pathogenic strains were described.

Polymeric biomaterials in the biomedical field

Medical grade polymers are biocompatible materials that can be either bioresorbable or biostable, depending on their ability to degrade or not to degrade once implanted *in vivo*. In the following subparagraphs, these two classes of medical grade polymers are described.

Bioresorbable polymers

Bioactive biomaterials are typically designed to elicit an effective interaction with tissues, provoking physiological responses such as cell growth and/or cell differentiation at the site of implantation.[9] Bioactive biomaterials are generally bioresorbable as they degrade *in vivo* through progressive reduction of their molecular weight, triggered by the biological environment (*e.g.* the presence of water and/or specific degradative enzymes), and further bioresorption, as the degradation residues are eliminated by the metabolic pathways of the organism.[9]

Bioresorbable polymers can be synthetic, *i.e.* industrially synthesised, or of natural origin, *i.e.* derived from natural sources. One of the most common bioresorbable synthetic polymers is poly(lactic acid) (PLA), also known as polylactide, which is synthesised from lactide monomers.[10] When in the body, PLA degrades into lactic acid, a non-toxic chemical product, which occurs naturally in the body.[10] For its degradation into non-toxic products, PLA has been used in medical implants in the form of anchors, screws, plates, pins, rods, and as meshes.[11] Depending on the PLA stereochemistry, the polymer may degrade inside the body within 6 months (amorphous polymer) to 2 years (semi-crystalline polymer). A gradual degradation rate is desirable to support structures, as they gradually transfer the load to the local tissues (*e.g.* bones in case of bone remodelling) while the treated area heals.[12] PLA is subjected to a bulk hydrolytic degradation mechanism that means that the degradation rate is faster inside the polymer bulk than on its surface; hence, the implant keeps its shape, up to the last steps of its life-time before fragmentation and complete degradation.

Polycaprolactone (PCL) is a semi-crystalline polyester with good organic solvent solubility, a melting temperature of 55-60°C and glass transition temperature of −54°C. Due to a low *in vivo* degradation rate and high drug permeability PCL can be used in long-term implant delivery devices.[13] Current research is being conducted into the development of micro- and nano-sized drug delivery vehicles, but the average degradation rate (2-3 years) is a significant issue for pure PCL products to be approved by the Food and Drug administration (FDA) for this issue.[13] PCL is often blended or copolymerized with bioresorbable polymers like other polyesters or polyethers to expedite polymer erosion.[13]

Polyglycolic acid or polyglycolide (PGA) which degrades into glycolic acid is another type of bioresorbable polymer usually used for bioresorbable sutures.[10] The material can be copolymerised with lactic acid to form poly(lactic acid-co-glycolic acid), with ε-caprolactone to form poly(glycolide-co-caprolactone), and with trimethylene carbonate to form poly(glycolide-co-trimethylene carbonate).[10] PGA is highly semi-crystalline and relatively more hydrophilic, and degrades rapidly through a bulk degradation mechanism by hydrolysis, generally within 6-8 months, depending on the molecular weight and the crystallinity degree.

Polyhydroxylalkanoates (PHAs) are polyesters synthesized by many gram-positive and gram-negative bacteria from at least 75 different strains.[14] These polymers are accumulated within cells to levels as high as 90% of the cell dry weight under conditions of nutrient stress and act as a carbon and energy reserve.[14] As they are generally biodegradable, PHAs are attractive as biomaterials for applications in both conventional medical devices and tissue engineering.[15] PHAs generally degrade through a surface erosion mechanism by hydrolysis due to their hydrophobicity.

Polyurethanes (PUs) are a large class of polymeric materials that contain a urethane moiety in their chemical repeating structure. PUs are composed by three main monomers: a diisocyanate, a macrodiol (which is an oligomeric macromonomer) and a chain extender. These monomers react to form linear copolymers, showing a two-phase structure in which hard segment-enriched domains (derived from the reaction of the diisocyanate and the chain extender) are dispersed in a matrix of soft segments (macrodiol moieties). The particular molecular architecture and the intrinsic properties of each constituent influence PU degradation rate.[16] PUs are known to undergo hydrolytic degradation due to the susceptibility to hydrolysis of urethane and urea linkages present in the main chain.[17,18] Particularly, the polyol (macrodiol) chemical structure which forms the soft segments is the main responsible for the hydrolytic degradation of biodegradable PUs: PCL diols, poly (ethylene glycol) (PEG) diols, poly (propylene glycol) diols, or polyols based on hydroxy acids such as glycolic acid, lactic acid and their copolymers are generally employed for biodegradable PU synthesis. The PU degradation kinetics is affected by hydrophilic (*e.g.* PEG) or hydrophobic (*e.g.* PCL) nature of polyols: the higher is the content of hydrophobic polyols, the lower is the water uptake and the degradation rate.[19] Moreover, when using polyether polyols in PU synthesis, PUs are subjected to oxidative degradation of their ether linkages because of several biological events (*i.e.* monocyte recruitment, differentiation into macrophages and release of biologically active molecules).[20] Additionally, by incorporating chain extenders based on amino acids or enzymatically cleavable peptides into the PU structure, PUs with degradable hard segments have been developed to enhance enzyme mediated degradation.[21,13]

