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Antibiotic Drug Delivery Systems for the Intracellular Targeting of Bacterial Pathogens

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Additional information is available at the end of the chapter

Abstract

Intracellular bacterial pathogens are hard to treat because of the inability of conventional antimicrobial agents belonging to widely used classes, like aminoglycosides and β-lactams, fluoroquinolones, or macrolides to penetrate, accumulate, or be retained in the mammalian cells. The increasing problem of antibiotic resistance complicates more the treatment of the diseases caused by these agents. In many cases, the increase in therapeutic doses and treatment duration is accompanied by the occurrence of severe side effects. Taking into account the huge financial investment associated with bringing a new antibiotic to the market and the limited lifetime of antibiotics, the design of drug delivery systems to enable the targeting of antibiotics inside the cells, to improve their activity in different intracellular niches at different pH and oxygen concentrations, and to achieve a reduced dosage and frequency of administration could represent a prudent choice. An ideal drug delivery system should possess several properties, such as antimicrobial activity, biodegradability, and biocompatibility, making it suitable for use in biomedical and pharmaceutical formulations. This approach will allow reviving old antibiotics rendered useless by resistance or toxicity, rescuing the last line therapy antibiotics by increasing the therapeutic index, widening the antimicrobial spectrum of antibiotics scaffolds that failed due to membrane permeability problems, and thus reducing the gap between increasingly drug-resistant pathogens and the development of new antibiotics. Different improved drug carriers have been developed for treating intracellular pathogens, including antibiotics loaded into liposomes, microspheres, polymeric carriers, and nanoplexes. The purpose of this chapter is to present the limitations of each class of antibiotics in targeting intracellular pathogens and the main research directions for the development of drug delivery systems for the intracellular release of antibiotics.

Keywords: Intracellular bacterial pathogens, drug delivery systems, drug carriers, liposomes, polymeric carriers, nanoplexes

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1. Introduction

Infections with intracellular bacterial pathogens are hard to treat due to the inability of conventional antimicrobial agents to penetrate, accumulate, or be retained in the mammalian cells [1]. The increasing problem of antibiotic resistance complicates more the treatment of the diseases caused by these agents.

Taking into account the huge financial investment associated with bringing a new antibiotic to the market and the limited lifetime of antibiotics, the design of drug delivery systems to enable the targeting of antibiotics inside the cells, to improve their activity in different intracellular niches at different pH and oxygen concentrations, and to achieve a reduced dosage and frequency of administration could represent a prudent choice [2]. This approach will allow reviving old antibiotics rendered useless by resistance or toxicity, rescuing the last line therapy antibiotics by increasing the therapeutic index, widening the antimicrobial spectrum of antibiotics scaffolds that failed due to membrane permeability problems, and thus reducing the gap between increasingly drug-resistant pathogens and the development of new antibiotics.

The purpose of this review is to present the limitations of each class of antibiotics in targeting intracellular pathogens and the main research directions for the development of drug delivery systems for the intracellular release of antibiotics.

2. Microbial adaptation to the intracellular lifestyle

Invasion (aggressiveness or invasiveness) represents the ability of pathogens to overcome epithelial barriers through specific mechanisms, to penetrate the host tissue, and to multiply, producing pathological effects [3]. Invasive microorganisms have the ability to penetrate into the host tissues or to stimulate the endocytic function of the substrate and to maintain their viability in the host cell [4].

Many pathogenic bacteria are capable of surviving inside eukaryotic, normally nonphagocytic cells (mucosal cells and blood vessel endothelial cells) [5, 6]. The intracellular medium offers protection to the microorganisms that could thereby multiply or persist [7].

Some pathogenic bacteria are facultative intracellular (e.g., *Mycobacterium* spp., *Listeria monocytogenes*, and *Salmonella* spp.) going through an intracellular phase during infectious cycle without being strictly dependent on the cellular medium, while others are obligate intracellular parasites (*Chlamydia* spp. and *Rickettsia* spp.), which do not survive in the extracellular medium of the host. They infect endothelial and epithelial cells and also mono-cytes.

Generally, invasive organisms adhere to the host cells using a class of molecules represented by proteins with adhesion function associated to the cell surface called invasins, which direct the entry of the bacteria into the cells [8]. The mechanisms of adherence trigger or promote cellular signals, which directly or indirectly facilitate the bacterial penetration [9].

After bacterial adherence, invasion is produced in two ways: (a) the "zipper" mechanism—after binding to the host cell, the adherent bacteria induce changes of the cytoskeleton, in particular, actin filaments, resulting in the embedding of the bacteria, and (b) internalization by a pathway independent of the membrane molecules that mediate adherence. In this case, the interaction of the bacteria with the host cell membrane produces localized intrusion (ruffling), followed by endocytosis [10].

Professional phagocytes express membrane receptors for conserved structures of the microbial pathogens called pathogen-associated microbial patterns (PAMP), which are missing from mammalian cells. The broad spectrum of microbial pathogens is therefore recognized by a limited number of receptor molecules of the host (pathogen recognition receptors [PRR]) belonging to the following groups: *receptors of TLR family* (*Toll-like receptors*), which recognize different structures of the microorganisms—glycoproteins, lipoproteins, heat shock proteins, and flagellar proteins; *lectin*-type receptors with specificity for common carbohydrates expressed on the surface of bacteria, i.e.: (a) receptors for mannose (MBL) that could also recognize other carbohydrates (*N*-acetylglucosamine, glucose, and L-fucose), (b) receptors for galactose recognizing *N*-acetyl-galactosamine and galactose, (c) receptors for fucose and specific membrane molecules, such as CD14 with specificity for glycolipids (LPS) and for the lipoarabinomannans (LAM) of mycobacteria.

The fact that adhesion of bacteria to eukaryotic cells requires the recognition of specific oligosaccharides or glycoproteins [11] was demonstrated by the *in vitro* experimental results, showing that oligosaccharides are the most potent inhibitors of the interaction between the bacteria and the eukaryotic cell surface.

Invasion is an active event, sustained by normal cell functions [12], with the host cell cytoskeleton supporting the invasion and embedding process [13].

Pathogenic bacteria, such as *L. monocytogenes*, *Shigella* spp., and *Rickettsia* spp., possess mechanisms that induce cytoskeletal rearrangements (actin condensation with the formation of a propulsion actin comet behind the bacterial cells) of the host cell to assure their cytoplasmatic and intercellular transit [13].

Some bacterial strains could modulate the host cell apoptosis during the infectious cycle [14]. This proapoptotic effect could facilitate the endocytosis of the apoptotic bodies containing bacterial cells by the adjacent cells, without the occurrence of an inflammatory process, but bacteria could spread into the healthy tissues at the same time. The bacteria-induced apoptosis is mediated by the activation of caspases or Fas/FasL pathways correlated with the inactivation of antiapoptotic proteins (e.g., NFkB and MAP kinases). *Pseudomonas aeruginosa* could induce the lung epithelial cells apoptosis, a process by which the bacterial cells are cleared from the lung or other infected organs. However, *P. aeruginosa* could invade, survive, and multiply in the host cells and induce an antiapoptotic effect in order to maintain its host and protect itself from the immune response effectors [15].

By using microscopy evaluation and viable cells count assays, we have demonstrated that *P. aeruginosa* clinical strains could survive and multiply in nonphagocytic epithelial cells [5], inducing changes of cellular morphology (cytoplasm wrinkling, the formation of long, lamellar

pseudopodes). The respective *P. aeruginosa* strains were also able to modulate the apoptosis of the infected cells by increasing the expression of the proapoptotic caspase 3 and Bax genes and by decreasing the expression level of the antiapoptotic Bcl-2 and Mcl-1genes [16].

Selective adherence to the microfold (M) cells is an effective way of invasion. Bacteria and viruses that use M cells transport pathways can infect the gastrointestinal mucosa and may disseminate systemically.