Natural polymers, such as collagens, cellulose, chitosan, *etc*. may also be used for antibacterial applications. Chitosan, a hydrophilic biopolymer industrially obtained by N-deacetylation of chitin, found in shrimp and other crustaceans, can be applied as an antimicrobial material.[22] Cellulose can be extracted from plants and it is composed of beta-linked D-glucose units.[23] Collagens are the main structural proteins in animal connective tissue.[23,24] Antibacterial properties can be conferred to polymers, for example, by functionalizing polymers with antibacterial agents such as silver nanoparticles preparing polymer composites, or by conjugating antibacterial groups to synthetic polymers.[22,25-27] Table 1[28] illustrates advantages and disadvantages of natural polymers.

Table 1. The advantages and disadvantages of natural polymers.[28]

Advantages	Disadvantages
No severe systemic toxicity	Possibility to transmit animal pathologies
Bioactivity	High natural variability
Rapid degradation by enzymes Crosslinking can slow down degradation rate	Material structural complexity

Current food and drug administration-approved and commercially used bioresorbable polymers

Medical devices made from bioresorbable polymers have already reached a good deal of commercial success.[29] Capronor® is a commercial contraceptive PCL product that is able to deliver levonorgestrel *in vivo* for over a year and has been on the market for over 25 years.[30] For example, products like Ethicon's Securestrap®, a device used for mesh fixation in endoscopic procedures, have already provided bioresorbable technology to patients.[29] Another company, Meredian Inc., is positioned as the world largest manufacturer of PHAs and the only one worldwide whose medium-chain-length PHA is approved for food substance contact by the United States FDA.[31]

Biostable polymers

Biostable polymers including nylon, polyethylene terephthalate, poly(1,4-butylene terephthalate), and some types of polyurethanes are used extensively in different biomedical applications, such as non-absorbable surgical sutures, tissue engineering scaffolds, films, foams, short-term medical devices (catheters, endotracheal tubes, cannulas), long-term implantable devices (vascular prostheses, intra-aortic balloons, cardiac pacemakers), and drug infusion pumps, and are considered safe.[32]

The biostability of PUs in biological environment is mainly affected by the chemical composition of soft and hard segments as well as the associated morphology. PUs with a high level of soft segments (softer grades) tend to degrade significantly more than the harder grades.[33] Polyester PUs are subjected to hydrolytic degradation and are not used in long-term implanted devices. Polyether PUs are hydrolytically stable but they are subjected to oxidative degradation in several forms including environmental stress cracking and metal ion oxidation. To increase the PU stability in biological environment, the main approach has been replacement of the polyether or polyester soft segments with macrodiols with chemical functional groups less susceptible to oxidative and hydrolytic degradation. Hence, macrodiols based on polycarbonate, hydrocarbon and siloxane functionalities have been used in the soft segment of biostable PUs.[18,34-36]

Bacterial infections on implanted biomaterials

Bacterial biofilms produced on the surface of medical materials (both temporary and permanent implants) are a considerable issue that may turn a successful treatment into a significant risk for patients' health, causing infectious diseases that in a long-term may also lead to death. As represented in Figure 1, from a real case of a patient that unfortunately developed a late periprosthetic joint infection, most free-living bacteria (planktonic) species have the capacity to grow in groups attached to a surface in a remarkably complex community of microorganisms encapsulated within a growing biopolymeric matrix, known as biofilm.[37,38] This adaptation allows them to tolerate external unfavorable environments.[5,39] In these structures, bacterial organisms communicate, coordinate their activity and cooperate with each other.

Biofilm formation is initiated and maintained when bacteria attach more firmly to a surface secreting self-expressed biomacromolecules and creating a matrix of hydrated extracellular polymeric substances (EPS) that forms their own immediate environment (Figure 1E).[37] During the biofilm development, a high variety of genes are up-regulated or down-regulated, hence bacteria in a biofilm (sessile phenotype) express different genes when compared to their planktonic phenotype. One gene that is clearly up-regulated is the EPS gene that induces the production of polymeric substances to form a matrix that protects the bacterial cells and enhances gathering of nutrients.[40] The confining scaffold formed by EPS is composed by polysaccharides, lipids, proteins, extracellular enzymes (e-enzymes) and extracellular DNA that is continuously secreted by plasmids (small pieces of DNA carrying specific genes) and cells, or released by lysed cells. The EPS matrix provides mechanical stability to biofilms, mediates their adhesion to surfaces and forms a cohesive three-dimensional polymer network that interconnects and transiently immobilizes biofilms to cells.[37] As a consequence, bacterial cells can exchange plasmids and free DNA very easily and this capacity prepares bacteria with genetic machinery to persist through external stresses (*e.g.* immunity response, antibiotics, *etc.*). Another important characteristic within biofilms is the chemical communication performed by bacteria in a process known as quorum sensing.[41] In this way, these microorganisms coordinate their metabolism by sending chemical signals to other cells nearby and this process increases the efficiency and resilience of the community. The majority of all chronic infections are due to the bacterial biofilms that colonize either biological surfaces (*i.e.* bradytrophic tissue, necrotic tissue) in the