M cells, which cover the Peyer's patches, separate the epithelium-associated lymphoid follicles from the gut lumen. They are coated with a thin mucous layer, have short microvilli, but are very active in terms of pinocytosis compared with columnar epithelial cells. The Peyer's patches, consisting of aggregated lymphoid follicles, are the major component of the mucosal immune system and have a precise function: to exclude exogenous antigens, before they enter into the internal medium, and to avoid or minimize the exposure of the systemic immune apparatus to molecular antigens or cells that reach the internal medium. At the same time, mucosa-associated lymphoid tissue (MALT) should remain insensitive to normal mucosal microbiota. MALT is therefore a "control zone" of the body in contact with antigens and also has a regulatory role on the functionality of systemic immune response. This explains the fact that oral administration of an antigen in human or animals, in essence, does not produce a systemic immune response, but typically a mucosal immune response. The mechanism is unknown, but the mucosal immune system prevents an extensive immune response after the contact with a large number of intestinal antigens, especially with food origin. Bacterial or viral complex antigens can initiate a complex immune response through mucosal immune apparatus. MALT functional deficiencies expose the organism and systemic immune apparatus to a permanent state of activation, which exceeds the physiological limits, with the possible occurrence of autoimmune diseases [17].

M cells can uptake by pinocytosis soluble luminal material and transfer it to the underlying macrophages. Macrophages process the antigens and present them to the adjacent lymphocytes. They have few lysosomes, and the embedded materials are not submitted to degradation. M cells are carrying macromolecules, particles, and microorganisms directly into the cellular environment of the mucosal lymphoid follicles.

M cells have no receptors for polymeric immunoglobulins, indicating that they do not transfer IgA, which favors the access of the antigens to the mucosal surface. They are specialized for the transepithelial transport. The basolateral surface of M cells is deeply intrusive, presenting extensions of about 10-μm dimension, which are forming a big intraepithelial "pocket" and extending in the underlying lymphoid tissue, in which transported macromolecules and particles are released. Below the epithelial M cells, there is a rich population of macrophages and dendritic cells in close spatial relationship with CD4 T cells harboring the $\alpha\beta$ type receptor and B cells. Few lymphocytes are memory T cells or uncommitted (naive) cells. The folds of M cell are the site of interaction between T cells and antigen-presenting cells (B cells and macrophages).

Through follicular epithelium, microorganisms gain access to the lymphoid follicle structures. The consequence is beneficial because it initiates protective immune response against luminal

microorganisms. M cells are therefore regarded as an early warning system of the immune system. Although M cells have evolved as a strategic protective system, their functional properties are qualifying them as true gateways—the Achilles heel of the intestine, because the pathogenic bacteria could gain in this way access to deeper structures.

The bacterial cells interact with M cells, probably via carbohydrates [18]. M cells possess a wide range of glycoconjugates that modulate their capacity to uptake microorganisms [19]. Further, macrophages and dendritic are involved in the embedding pathogens transported by M cells, in processing and storing antigens [20].

Certain *Escherichia coli* pathogenic strains that colonize the intestinal mucosa could selectively adhere to the epithelial cells and interact with the M cells. The adherence to intestinal epithelial cells and to M cells induces the disintegration of the microvilli and M cells folds and also the appearance of some special structures called pedestals—a consequence of actin filaments reorganization at the adhesion site [21].

Shigella sp., a facultative intracellular pathogen induces severe damages of the small intestine and colon mucosa, accompanied by the loss of epithelial barrier function. *Shigella* sp. cells adhere to the cellular membrane, are phagocytosed and released into the cytoplasm after the degradation of the phagosome membrane, where they multiply, induce the assembly of a tail of actin filaments, and are eliminated in a vacuole with membranar origin, which is subsequently phagocytosed by the neighboring cells [22].

In vitro experiments with enterocytes have shown that *Shigella flexneri* does not invade the apical surface, if the epithelial tight junctions are intact. The invasion is possible only through the basolateral membrane. *In vivo*, *Shigella* invades the mucosa, first of all, through M cells, followed by the invasion of epithelial cells through the basolateral surface. Mucosal ulcerations have the highest frequency in the ileum and colon, where lymphoid follicles and M cells are more numerous. We have demonstrated that *S. flexneri* and *S. boydii* strains modulated the expression of different anti- and proinflammatory cytokines in HeLa cells, by decreasing the production of IL-1β, IL-6, TNF-α, and IL-17 [22, 23].

Studies on the relationship between viral and bacterial infections showed that the immunity of host organism is reduced temporarily in the context of viral infections, increasing the incidence of bacterial infections, probably by increasing the level of expression of epithelial cell receptors for bacterial adhesins. Virus-infected host cells could also undertake changes of the cytoskeleton [24], which may result in the increase/decrease of the bacterial invasion capacity. Therefore, we have investigated the influence of viral preinfection using vaccinia, measles, echovirus 32, and herpes simplex virus 1 strains on the ability of an enteroinvasive *E. coli* strain to colonize the HeLa cells, and we have demonstrated that the viral preinfection of the cellular substrate induced a decrease of the invasive ability, pleading for an increased incidence of infections with extracellular pathogenic organisms after viral infections [6].

In the small intestine, *Vibrio cholerae* expresses a group of pilliary adhesins used for the adherence to the enterocytes. The pilli maintain the vibrions adhered on the surface of the mucosa, and the cholera toxin induces the secretion of chloride ions from the intestinal cells into the lumen. *V. cholerae* also interacts closely with extensive areas of the apical membrane

of the M cells. The activating signal induces actin reorganization, and bacterial cell is phago-cytosed, without the M cell damaging. However, bacteria embedding in the M cells does not cause the disease in this case [25], but on the contrary, the process activates a protective immune response of the mucosa, mediated by the secretion of anti-toxin and anti-LPS sIgA, which can prevent mucosal colonization by *V. cholerae* and by default, prevent diarrheal disease.

The ingestion of *Salmonella* cells induces the infection of Peyer's patches. *S. typhi* and *S. typhimurium* adhere rapidly and selectively to M cells and also directly invade through epithelium villi. *Salmonella* sp. cells are embedded in a large endocytic vacuole by a phagocy-tosis mechanism induced after apical microvilli disassembling and cytoskeleton reorganiza-tion.

The experiments with the ligated intestinal loops showed that after 30 min of injection, *Salmonella* sp. cells induce the growth of M cells volume; and a rapid incorporation of the bacteria was observed, followed by the degeneration of M cells and the access of the infectious cells to the mucosa structure.

Yersinia sp. cells penetrate the intestinal mucosa through M cells to which bacteria adhere preferentially, being embedded and crossing the cytoplasm by transcytosis.

A few bacterial species are able to force the entrance directly into the host cells, after adherence, by the local enzymatic digestion of the host cell membrane. For example, *Rickettsia prowazekii* secretes phospholipases that determine the localized and controlled degradation of the host cell membrane. Through the membrane lesions, the pathogen enters directly into the cyto-plasm [26].

Most bacteria, including many pathogenic bacteria, are killed after their phagocytosis by macrophages or neutrophils (PMNN). Some other species have developed some strategies that allow them to survive and multiply inside phagocytes. *S. flexneri*, *L. monocytogenes*, and *Rickettsia* sp. dissolve the initial membrane vacuole and thus gain access to the cytoplasm rich in nutrients. *Salmonella* spp., *Mycobacterium* spp., and *Legionella* spp. could inhibit the fusion between phagosome and lysosome and thus escape phagocytosis.

The virulence factors that determine the bacterial resistance to lysosomal enzymes and increase the intracellular survival capacity are cell surface protective envelopes (capsule and LPS), bacterial enzymes that neutralize the toxic free radicals and reactive oxygen species, and proteolytic enzymes that degrade the lysosomal enzymes of the host [13].

3. Efficiency of different classes of antibiotics against intracellular pathogens

Antibiotics are low molecular weight substances produced by microbial biosynthetic processes or by chemical synthesis, which can be used in low concentrations to specifical-ly inhibit the proliferation or to kill microorganisms [27]. Because of their high specificity, antibiotics exhibit different efficiencies against various microbial species. Antimicrobial

drugs act by different mechanisms, such as inhibition of cell wall synthesis, inhibition of cell membrane functions, inhibition of protein synthesis at different stages (translation or transcription), inhibition of nucleic acid synthesis, and blockage of metabolic pathways by competitive inhibition (Figure 1) [28].

Depending on the number and diversity of affected microbial species, the activity spectrum of the antibiotics can be broad (i.e., the spectrum of tetracycline is represented by Gram-negative bacteria, including *Chlamydia* sp. and *Rickettsia* sp. and Gram-positive species; penicillins are active especially against Gram-positive species, and some Gram-negative bacteria, including *Chlamydia* sp., nitrofurans, rifampin, and sulfonamides are active on a large number of Gram-positive and Gram-negative bacteria species), narrow (novobiocin is active on Gram-positive bacteria, especially staphylococci, but also on Gram-negative species, such as *Haemophilus* sp. and *Pasteurella* sp., glycopeptides and bacitracin are active against Gram-positive bacteria), and limited (nitroimidazoles are active only against anaerobic microorganisms).

Even within the same microbial species, there can be large differences regarding the susceptibility of different strains to a particular antibiotic; thus, antibiotic treatment in the clinical setting requires the isolation of the microbial strain, which is the etiologic agent of a specific infection (especially if it belongs to genera and species with a high ability of acquiring clinical resistance) and determining its antibiotic susceptibility spectrum.

The *in vivo* antimicrobial activity is more complex, involving different host-related factors along with the impact of the antibiotic and the nature of the antimicrobial agent. Thus, in the host, a number of local factors (partial pressure of O_2, pH etc.) could influence the activity of the antibiotic. On the other hand, antibiotics are absorbed within the intestinal tract and distributed unevenly in various tissues and body fluids, and very few reach active concentrations in the central nervous system (CNS) or inside most eukaryotic cells. It is also very difficult to maintain an active concentration of the antibiotic for a prolonged period of time, so the interval between doses should be rigorously respected. Some antibiotics have postantibiotic effects (such as observed in the case of carbapenems activity against the Gram-negative bacilli) and may modulate the inflammatory response by indirectly inducing the chronicity of inflammatory reactions due to the accumulation of bacterial fragments. The combination of two or more antimicrobials is recommended for the treatment of severe and chronic infections to avoid the appearance of resistant mutants (i.e., tuberculosis) and also in mixed infections, for obtaining a synergy of action and a strong bactericidal effect (i.e., beta lactams and aminoglycosides, trimethoprim and sulfamethoxazole, amphotericin and flucytosine, beta-lactamase inhibitors and beta-lactam antibiotics).

Some antibiotic classes, such as macrolides, fluoroquinolones, tetracyclines, and ansamycins, are known to be active against obligate intracellular and facultative intracellular organisms, while others, such as beta-lactams and aminoglycosides, show no or only a poor intracellular activity. However, these antibiotics are active against facultative intracellular organisms, including *Mycobacterium tuberculosis*.
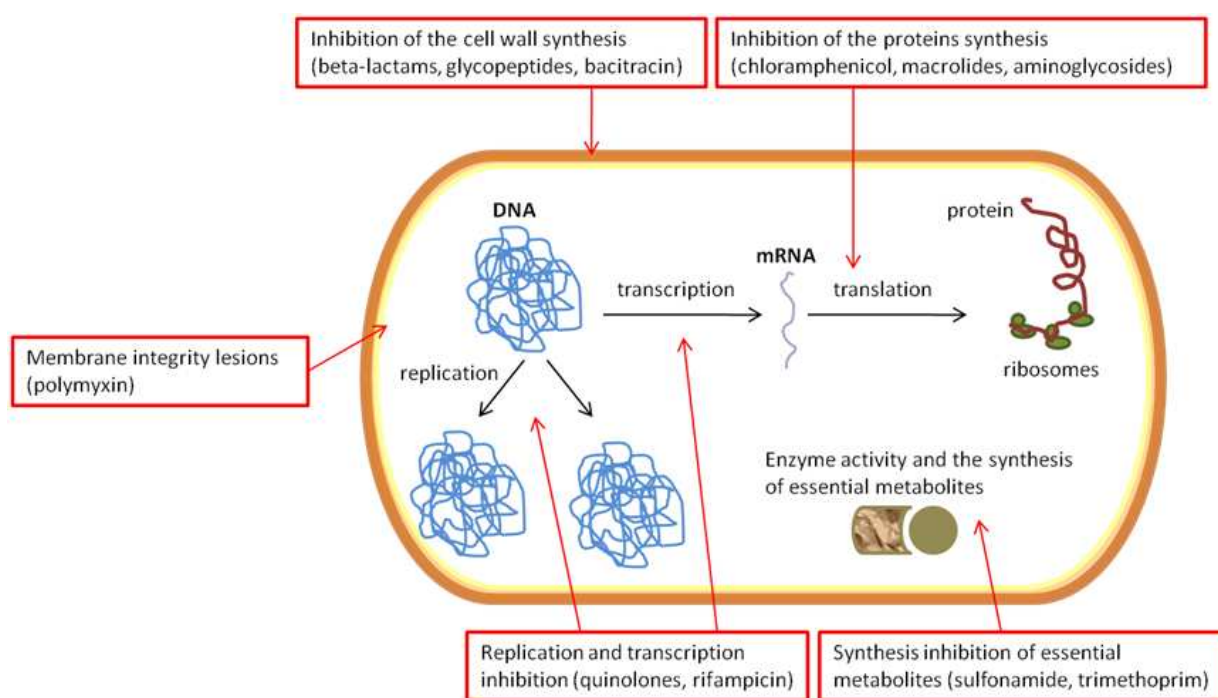
Figure 1. The main mechanisms of action and cellular targets of antibiotics within the bacterial cell.

3.1. Inhibition of cell wall synthesis

Peptidoglycan cell wall is a closed structure, composed by covalently linked units, which allow the sequential addition of new units on the external side of the cytoplasmic membrane, while the old units from the peptidoglycan's structure are shifted outward and released by the action of autolysins.

The synthesis of the peptidoglycan takes place in three stages: (i) low-molecular-weight-soluble precursors (GlcAc UDP and UDP-MurNAc-L-Ala-D-Glu-mezoDap-D-Ala-D-Ala) are synthesized in the cytoplasm; (ii) the nonnucleotide region of the previously synthesized molecular precursor (intermediate *N*-acetyl glucosamine and *N*-acetyl muramic acid-penta-peptide) is attached to a lipid carrier, integrated in the membrane and subsequently modified by adding GlcNAc and pentaglycine, resulting undecaprenol-pyrophosphate MurNac (L-Ala-D-Gln [NH$_2$ [Gly5] L-Lys-D-Ala-D-Ala]-[beta1-4]-GlcNac), that will be translocated across the plasma membrane and serves as a substrate for a transglycosylation reaction, polymerizing the bacterial glycan chains of the cell wall, to form a repetitive disaccharide (MurNac-GlcNAc)$_n$; and (iii) the subunits of the peptidoglycan are polymerized by their insertion in the preexisting cell wall, by the reaction of transpeptidation, which takes place at the terminal D-ala-D-ala residues.

The polymerization and cross-linking of the sugar tetrapeptide chains are catalyzed by penicillin-binding protein enzymes (PBP), located in the cytoplasmic membrane and the periplasmic space.

Some antibacterial agents interfere with the early steps of the cell wall synthesis (vancomycin, bacitracin, and cycloserine), while others (β-lactam antibiotics, penicillins, cephalosporins, monobactams, and carbapenems) inhibit the last steps of peptidoglycan synthesis, such as the formation of interpeptidic links, because these antibiotics have structural analogy with terminal D-ala-D-ala dipeptide [29].

Glycopeptides (vancomycin and teicoplanin) inhibit the early stage of the peptidoglycan synthesis by binding to the carboxy-terminal dipeptide D-Ala-D-Ala. It has been revealed that vancomycin shows a slow uptake and modest accumulation into macrophages, especially in the lysosomes compartment (up to eightfold in 24 h) [30, 31], while teicoplanin, a more lipophilic compound, shows a more extensive and faster intracellular accumulation (40- to 60-fold) [32, 33].

A newly investigated glycopeptide antibiotic called oritavancin (LY333328) proved to be avidly accumulated by J774 and THP-1 macrophages and rat fibroblasts and to a lesser extent by LLC-PK1 and Caco-2 cells. The intracellular pharmacokinetic and pharmacodynamic results demonstrated that the level of accumulation reached a plateau (at 370-fold the extracellular concentration) within 24 h, and the effect was partly defeated by a rise in serum protein levels [34].