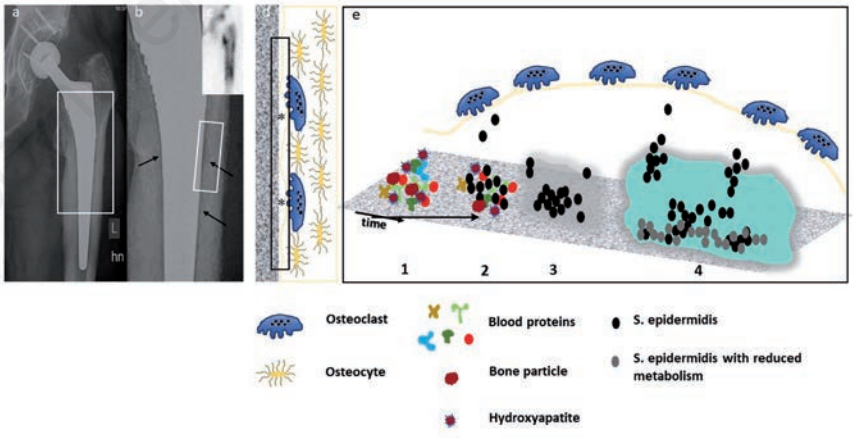


Figure 1. Late periprosthetic joint infection of a male patient with increasing problems one year after a primary total hip arthroplasty. A) X-ray image showing the left hip region with the implanted total hip endoprosthesis composed of acetabular cup fixed into the socket of the hip bone by two pins and three screws, femoral head made of ceramic and an intramedullary uncemented press-fit stem. The surface of the femoral stem is based on hydroxyapatite-coated titanium dioxide; B) Enlarged secation of the X-ray image showing the interface between cortical bone and femoral stem surface. Black arrows are indicating a radiolucent line at the interface of the femoral component as a sign of osteolysis, a typical radiological sign of loosening of the femoral stem; C) Three-phase bone scintigraphy demonstrates the pathological uptake around the total hip replacement in the additional blood pool image as positive sign of infection and septic loosening; D) Scheme of the femoral stem – bone interface with the bone resorption zones (✱) showing the typical arrangement of osteocytes in cortical bone and activated osteoclasts responsible for the bone resorption. Microbiological analysis of *Staphylococcus epidermidis* confirmed the osteoclast activation and thus the PJI; E) Scheme demonstrating the initial step and following sequences of biofilm formation, its maturation and the *circulus vitiosus* of biofilm-associated implant infections.

body or the implant surfaces.[42-44] The most commonly affected medical devices by biofilm formation include prosthetic heart valves, orthopaedic devices, tissue fillers, cardiac pacemakers, intravenous catheters, *etc*.[42-45] Almost 60 to 70% of nosocomial infections are due to biofilm formation on implants.[46] Most reported cases are caused by *Staphylococci* spp., particularly *S. aureus* and *Staphylococcus epidermidis* followed by infection with *Pseudomonas aeruginosa*.[42-45] Biofilm of *S. aureus* species have been found in middle ear, bones, sutures, central venous catheters, prosthetic heart valves and joint protheses, and it leads to implications such as otitis media, bone infections (*i.e*. osteitis, osteomyelitis) and nosocomial infections. Then, other opportunistic bacteria also get the chance to infect the host compromised with medical intervention.[5]

The bacteria in biofilms show enhanced resistance to antibiotics and evade the host immune response that leads to the chronicity and recurrence of infection. In biofilm bacteria undergo a set of genetic alterations that indeed promote biofilm formation. For example, *S. aureus* secretes thermonuclease that acts as a regulator for biofilm formation.[47] The altered genetic program includes the production of extracellular matrix, which gives the structural stability as well as protection to the embedded bacteria against antibiotic agents by accumulating the antibiotic degrading enzymes. Other mechanisms involve the production of the efflux pumps to excrete out the toxic compounds synergistically with other mechanisms like the decrease in outer membrane permeability. For example, a novel efflux pump in *P. aeruginosa* in biofilms was found to be involved in strong resistance to aminoglycosides and fluoroquinolones.[48] This change in metabolic processes is propagated among the whole bacterial community by quorum sensing and by developing linkages with the surfaces.[5] As mentioned previously, this ability of bacteria to resist to the effect of antibiotics is intrinsic to the nature of biofilms. Significant efforts have been made to understand these enduring mechanisms, because this information is important to develop drug-free antibacterial polymers and implants as novel therapeutic strategies.