Bacitracin (a cyclic peptide) prevents the dephosphorylation of the lipid carrier molecule, which transfers a newly synthesized peptidoglycan molecule to the cell membrane during the synthesis of the cell wall. This antibiotic is toxic to kidneys and is not systemically administered but is applied topically to treat skin and mucosa infections.

Cycloserine competitively inhibits the formation of D-ala from L-ala and thus stops the synthesis of the dipeptide D-ala-D-ala. This antibiotic is relatively toxic and is used for the treatment of *M. tuberculosis* infections resistant to other drugs.

Fosfomycin is a pyruvyl-transferase inhibitor, which blocks the synthesis of *N*-acetyl-muramic acid.

Cycloserine and fosfomycin act as peptidoglycan precursor analogues. They are very hydrophilic molecules and enter the cytoplasm following the path of transport systems usually utilized for some related metabolites; i.e., fosfomycin is structurally analogous to the phospho-enol-pyruvate and cycloserine is similar to D-alanine.

Beta-lactam antibiotics act as pseudosubstrates and perform the acylation of the active sites of the PBP transpeptidases, which are thus unable to catalyze the polymerization of the peptidoglycan. The acylation reaction of the PBP is very slowly reversible. PBP-deacylated enzymes are unable to catalyze the cross-linking of peptides (Figure 2). Antibiotic-PBP complexes stimulate the release of autolysins, which produce the degradation of the cell wall, leading to osmotic bacterial cell lysis.

The inhibitors of beta-lactamase enzymes, such as clavulanic acid, sulbactam, tazobactam, have a high affinity for the respective antibiotic-inactivating enzymes, inducing their acylation and formation of stable, unefficient complexes (Figure 3).
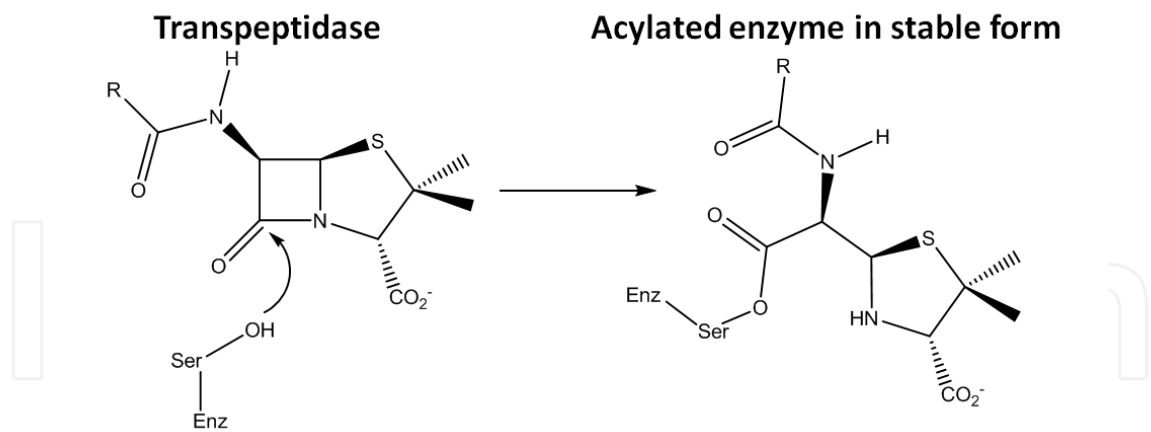
Figure 2. Beta-lactam antibiotics mediate the inactivation of PBP by the acylation reaction.
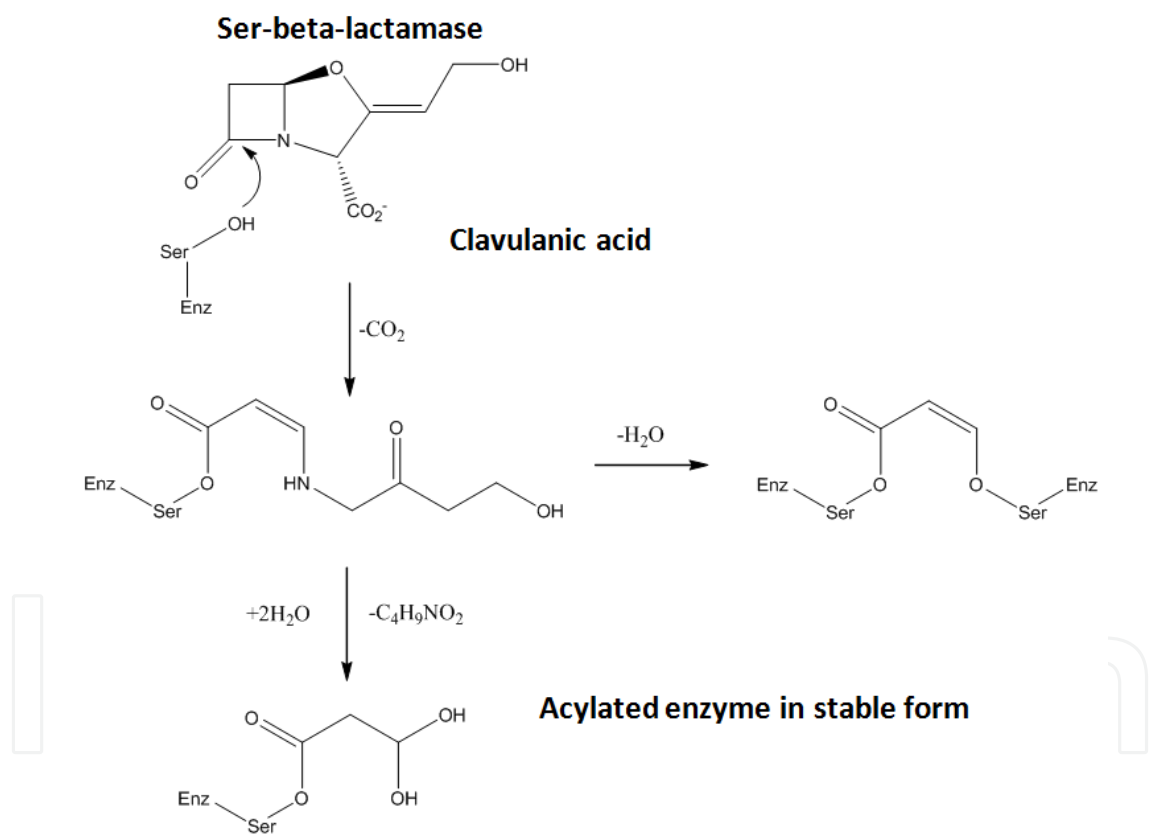

Figure 3. Beta-lactamase enzymes inactivation by acylation in the presence of clavulanic acid.

Penicillin G is the drug of choice for meningococcal and gonococcal intracellular infections. Third-generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefotetan, ceftizoxime, cefoperazone, and cefixime) may cross the blood–brain barrier; therefore, cefotaxime, ceftriaxone, and ceftizoxime are usually intravenously administrated for the treatment of meningitis caused by Gram-negative bacteria. The intracellular concentration of beta-lactams is usually

lower than the extracellular amount, as revealed for both phagocytic and nonphagocytic cells [35, 36]. This could be explained by the weak acidic character of these molecules affecting their accumulation in acidic cytosol milieu [37].

3.2. Inhibition of the cytoplasmic membrane function

Cytoplasmic membrane acts as a selective permeability barrier for ions and nutrients and is also the headquartered structural transport system that controls the chemical composition of the cytoplasm. The disruption of structural integrity and/or functional parameters entails the amendment of ion-selective permeability and loss of ionic or macromolecular balance.

Bacteria and fungi have a slightly different cellular membrane as compared with the animal cells, this being injured faster by different therapeutic agents, which enables selective therapy. Biological membranes are comprised of a lipid matrix, wherein the randomly distributed globular proteins penetrate the lipid layer. Cationic, anionic, or neutral detergents may disrupt biological membranes. The effect of polymyxins is similar to the cationic detergents. Their molecule contains hydrophilic and hydrophobic groups. These antibiotics are positively charged at neutral pH and actin similar with the cationic compounds, which are active against the polyanionic outer membrane of Gram-negative bacteria (the load offered by the lipopolysaccharide). Detergents (which are molecules containing lipophilic and hydrophilic group) act by disorganizing the double lipid layer leading to cell disruption.

Polymyxins are polypeptide antibiotics (octapeptides of high molecular weight) with specific chemical and biological properties. There are five known major chemically distinct polymyxins, designated as polymyxin A, B, C, D, and E. All polymyxins are synthesized by *Bacillus polymyxa* and have the same antibacterial spectrum. Polymyxins A and D are nephrotoxic and therefore cannot be used *in vivo*. The most representative are polymyxin B and colistin (or polymyxin E), which are also the less cytotoxic (in concentrations up to 5 μg/ml), being most active against Gram-negative bacteria, such as *Brucella* sp., *Yersinia* sp., *Salmonella* sp., *Shigella* sp.), probably due to their negative lipid charge.

Polymyxins are not absorbed from the intestine, and also they do not accumulate in active concentrations in soft tissues, being not efficient in the treatment of systemic or internal organs infections. In exchange, they can be used for the treatment of superficial, cutaneous, or mucosal infections (wounds, burns, intestinal tract mucosa, and pleural cavity infections), as well as for the prophylaxis of transplant or digestive tract surgery. Polymyxins are used with great effectiveness in the treatment of meningeal, lung, and urinary tract infections. They are often associated with other antibiotics to extend the antimicrobial activity spectrum.

The fixation of the antibiotics within the membrane structure depends on the concentration of divalent environmental cations: their deficiency or excess inhibits the action of polymyxins. Also, the LPS composition, bacterial membrane phospholipids, and proteins influence the sensitivity or resistance of various bacterial species to the action of polymyxins.

Polymyxin and other polycationic molecules bind to the lipid A of LPS in a stoichiometric ratio and are inserted into the membrane structure. Such a molecular patchwork disorganizes the lipid layers so that the cell membrane does not function normally as effective osmotic barrier.

Due to structural deterioration of external and internal membranes, osmotic balance is disrupted by the loss of K^+ ions. The permeability changes are associated with the loss of soluble cell constituents and viability. The mechanism is the same as that proposed for hemolysis by the action of ionic detergents. The hemolytic effect occurs due to the disruption of cholesterol–phospholipid–lipoprotein complex from the erythrocyte membrane. Other agents acting on the cytoplasmic membrane are amphotericin B, imidazoles, and triazoles.

3.3. Inhibition of the protein synthesis

Several classes of antibiotics are active specifically on the 70S ribosomes, thus blocking protein synthesis at different levels [38].

Aminoglycosides are low molecular weight cationic molecules, but very hydrophilic, which accumulate in the cell only through an energy-dependent transport process. Their accumulation in the bacterial cell occurs through two phases: Phase I is slow and depends on the transmembrane gradient of the electrical potential and thus the oxidative respiratory sinergon, and phase II is quick and translates into an important intracellular accumulation. Intracellular concentrations are approximately 100 times higher than those from the external environment. Slow accumulation causes a bacteriostatic antibiotic, while rapid accumulation produces bactericidal effects. The accumulation rate is conditioned by the size of the electric component ($\Delta\psi$) and the proton-motive force. This explains why microorganisms with transportation systems deficiencies, such as anaerobes, are intrinsically resistant to aminoglycosides. For the same reason, enterococci and other facultative anaerobes are resistant to low concentrations of aminoglycosides [39].

An important role in the intracellular accumulation of aminoglycosides is attributed to the periplasmic proteins whose synthesis is induced by the antibiotic.

Aminoglycosides induce the pleiotropism phenomenon (i.e., simultaneous changes in the expression of many genes) and also produce mRNA reading errors.

Aminoglycosides are rapidly acting antibiotics with broad spectrum of action being active against strict and facultative Gram-positive and negative aerobic bacteria.

Aminoglycosides could slowly accumulate through endocytosis in the lysosomes of the eukaryotic cells to an apparent cellular-to-extracellular ratio of 2 to 4, excepting some tissues like kidney proximal tubular cells, exhibiting binding sites such as megalin and acidic phospholipids where the accumulation is faster [40–43].

Spectinomycin is an aminocyclitol antibiotic related to the aminoglycosides, manifesting bacteriostatic action, usually used for the treatment of gonorrhea produced by penicillin-resistant *Neisseria gonorrhoeae* strains.

The encapsulation of aminoglycosides in liposomes could increase the therapeutic index of the drug by reducing the level of drug delivered at the sites where the antibiotic is toxic to the therapeutic amounts necessary for the treatment of infection. This procedure also increases the aminoglycosides efficiency against intracellular bacteria.

Tetracyclines represent a family of antibiotics inhibitory for the protein synthesis through a mechanism of blocking the attachment of aminoacyl–tRNA complex to the ribosome acceptor site (site A) [44]. These antibiotics have a broad spectrum bacteriostatic effect, being active against Gram-positive Gram-negative bacteria and protozoa, but they also kill normal gut microbiota and produce gastrointestinal disorders.

Tetracyclines have the ability to accumulate in eukaryotic cells, including neutrophils [45].

Tetracyclines are strong chelating agents, their pharmacological properties being influenced by the presence of metal ions. Each of the rings of the tetracycline core can contain only linear carbon atoms in order to keep the antibiotic activity.

Atypical tetracyclines and some of their analogues disrupt the cytoplasmic membrane structure and manifest a bactericidal effect, which contrasts with the bacteriostatic effect of tetracyclines, reversibly inhibiting protein synthesis. Atypical effects of membrane disruption are likely a consequence of the lipophilic nature of the molecule. Because of many side effects derived from their nonspecific interaction with prokaryotic and eukaryotic cell membranes, atypical tetracyclines present no current therapeutic interest.

The use of this antibiotic during pregnancy or in the first 5 years of life results in the deformation of the fetus skull bones and permanent teeth staining due to their ability to bind Ca^{2+}.

In the 80s, before the emergence of resistant strains of *N. gonorrhoeae*, tetracycline was used to treat sexually transmitted infections and is currently used in the treatment of non gonococcal urethritis and chlamydial infections

Tetracycline resistance is widespread in Gram-positive cocci and is present also in *Mycoplasma* sp.

Although the main action of tetracycline is antibacterial, this antibiotic is also active against protozoan parasites, inhibiting *Giardia lamblia*, *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Plasmodium falciparum*. The effectiveness of tetracycline derivatives to parasitic protozoa is correlated with the degree of penetration into the cell. The most effective are lipophilic compounds that cross quickly the cytoplasmic membrane (such as the tiotetracycline derivative).

Glicil-tetracyclines are represented by tigecycline, used to treat skin and abdominal infections. The structural feature of tigecycline is the substitution in the position 9 of the tetracycline with a glycine residue. These are broad-spectrum antibiotics against *N. gonorrhoeae, Legionella pneumophila*, and also for fast growing non tubercular mycobacteria.

They are active agents to be used for the prophylaxis of malaria and strains of *P. falciparum* resistant to specific chemotherapeutic agents.

Macrolides, lincosamides, and streptogramins (MLS) have a different chemical structure, but they act in a similar manner on a variety of intracellular bacteria (e.g., Gram-positive, Gram-negative cocci, *Chlamydia* spp., *Mycoplasma* spp., and *Legionella* spp.).

MLSs prevent bacterial ribosomes to translate the RNA in two different ways, either by the inhibition of translocation of peptidyl-tRNA from the acceptor site (A) and the peptidyl donor site (P) or by inhibiting the initial steps of the assembly of the 50S ribosomal subunit.

Macrolides have a marked intracellular accumulation in almost all cells, explained by their weak basic character favoring the accumulation in the acidic cytosol compartment, particularly in the lysosomal apparatus, with a rate depending on the derivative structure, lower for erythromycin and higher for those carrying two basic functions [46, 47].

Regarding their structure, macrolides are characterized by a multiunitar lactone ring with 12, 14, 15, or 16 carbon atoms, with few double bonds, which contain attached 1–3 glucidic residues by glycosidic linkages.

Macrolides are active against Gram-positive and Gram-negative bacteria (*Mycobacterium* spp., *Treponema pallidum*, *Mycoplasma pneumoniae*, *Chlamydia* sp., *Rickettsia* sp.).