Antibacterial functionalities for polymeric biomaterials

Antimicrobial polymers represent a promising class of biomaterials showing not only antibacterial high efficacy but also less susceptibility to the development of bacterial resistance. According to the type of polymeric system, antibacterial polymers can be classified into: i) polymers with intrinsic antimicrobial activity or ii) polymers in which the antimicrobial function is achieved by the conjugation of the antibacterial functionalities onto the polymer backbone or by loading an antibacterial filler into the polymer matrix. There are some general principles to introduce antibacterial properties to the polymeric surfaces (Figure 2).[49]

Cationic polymers, silver ions, quaternary ammonium moieties, silica- and carbon-based materials, reactive oxygen-species generating conjugated polymers, antimicrobial peptides, *etc*. have been widely studied as new antimicrobial agents.[50] Among them, cationic natural and synthetic polymers have gained an increasing interest as they offer several advantages: i) they minimize the environmental concerns and ii) they show flexible properties, robustness and proven efficacy against resistance development.[50,51]

Natural cationic polymers generally possess a high biocompatibility while synthetic polymers allow a precise control of their properties and changes, among which the molecular weight distribution, polarity and the degradability of the chains. The main mechanism involving both natural and synthetic cationic polymers to kill bacteria is based on: i) the adsorption of the antibacterial agent on the walls of the bacteria; ii) the diffusion through the cell wall and iii) the disruption of the cytoplasmic membrane.[52] Common investigated cationic polymers are chitosan (CS), poly(ethyleneimine) (PEI), poly-L-lysine (PLL), poly[2-(N,N-dimethylamino)ethyl-methacrylate] (PDMAEMA) and polyamidoamine (PAA).[50,52]

CS is obtained by deacetylation of chitin that is the second most abundant natural biopolymer commonly found in the exoskeleton of shrimps and crabs or even on the cell walls of fungi. It is a linear, semi-crystalline polysaccharide composed of randomly or block distributed N-acetylglucosamine and D-glucosamine units through the CS chain.[53] CS has found many applications in biomedical formulations over recent decades, being a non-toxic, biodegradable and biocompatible polymer with antioxidant and antibacterial properties.[54,55] The degrees of deacetylation and molecular weight affect the cationic properties of CS by varying the positive charge density and as a consequence its antibacterial activity.[55] CS is soluble in diluted acidic aqueous solutions (pH<6) by protonation of the $–NH_2$ function on the C2 position of the D-glucosamine repeating unit, allowing the cationic nature to the polymer.[56] CS can be processed into various forms[57-65] and can form ionic complexes with a wide variety of natural or synthetic anionic species, such as metal ions,[66-68] proteins,[69,70] DNA,[71,72] and some negatively charged synthetic polymers.[73,74] However, the mechanism by which CS exerts its antimicrobial activity is still unknown. Three different approaches have been proposed: i) the $–NH_3^+$ groups of CS interact with the negatively charged components (*e.g*. lipopolysaccharides and proteins) of bacterial cell wall changing the permeability barrier properties and inducing the disruption of intracellular components;[75,76] ii) CS interaction with the DNA of the cell and subsequent inhibition of DNA transcription and protein synthesis;[77] iii) the microorganism growth inhibition associated to the chelating capacity of CS to trace metals (*e.g*., iron, copper or zinc).[77]

PLL is a cationic homopolymer of the amino acid L-lysine and is composed of a large number of primary amines which enable efficient complexation of polyanions

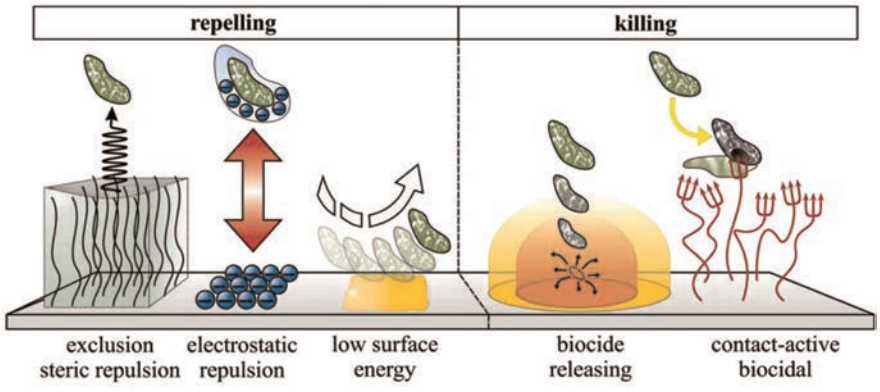


Figure 2. Strategies to induce antimicrobial surfaces. Adapted from Siedenbiedel, 2012.[49]

through electrostatic interaction when protonated (under pI, which is around 9). The electrostatic interaction disturbs the cell membrane, leading to the formation of pores and the entrance of PLL into the cell cytoplasm, with reactive oxygen species generation and, finally, cell death. However, PLL molecular weight directly affects the antimicrobial activity and cytotoxicity.[78] Among PLL, epsilon-poly-l-lysine, with 25-35 lysine residues, shows a wide range of antimicrobial activity against different pathogens including both Gram-positive and Gram-negative bacteria,[79,80] heat stability and lack of toxicity and is generally regarded as safe as food preservative.[81]