Novel molecules, such as azithromycin and clarithromycin, have a superior antibacterial activity as compared with erythromycin because they have higher coefficients of intracellular penetration and are more stable, being more easily absorbed and manifesting lower incidence gastrointestinal side effects. Azithromycin is active against *Mycoplasma* spp. and *Chlamydia* spp. Clarithromycin has significant antibacterial activity *in vitro* against mycobacteria.

Streptogramins are represented by two synergistic components (A and B). Similar with macrolides and lincosamides, the A and B compounds of the streptogramin set at the ribosomal subunit 50S, 23S, and to rRNA.

Lincomycin and clindamycin are macrolides, but many of their biological properties are similar to erythromycin. They consist of an amino acid linked to an amino-sugar. Ketolides (telithromycin) are new chemical entities, characterized by the replacement of L-cladinose in the erythronolide A ring, with a 3-keto function and the C11–C12 carbamate [48]. ABT 773 represents the latest generation of drugs, characterized by 3-keto group that substitutes the sugar rest of the 3-cladinose from erythromycin and clarithromycin [49].

Oxazolidinones (eperezolid and linezolid) represent a unique class of synthetic antimicrobial agents with a unique mechanism of action, which eliminates the risk of extending existing resistance to the available antimicrobial agents. The oxazolidinones are inhibitors of ribosomal protein synthesis in bacteria, preventing the formation of the 70S initiation complex comprising fMet RNA, mRNA, and the two ribosomal subunits.

Linezolid has a good *in vitro* activity against *N. gonorrhoeae, N. meningitidis*, and *M. tuberculosis.*

There are few studies showing the capacity of oxazolidinones (i.e., linezolid) to preferentially and rapidly accumulate intracellularly (in concentrations 1.2 times higher than the extracellular ones within 20 min), both in human phagocytic (PMNs) and in nonphagocytic (McCoy) cells. However, the efflux of the antibiotic is also very rapid, the great amount of the intracellular antibiotic being released in less than 2 min [50].

Sulfonamides are synthetic chemotherapeutic agents, very similar to sulfanilamide (para-amino-benzenesulfonamide). The bacteriostatic action of this antibiotic is due to the interference with the folic acid synthesis pathway.

The best known sulfonamides are sulfadiazine and sulfamethoxazole (cotrimoxazole). As sulfonamides, para-aminosalicylic acid (APAS) and dapsone obtained by chemical synthesis are competitive inhibitors for the para-aminobenzoic acid metabolism and inhibits the synthesis of folic acid, being active against *M. tuberculosis* and *Mycobacterium leprae*.

The family of the diaminopyrimidine derivatives includes trimethoprim and tetroxoprim.

Trimethoprim is an analog of dihydrofolic acid, which competitively inhibits dihydrofolate reductase, an enzyme that converts dihydrofolate to the active cofactor—tetrahydrofolic acid [51].

The blockage of the same biosynthetic pathways sequence under the action of sulfonamides and trimethoprim provides a high degree of synergistic activity against a broad spectrum of microorganisms.

3.4. Chemotherapeutic agents acting by inhibiting DNA replication and transcription

Quinolones (also known as 4-quinolones) are the first antimicrobials produced synthetically and form a family of compounds that resemble the core quinolinic existence.

Along with the β-lactam antibiotics and macrolide antibiotics, quinolones represent one of the three major families of antimicrobial agents used in human therapy [52]. Nalidixic acid is an intermediate for the synthesis of quinolones. Subsequently, quinolones have diversified by introducing a fluorine (F) in position 6 and position 7 of a heterocyclic ring (piperazine, pyrrolidine etc.), which generated fluoroquinolones.

Fluoroquinolones (norfloxacin, pefloxacin, ofloxacin, ciprofloxacin etc.) have a broad spectrum of activity against intracellular bacteria, including *Chlamydia* sp., *Rickettsia* sp. and mycobacteria. These molecules penetrate the bacterial cell by passive diffusion and act on specific targets represented by topoisomerases: DNA gyrase (topoisomerase II) and topoisomerase IV, probably inducing lethal effects such as bacterial DNA damage.

Rifampicin B is naturally synthesized, but in the recent years, the semisynthetic derivatives of rifampicin are the most extensively used. Rifampicin belongs to a group consisting of an aromatic chromophore, which is included in the aliphatic chain. It is associated with the B subunit of DNA-dependent RNA polymerase, thus blocking transcription and RNA synthesis initiation. The antibiotic is widely used in the combinatory therapy of tuberculosis.

It was demonstrated that quinone and hydroquinone forms of rifampin can accumulate in PMNs from normal and chronic granulomatous disease individuals and be active against intracellular staphylococci invading the chronic granulomatous disease PMN [53].

3.5. Other synthetic chemotherapeutic agents

Nicotinic acid hydrazide (isoniazid, INH), introduced in the clinic before 1950, together with rifampin, forms the basis of antituberculosis chemotherapy. Isoniazid is a nicotinamide derivative. The mechanism of action is not known, but it influences the synthesis of lipids, nucleic acids and mycolic acid from *M. tuberculosis*.

It is assumed that isoniazid is active by competing with pyridoxine (vitamin B6) necessary for the growth of *M. tuberculosis* cells or by inhibiting mycolic acid synthesis. It is bactericidal to the growing cells and has a bacteriostatic action against the cells that do not replicate. Together with PASA and dapsone, isoniazid is used to treat infections with *Mycobacterium* sp.

Ethambutol, pyrazinamide, and ethionamide block the enzymatic reactions in the bacterial cell because they are similar but not identical to bacterial vitamins.

Ethambutol inhibits arabinosyl chloride-transferase enzyme involved in the biosynthesis of arabinogalactan and lipoarabinomannan. Other effects attributed to metabolic inhibition action of ethambutol are RNA and phospholipids synthesis, inhibiting the transfer of mycolic acids linked to arabinogalactans of the murine cell wall and also inhibiting the synthesis of spermidine at early stage conversion of glucose into monosaccharides used for the synthesis of parietal polysaccharides and peptidoglycan. It is a very specific and effective drug used in association with isoniazid for tuberculosis treatment. It has a good bacteriostatic effect [54].

Pyrazinamide is a synthetic derivative of nicotinamide, which is metabolized to the pyrazino acid, antibacterial active intermediary.

Ethionamide, a derivative of the isonicotinic acid, is active against *M. tuberculosis* and other mycobacteria, acting through inhibition of mycolic acid synthesis.

Despite the massive amount of literature on the intracellular activity of antibiotics and on its relation to cellular accumulation and disposition, the relationship between drug concentration (or dosing), time of exposure (or other pertinent pharmacokinetic parameters), and chemotherapeutic response (in terms of quantitative measurement of the variation in the bacterial population) is incompletely elucidated [55]. Clinical studies, in this context, are particularly difficult due to the complex extracellular and intracellular pharmacokinetic variables, microbial and host-response variables, and simultaneous presence of extracellular and intracellular foci of infection. Some classes of antibiotics, such as ansamycins, macrolides, tetracyclines, and fluoroquinolones, are generally considered as being active against intracellular pathogens, being already clinically used for the treatment of bacterial infections with obligate and facultative intracellular bacteria. Conversely, there is a consensus over the fact that beta-lactams and aminoglycosides show no or only a poor intracellular activity. However, beta-lactams could exhibit a time-dependent activity against intracellular bacteria when administered in prolonged treatments at the maximal dose to compensate for the lack of accumulation, whereas aminoglycosides, which are concentration-dependent, could be active a high concentrations [56]. For macrolides, activity is clearly observed against phagosomal organisms (phagosomes are neutral or only slightly acidic) at a sufficiently high concentration to cope with the loss of activity caused by low pH or binding to cell constituents. Although

some organisms, like *Chlamydia* sp. and *Legionella* sp., are quite sensitive, this may not be the case for others, such as *Staphylococcus aureus*.

The balance between influx and efflux, metabolism, and binding properties determines the intracellular concentration of free active drug; bacterial responsiveness, physico-chemical conditions prevailing at the site of infection, and degree of cooperation (or hindrance) with the host defenses are affecting the intracellular activity of antibiotics.