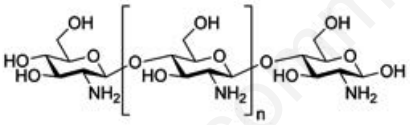
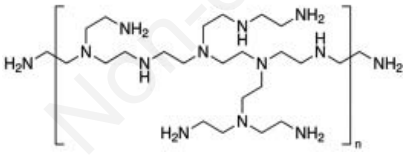
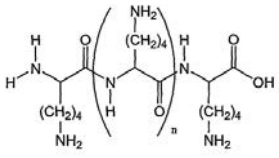
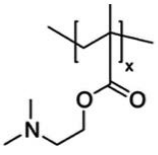
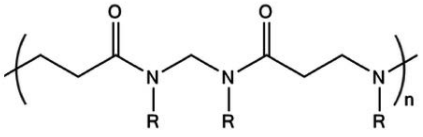
PEI is a polycationic aliphatic polymer characterized by the presence of primary, secondary, and tertiary amino groups. Since PEI does not contain quaternary amines, cationic charges are generated by protonation of the amine groups in the biological environment, showing a correlation between environmental pH and cationic charge density.[82] Cationic linear or branched PEIs have been used as drug carriers in biomedical applications because of their highly positively charged nature and their condensing ability for anionic structures, such as DNA and siRNA.[83,84] However, it has been shown that PEI based materials induce cytotoxicity.[85-87] PEI antibacterial activity has been primarily investigated for alkylated permanently quaternized PEIs used as film coatings or as nanoparticles integrated in resins, showing antibacterial effects against both Gram-positive and Gram-negative bacteria.[88-90]

pDMAEMA is a mucoadhesive polymer, that is cationic when dissolved into an acidic media or quaternized by using an alkylating agent.[91] pDMAEMA has been shown to exhibit antibacterial activity through the destabilization of the bacterial membranes by exchange with divalent cations causing cell death.[92,93] In addition, grafting pDMAEMA to various flat substrates (*e.g.* glass, polystyrene, silicone) has been shown to have an antibacterial effect which is directly correlated to the graft density of pDMAEMA.[91,94,95]

PAAs are a new class of hyperbranched, monodisperse, three-dimensional polymers that are water-soluble, non-immunogenic and biocompatible compounds, and their cytotoxicity is surface charge and concentration dependent.[96] Due to their unique properties, PAAs have been studied as antibacterial and antifungal drug carriers, with capacity to improve the drug solubility, therapeutic efficiency, and permeation.[97-99] Moreover, the amino-terminated PAAs dendrimers have been shown to intrinsically possess a high antibacterial efficacy, associated to the electrostatic interaction between the cationic dendrimer and the anionic bacterial cell surface with resultant disruption of the lipid bilayer and subsequently cell lysis.[100]

Studies have shown that cationic polymers suffer from their high charge-associated toxicity.[85-87,101,102] To overcome this drawback, amphiphilicity is generally introduced in the cationic polymers mimicking host defense peptides, which also contain hydrophobic and cationic domains. Table 2 reports a summary of natural and synthetic cationic polymers used in therapeutic applications.

Table 2. Common natural and synthetic cationic polymers used in therapeutic applications.

Cationic polymer	Structure	Nature	References
Chitosan		Polysaccharide: *N*-acetyl glucosamine and *D*-glucosamine	54, 55, 57-72, 74, 75, 102
Poly(ethyleneimine)		Linear poly(ethyleneimine) contains secondary amines. Branched PEIs contain primary, secondary and tertiary amines.	83-87
Poly-L-lysine		Homopolymer of the amino acid L-lysine	78-81
Poly[2-(N,N-dimethylamino) ethylmethacrylate]		Synthetic cationic polymers containing tertiary amino groups	91, 93, 95
Polyamidoamine		Polymers with amine and amide functionalities.	97-100

Polymer composites with antibacterial properties

An antibacterial polymer is a material consisting of a polymer matrix and an antibacterial functionality that inhibits the growth of targeted microorganisms.[103] As described above, the antibacterial properties of a polymer can be intrinsic or achieved by direct incorporation of the antibacterial functionality in the polymer backbone or through the immobilization of the antimicrobial agents on a carrier and its subsequent incorporation into a polymer matrix, obtaining an antibacterial polymer composite.[103] Polymer composites are generally prepared by melt-compounding the thermoplastic polymer and the filler in a desired relative proportion.

Table 3 reports several recent studies on antibacterial polymer composites and highlights the key findings.

In recent studies, several polymer composites have been obtained to enable them to slowly release metal ions, such as silver, zinc, magnesium, copper ions, or a combination of them, to act as antibacterial agents being extremely toxic to most bacteria and yeast at exceptionally low concentrations.[104-108] Unlike other antimicrobial agents (*i.e.* peptides), metals or inorganic compounds (*i.e.* AgO, ZnO) are stable under conditions currently found in the industry allowing their use as additives.[107] These metal-based additives are used as: particles, ions absorbed/exchanged by different carriers, salts, hybrid structures *etc*.[107]

In other cases, composites have been obtained with the main aim to improve the mechanical properties of the polymer matrix consisting of an antimicrobial polymer, as in the case of composites based on biocidal poly(vinyl alcohol) (PVA) (*i.e.* PVA containing methylated melamine grafted polyvinyl benzylchlori as biocidal additive) with graphene nanosheets.[108,109] Zeolites (loaded with metal ions), multiwalled carbon nanotubes and nanocrystalline cellulose may also act as fillers and modify the mechanical properties of the antibacterial polymer.[108,110,111]

The main requirements of the antibacterial polymer composites collected in Table 3 are uniform dispersion of the antibacterial agent in the polymer matrix, with the prevention of aggregate formation, and the controlled release of the antibacterial agent. A study by Shi *et al*. addressed the issue of aggregate formation by immobilizing silver nanoparticles in cellulose nanocrystals (CNC).[112] CNC is one example of a capping agent, a material used to inhibit aggregation by electrostatic and steric repulsion.[112]

Table 3. Collection of recent relevant studies on antibacterial polymer composites.

Author and Year	Polymer Material	Antibacterial Agent and Composite	Remarks
Shoja *et al.* 2015[104]	Polycaprolactone	Zinc oxide microparticles	- Octadecylamine enhanced the surface adhesion of the ZnO microparticles. - The Polycaprolactone/Zinc oxide composite films containing surface-modified microparticles showed superior antibacterial properties against *Bacillus subtilis* compared to films with unmod fied microparticles
Urbankova *et al.* 2015[103]	Low-density Polyethylene	Essential oils (Linalool, Allylanisole, trans-Anethole) immobilized on Molecular Sieves or Wood Flour, or Talc	- Uniform dispersion of molecular sieves and talc in the low-density Polyethylene matrix; poor dispersion of the wood flour possibly due to its hydrophilic nature in nonpolar low-density Polyethylene - Composites showed enhanced Young's modulus - Pure low-density Polyethylene and essential oil-free composites showed no antibacterial activity compared to various essential oil-immobilized composites, showing antibacterial activity on either Gram-positive or Gram-negative bacteria depending on the essential oil and com posite composition
Altan *et al.* 2014[105]	High-density Polyethylene	Titanium dioxide or Zinc oxide	- Silane coating was applied on th Titanium and Polypropylene dioxide or Zinc oxide fillers before melt mixing with polymers to distribute particles homogeneously in the matrix - Titanium dioxide showed slightly better antibacterial efficiency
Kiriyama *et al.* 2013[106]	Self-cured acrylic resin	Coating based on silver -containing organic composite (70.0 wt% zirconium phosphate ceramics containing silver ions, 29.7 wt% trimethylolpropane trimethacrylate, 0.3 wt% Azo-bis-isobutyronitrile)	- Polymer particles with the coating were prepared using a polymer processing technique known as *surface uniformity revolutionary fixation technology* - Antibacterial activity increased as a function of silver ions content - Composites showed antibacterial effect and inhibited biofilm formation against four representative types of bacteria contributing to biofilm on acrylic resin and tooth surfaces

In vitro tests assessing antibacterial activity of biomaterials

To evaluate the antibacterial potential of new biomaterials, the first approach is to perform *in vitro* testing against the pathogenic bacterial strains related to the specific application of the developed material. Different *in vitro* tests have been reported, but in general they should follow the international standard operating protocols from International Organization of Standardization (ISO for biological characterization), European Committee on Antimicrobial Susceptibility Testing (EUCAST), Clinical and Laboratory Standards Institute (CLSI), Japanese Standards Association (JIS), and American Society for Testing & Materials (ASTM). As advised, these guidelines provide a uniform procedure for practical testing in most clinical microbiology laboratories.

The selection of antimicrobial testing methods depends on the mode of action of antibacterial agents and how it is affected by their addition to the polymers. It also depends on whether the antibacterial agents are physically incorporated and released, or chemically immobilized. There are different mechanisms of antibacterial activity: for example, bacteria are killed either from eluting antibacterial agent or by direct contact with the surface of the material through a biocidal effect or by inhibiting the adhesion to a surface.

The main *in vitro* tests that can be applied to assess the antibacterial ability of a polymeric material are as following.

Zone of inhibition

This assay is used in many clinical microbiology laboratories for a first assessment of the antimicrobial capacity of a drug/material. It involves the direct contact of antimicrobial biomaterials with bacterial culture, eluting the agents into media and inhibiting the bacterial growth in that zone. The size of the zone is important to define the clinical concentration required to inhibit bacteria. This test depends on the concentration of the antibacterial agent and its ability to diffuse.[113,114] For example, in the agar disk-diffusion method and the agar well diffusion method, after the established incubation endpoint, the diameters of inhibition growth zones are measured (CLSI standards). An approximate Minimum Inhibitory Concentration can be calculated for some microorganisms by comparing the inhibition zones with stored algorithms (EUCAST guidelines).