4. Antibiotics carriers for the intracellular delivery

Intracellular bacterial pathogens are hard to treat because of the inability of conventional antimicrobial agents belonging to widely used classes to penetrate the lipidic membrane. They accumulate in different compartments of the cells and face the limiting conditions of the phagocytic cells, such as the lysosomal acidic pH and inactivating enzymes, low oxygen pressure, etc. [57, 58], requiring the development of efficient delivery systems that could release the antimicrobial agent intracellularly in active concentrations, thus increasing its effectiveness while decreasing the required therapeutic doses, its systemic toxicity, and the probability of selecting resistance [59–61].

Different improved drug carriers have been developed for treating intracellular pathogens, including antibiotics loaded into liposomes and other lipid formulations, microspheres, polymeric carriers, fullerenes, dendrimers and nanoplexes [1, 61–63].

The advantages of using drug delivery systems are represented by the tunable surface/size/shape/functionalization properties, depending on the structure of the transported drug; evasion of the immune system; use of the same carrier to transport more than one drugs; improvement of the biodisponibility, biodistribution, and pharmacokinetics of the drugs; availability of drug carriers for different administration routes; and low probability of selecting resistance [64, 65].

This section will focus on drug delivery systems oriented toward treatment of intracellular infections.

4.1. Polymeric drug carriers

Polymeric (both natural and synthetic) biodegradable and/or biocompatible matrixes, such as poly (ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(γ-glutamic acid) (PGA), poly(L-lysine) alginate, gelatin, collagen, cellulose, albumin, fibrin, dextran, pectin, chitosan, agar, agarose, and carrageenans, could be used to embed/encapsulate/adsorb/conjugate a certain drug in order to protect it against enzymatic and hydrolytic degradation [66–79] to control the rate of drug release with an optimal maintenance of the biologically active drug level within therapeutic window [80, 81], to prevent toxicity, to target drugs to the site of action, and to improve absorption, bioavailability, and therapeutic efficacy [82, 83].

The polymeric carrier systems could be classified in four different categories: diffusion controlled (the drug is released by diffusion), chemically controlled (the drug molecules are linked to a polymeric backbone often by means of a spacer molecule, being released inside the host tissue by hydrolysis or by the enzymatic cleavage of the linkage between the polymer carrier and the drug, by polymer biodegradation or by bioerosion), solvent activated (controlled by swelling or osmosis allowing the drug inside the system to diffuse outward), and magnetically controlled (the polymer carrier is combined with magnetic microparticles of iron, cobalt, and nickel, which could be oriented inside the body by an externally applied magnetic field) [67, 84–87].

Responsive or smart polymers respond and modulate their properties in accordance with different external parameters (e.g., UV/visible light, electric charges, electromagnetic radiation, temperature, pH, ionic strength, ionic or metallic interactions), leading to degradation, drug release, dissolution/precipitation, swelling/collapsing, and formation of micelles and vesicles [88, 89].

Particulated micro- and nanospheres and capsules prepared from natural polymers have been synthesized by emulsion polymerization, solvent evaporation, ionic gelation, self-assembly, nanoprecipitation, and supercritical fluid technology for drug delivery [90–92].

While microparticles are likely to remain in time at the injection place and can be engulfed only by phagocytes, the smaller nanoparticles can diffuse from the injection place and cross biological barriers, including the cellular membrane of different cell types [93, 94].

Nanoparticle-based drug delivery systems were applied in the treatment of different infectious diseases with intracellular pathogens, such as acquired immune deficiency syndrome (AIDS), malaria, and tuberculosis [95, 96]. Polymeric nanoparticles represent also a promising solution for the local delivery of therapeutics to the central nervous system through the blood–brain barrier [96, 97].

The polymeric micelles consisting of a hydrophilic shell and a drug-containing core were used to incorporate extremely hydrophobic drugs, to assure prolonged drug circulation time, drug stability, and escape from the reticuloendothelial system due to their nanometer size [98]. Nanoparticles formulated using biodegradable polymers have been also shown to enhance the delivery of antibiotics intracellularly and to improve their effectiveness as revealed by the reduced microbial burden.

In comparison with other delivery systems, natural polymers are generally safer and more stable, easier to obtain, and offer better control over agent release [82, 99].

Dextran, a biodegradable, biocompatible, nonimmunogenic, and nonantigenic polymer of bacterial origin, composed essentially of α-1,6-linked D-glucopyranose units [100], proved to improve the activity of polar molecules such as penicillins, aminoglycosides, rifampicines, and quinolones and to efficiently challenge the drug delivery into the infected cells in an active form, leaving the host cells intact [101]. Dextran microspheres could act as macromolecular carriers for the small molecules antibiotics and induce endocytosis of the drug by the target cell *via* a specific receptor, followed by the subcellular distribution of the drug to sites where the microbial cells are localized [102].

Chitosan-dextran sulfate (CD) nanocapsules were assessed for their efficiency in delivering ciprofloxacin or ceftriaxone drugs against *Salmonella* using a murine salmonellosis model. CD nanocapsules proved to efficiently target and kill the intracellular pathogen at a significantly lower dose, as compared to the free antibiotic, assuring also a more increased retention time of ciprofloxacin in the blood and organs [103].

Cyclodextrin, a cyclic oligosaccharide consisting of six to eight glucopyranose units joined by α-(1 → 4) glucosidic linkages, exhibits an internal lipophilic cavity, which can be complexed with hydrophobic agents [104], improving drug solubility, stability, and bioavailability [105, 106].

Hyaluronic acid, a linear anionic polysaccharide of animal or microbial origin, belonging to the glycosaminoglycans family, consisting of alternating units of *N*-acetyl-D-glucosamine and glucuronic acid, is a promising delivery vehicle for antibiotics [107].

Alginate nanoparticles increased the activity of rifampicin, isoniazid, pyrazinamide, and ethambutol against *M. turberculosis* by assuring a higher drug payload and therapeutic efficacy as well as an improved pharmacokinetic [96].

Some pathogens can survive for a prolonged period of time in the first-line anti-infective defense cells represented by polymorphonuclear leukocytes (PMNs). The amorphous chitin nanoparticles with a size of 350 ± 50 nm in diameter proved to achieve a sustained release of rifampin till 72 h and significantly enhanced the drug accumulation into the intracellular compartments of PMNs [108].

Synthetic polymers are preferentially used for the development of drug delivery systems due to their excellent and tailor-made properties (biocompatibility; water compatibility; lack of immunogenicity; optimal degradation time coinciding with their function; appropriate mechanical properties in terms of toughness, flexibility, and swelling; generation of nontoxic degradation products that can be easily resorbed or excreted; flexibility for chemical modification to get increased biocompatibility and to enlarge the variety of the loaded agent; they do not need to be removed from the body being able to be degraded and excreted or resorbed; the existence of FDA and European Medicine Agency approval for drug delivery systems for parenteral administration; and protection of the loaded agent from degradation, assuring its sustained and targeted release) [99, 109–114].

Amoxicillin-loaded PLGA microspheres successfully eliminated *L. monocytogenes* from vital organs (kidney, spleen, and brain) and also increased the survival rate of treated animals in comparison with the free antibiotic, suggesting the targeted delivery of the antibiotic to the infected macrophages, as well as its sustained release over an prolonged period of time [115].

The PLGA nanoparticles proved to be efficient in encapsulating and releasing the rifampicin drug, showing an initial burst followed by the sustained release of this primary tuberculostatic agent [116, 117]. Also, rifampicin-loaded polybutylcyanoacrylate nanoparticles potentiated the *in vitro* and *in vivo* activity of rifampin and ciprofloxacin against *Mycobacterium avium* due to an effective delivery of drugs to macrophages [118, 119].

Gentamicin-loaded PLGA nanoparticles have been obtained for the treatment of brucellosis, proving to achieve high intracellular bactericidal activity of the antibiotic [120].

The PLGA microparticles proved efficient for the delivery of the antibacterial phosphorylcholine and of the dietary antigen beta lactoglobulin in a mouse model, inducing protective mucosal immunity against intestinal infection by *S. typhimurium* [121].