Immersion inoculation

In this method the sample is immersed in the bacterial inoculum, and then colony forming units (CFUs) are counted from the solution as described by ASTM for testing of immobilized antimicrobial agents.[115] A similar procedure is the broth dilution method that is one of the most basic antimicrobial tests. The procedure involves the preparation of serial dilutions of the antimicrobial agent in a liquid growth medium. With the aim to evaluate the percentage of viable microorganisms, several colorimetric methods based on the use of dye reagents can be used, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, 2,3-bis{2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide} and Alamar blue dye (resazurin).[116]

Direct inoculation

This method as described by JIS Z-2801 uses the bacterial inoculum in the form of droplet, placed onto the active surface of antimicrobial biomaterials. After the required inoculation time the bacterial cells are released and counted as CFU.[117]

Surface growth methods

It involves the aerosol inoculation of bacteria in the form of thin film over the activated surface that is then tested for the bacterial growth on agar. This test method is suitable for testing antimicrobial efficiency of surfaces.

Methods for viable but not culturable bacteria

Above mentioned methods use the approach where the bacteria can grow in the form of colonies that can be detected and quantified. However, bacterial strains can enter into viable but not culturable state and cannot be detected by the previous assays[118,119] but can be detected by other method (Figure 3). One of the methods is to measure the adenosine triphosphate (ATP, a chemical signal from living cells only) by using the ATP bioluminescence assay. Thus, there is a linear relationship between the living microbial cell population and luminescence signal. Other commonly used method is the Live/Dead staining that evaluates the membrane integrity.[120,121] A research group has developed a technique using this kit to determine whether the antimicrobial agent directly kills the cells at the surface or at distance.[122] This assay can be combined with flow cytometry.

In the last years, research on polymers with antimicrobial capacity has grown exponentially mainly due to the increase of biomaterial-associated infections. Some examples of antibacterial materials and the tests performed for their functional analysis are reported below.

Polymeric surfaces with covalently bound biocidal quaternary ammonium salts (QAS) were evaluated through the serial dilution method. For example, polyurethane (with QAS) was tested for contact of polymer with bacteria by successive addition of bacteria *(S. aureus,* or *E. coli)* and

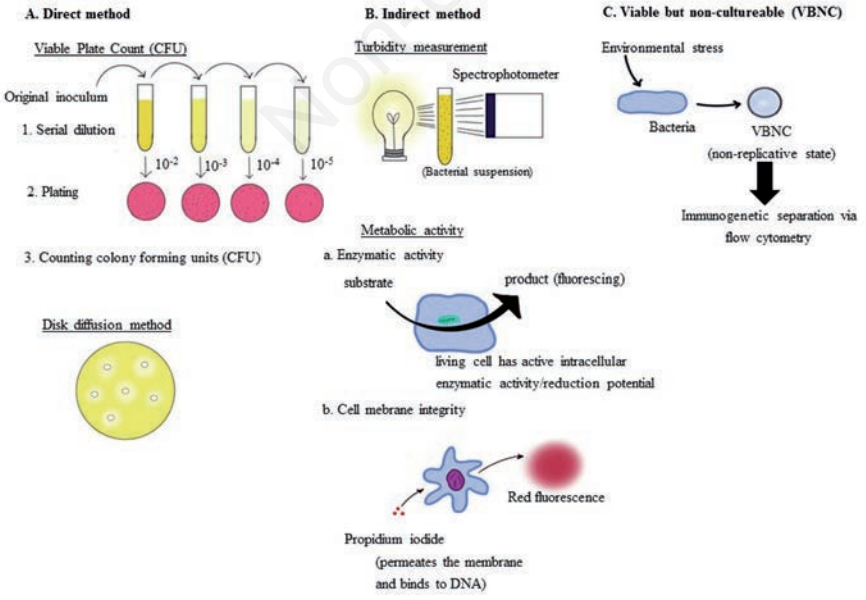


Figure 3. Examples of direct, indirect and viable but non-culturable bacteria assays to determine bacteria viability.

Saccharomyces cerevisiae to test whether the biocidal group retains its efficiency after repeated interactions with a high number of bacteria.[123,124] In an another study QAS was covalently bound on the polyethylene backbone through a hydrolysable ester linkage, and a slow release of the antibacterial agent was found to be effective against *S. aureus* and *E.coli* through serial dilution method.[125]

Polymers coupled with antimicrobial cationic peptides, for example, surface modified polystyrene, were found to be microbiocidal against *E.coli* O157:H7, *L. monocytogenes, S. aureus, P. fluorescens, K. marxianu* in a concentration and time dependent manner. To test the antimicrobial effect, samples were incubated with bacterial inoculum and evaluated at multiple time points by pour plate method.[126]

In another work, the 2-(2-methoxyethoxy)ethyl methacrylate-co-hydroxy-terminated oligo (ethyleneglycol) methacrylate non-adhesive copolymer brushes functionalized with natural antibacterial peptide (magainin) were tested against *L. ivanovii* and *B. cereus*. To test the number of adhered bacteria, samples were stained using LIVE/DEAD bacterial viability kit method. On the other hand for the sessile bacteria *L. ivanovii*, the adherent cells were recovered by sonication and tested by plate count method.[127]

The antibacterial activities of polymeric composites, for example, PCL/ZnO films were examined against *S. aesuis* and *B. subtilis* by agar disc diffusion method revealing the enhanced antibacterial activity with the increasing ZnO amount.[104] In another work, polypropylene and high density polyethylene with nano-sized ZnO and TiO_2 fillers were tested for antibacterial activity using JIS Z 2801 test that evaluates the antimicrobial surface properties of plastics, metal and ceramics.[105] In another study where the essential oils were used to confer antibacterial properties to low-density polyethylene, ISO 22196 method was used to evaluate antibacterial activity against *S. aureus* and *E. coli.*[103]

An antimicrobial dental resin was developed based on a self-cured acrylic resin, composed of a polymer coated with an antibacterial silver ions-releasing organic composite. The resin was tested for antibacterial activity through residual viable counts of the four bacteria *S. mutans, S. oralis, S. gordonii, A. naeslandii* and the yeast *C. albicans*, that are involved in initial biofilm formation on the surface of acrylic resin and denture stomatitis, respectively.[106]

A chlorinated coated polyester containing N-halamine moieties was also prepared and tested against *S. aureus* and *E. coli (O157:H7)* through the sandwich test method and biocidal activity analysis was performed.[124]

Recent studies have shown the development of *in vitro* biofilm models, especially chronic wound models for the evaluation of antibacterial properties. Lubbock Chronic Wound Biofilm was the first chronic wound biofilm model used to evaluate the inhibiting efficacy of various biofilm effectors.[128,129] Other examples of such models based on constant depth film fermenter[130] and colony-drip flow reactor were used to evaluate the antibacterial effect of wound dressings.[131]

Besides the testing methods described above, it is of great interest to evaluate the antibacterial potential of new biomaterials in more *in vivo* like situation. Recently, there has been development of human cells based three-dimensional *in vitro* systems with bacterial infection and biofilm formation mainly to unravel the poorly understood interactions between pathogenic bacteria and human tissue. For example, an *in vitro* model of oral and vaginal candidiasis was developed using reconstituted human epithelia to demonstrate the interaction between *C. albicans* and epithelial tissue.[132-135] Charles studied the biofilm formation by wound pathogens *S. aureus* and *P. aeruginosa* using the most advanced tissue engineered wound model based on Graftskin.[136] These advanced systems can be employed for *in vitro* screening of both antibacterial activity as well as cytocompatibility of novel antibacterial biomaterials to obtain more reliable preclinical data and better *in vivo* performances. Moreover, with the growing emergence of drug resistant infections worldwide, these systems can serve as a powerful platform to explore novel therapeutic approaches against infections and implant associated infections in a more *in vivo* like situation, by demonstrating the interactions between pathogen and human tissue.

Conclusions

New bacteria resistance mechanisms are continuously emerging and spreading globally, associated with an excessive use of antibiotics and leading to the formation of *superbugs*. Antimicrobial resistance threatens our ability to treat infections as well as increases healthcare costs.

As an alternative to antimicrobial drugs, a new intriguing possibility is the development of antimicrobial biomaterials for both temporary or permanent biomedical applications. Such biomaterials may be effective by preventing bacterial adhesion and biofilm formation or causing bacterial death (bactericidal effect). Hence, new lines of research are now emerging for the synthesis of novel copolymers (*e.g.* belonging to the polyurethane family) with intrinsic antibacterial properties or able to be conjugated with functional antibacterial moieties, *e.g.* cationic functionalities. Other possibilities include the incorporation of antibacterial fillers within a polymer matrix or onto its surface.

The development of new antimicrobial biomaterials is possible by a full comprehension of the mode of action of the antimicrobial functionalities as well as the properties of the biomaterials. New methods for antibacterial testing should be developed for an accurate prediction of the antimicrobial behaviour of new antibacterial biomaterials. In this context, as described above, the development of novel human cells based *in vitro* microbial infection models can not only be useful to understand the basic mechanisms of biofilm formation and infection persistence but also serve as a powerful tool for antibacterial testing. Although not described in this review article, new antibacterial promising strategies avoiding the use of drugs are represented by surface structuring of biomaterials with hierarchical micro-/nano-patterns, mimicking the bactericidal surface of cicada wings and dragon fly or the antibiofouling surface of shark skin, lotus and taro leaves.[51,137,138]

In conclusion, advancement in the knowledge of bacterial biology, as well as biomaterials science and engineering is continuously progressing with the aim to avoid or to treat biomaterial-associated infections. Novel strategies using drug-free polymeric materials may mitigate this challenging worldwide problem. The engineering of new biomaterial devices with intrinsic antimicrobial properties requires collaboration among clinicians (*e.g.* interventional specialists, surgeons, infection disease specialists) and research groups with complementary expertise in biomedical and materials engineering, biomaterials science, biology and biotechnology. Most of the authors of this review article collaborate in the HyMedPoly H2020-MSCA-ITN-2014 project, which represents a valuable example of such interdisciplinary collaboration, involving both academic and the industrial staff.[139]

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