PLGA nanoparticles have been shown to efficiently accumulate in inclusions in both acutely and persistently infected *Chlamydia*-infected cells, while the encapsulation of rifampin and azithromycin antibiotics in PLGA nanoparticles enhanced the effectiveness of the antibiotics in reducing microbial burden. The combination of rifampin and azithromycin was more effective than the individual drugs [122].

Gentamicin was ion-paired with the anionic AOT surfactant to obtain a hydrophobic complex (GEN-AOT) that was formulated as a particulated material either by the precipitation method or by encapsulation into PLGA nanoparticles. The *in vitro* studies against the intracellular bacteria *Brucella melitensis* demonstrated that the bactericidal activity of gentamicin was unmodified, proving their use for the treatment of infections caused by intracellular bacteria [123].

Rapamycin-loaded PLGA microparticles effectively released the active drug inside dendritic cells, under intra-phagosomal (pH 5) and extracellular (pH 7.4) conditions [124].

Amoxicillin-bearing human serum albumin and, more evident, amoxicillin-dopped PLGA microparticles proved to be efficient in combating *L. monocytogenes* infection in a mouse experimental model, as revealed by the decreased bacterial burden in various organs and reduced viable counts, the results clearly demonstrating that the respective microparticles successfully target the infected macrophages [106].

Poly(isohexylcyanoacrylate) (PIHCA) nanospheres improved the activity of ampicillin against *S. typhimurium* and *L. monocytogenes,* but the particles themselves exhibited also antimicrobial activity [125, 126].

Ampicillin-encapsulated poly(isohexylcyanoacrylate) nanoparticles prove to be more efficient than the free antibiotic against *L. monocytogenes* infecting mouse peritoneal macrophage, as revealed by the more drastic decrease of viable cell counts. However, the nanoparticles acted on the intracellular bacteria after a lag period of 6–9 h, probably due to a required period for the degradation of the polymer [127].

Poly-lactide-co-glycolide (PLG) nanoparticles enhanced the bioavailability and pharmacodynamic properties of rifampicin, isoniazid, pyrazinamide, and ethambutol against *M. turberculosis* [128].

Amphiphilic, cationic polymers with an amino moiety, a low molecular weight, and short alkyl chains designed to mimic the host secreted microbicidal peptides are considered promising candidates for potent and highly selective antimicrobial agents (acting on microbial walls or mitochondrial activity) with decreased risk to select resistance [129, 130].

The polyketal nanoparticles formulated from the hydrophobic polymer poly(1,4-phenyleneacetone dimethylene ketal) (PPADK) improved the activity of superoxide-dismutase to scavenge reactive oxygen species produced by macrophages [131, 132].

Poly-butyl cyanoacryle proved to increase the efficiency of the moxifloxacin fluoroquinolone against *M. tuberculosis* infecting the THP-1 cells [133].

Polyalkycyanoacrylate nanoparticles proved to improve the activity of ciprofloxacin and colistin against *S. typhimurium* at the early stages of the infection in mice and/or *in vitro* models. Ciprofloxacin-loaded nanoparticles induced a significant decrease of bacterial counts in the liver whatever the stage of infection and the form used. However, none of the treatments were able to sterilize the spleen or the liver. In the *in vitro* study, colistin was only active against bacteria recovered during the early phase of infection, whereas ciprofloxacin exerted its activity at all times postinfection [134].

Amphiphilic block copolymers could self-assemble, resulting in vesicles called polymersomes [135]. Polymersomes of (poly[2-[methacryloyloxy]ethyl phosphorylcholine] [PMPC]–poly[2-[diisopropylamino]ethyl methacrylate] [PDPA] block copolymers) proved to successfully deliver metronidazole and doxycycline in *Porphyromonas gingivalis*-infected oral keratinocytes significantly increasing their activity [136].

4.2. Liposomes

Liposomes are small spherical, uni- or multilamellar vesicles in which the central aqueous cavities are surrounded by amphipatic molecules, being thus able to entrap both hydrophilic and hydrophobic drugs [137]. After intravenous injections, liposomes are taken up by macrophages in the liver and in the spleen, representing thus a promising option for fighting infections due to facultative intracellular bacteria, parasites, or viruses [138].

The incorporation of different tuberculostatic agents in liposomes (such as ciprofloxacin) has shown good antibacterial efficacy both in both macrophage cell lines and in animal tuberculosis models [139, 140].

Streptomycin inclusion in phosphatidyl glycerol, phosphatidyl choline, and cholesterol-containing liposomes showed an increased antimicrobial activity against *Mycobacterium avium* [141].

The encapsulation of antibiotics in liposomes could represent a viable solution for the drug penetration into the systemic circulation through the alveolar-capillary barrier, followed by its accumulation in different organ tissues or for the direct administration to the lung [142].

Phosphatidylcholine, cholesterol, dicetylphosphate, *O*-steroyl amylopectin, and monosialo-gangliosides/distearylphosphatidylethanolamine-poly (ethylene glycol) 2000 liposomes have been shown to act as a promising targeted delivery systems for isoniazid and rifampicin to the lung in mice experimental model [143].

The liposomal encapsulation of membrane-impermeative antibiotics, like gentamicin is among the most used approaches to achieve intracellular antibiotic delivery and therefore increase the drug's therapeutic activity against intracellular pathogens.

Gentamicin, encapsulated in plurilamellar liposomal vesicles, proved to be active against intracellular *Brucella abortus* infecting murine monocytes [144].

Gentamicin entrapped within stable multilamellar liposomes was used to treat mice orally infected with *Salmonella dublin* and proved to achieve high and persistent (up to 10 days) concentrations of gentamicin in the spleen, while bacterial counts in the lymph nodes decreased. Also, gentamicin entrapped in liposomes was less toxic in mice than its free form [145]. Ciprofloxacin encapsulation in dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and cholesterol containing liposomes also proved increased anti-*Salmonella dublin* activity demonstrated by the decreased mortality of animals and good distribution of liposomes to all areas of infection [145].

The encapsulation of ampicillin in liposomes reduced the *L. monocytogenes* viable counts in mice peritoneal macrophages [146]. The DOPE (dioleoylphosphatidylethanolamine) liposomes, sensitive to pH proved to be efficient in the ampicillin uptake by the macrophages infected with *L. monocytogenes*, correlated with an increase in the microbicidal activity. The efficiency of this drug delivery system was also proven in an *in vivo* mouse infection model, as revealed by the decrease of viable cell counts in the liver and spleen [147].

Liposomes, as well as nanoparticles coated with a lipid bilayer have been used to encapsulate drugs for the passive transport through the blood–brain barrier due to the enhanced lipophilic transport of drugs to the target tissues [148–150].

Streptomycin and doxycycline were entrapped into macromolecular nanoplexes with anionic homo- and block copolymers enabling the simultaneous binding of both antibiotics into the nanoplexes, which significantly reduced the *B. melitensis* load in the spleens and livers of the infected mice [151].

It was demonstrated that gentamicin can be easily introduced into membrane vesicles of Gram-negative pathogens that naturally bleb off the bacterium throughout its growth cycle and delivered directly not only to other Gram-positive and Gram-negative pathogens but also to mammalian cells [152].

4.3. Niosomes

Niosomes or nonionic surfactant vesicles are microscopic, spherical, uni- or multilamellar, and polyhedral vesicles of 10–1000 nm formed by self-assembly of a mixture of cholesterol and a single alkyl chain nonionic and nontoxic surfactant with subsequent hydration [153]. Niosomes can be used for the delivery of hydrophilic, lipophilic, and amphiphilic drugs, irrespective of their degree of solubility [153]. Niosomes improve the activity of isoniazid and rifampicin against *M. tuberculosis* infecting the J774 macrophage cell line [154, 155].

4.4. Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles (SLN) (50 nm–1 nm) are colloidal carriers for lipophilic and hydrophilic drugs, containing natural lipids dispersed in water or in the aqueous surfactant solution [156].

Mannose-conjugated SLNs were successfully used to selectively deliver rifabutin, isoniazid, and pyrazinamide to alveolar and lymphatic tissues [157]. Stearic acid SLN improved the activity of rifampicin, isoniazid, and pyrazinamide against *M. tuberculosis* by increasing the residence time and the drug bioavailability while decreasing the administration frequency [158].

Nanosuspensions could be used for nebulization procedures for delivering drugs poorly soluble in the lung secretions [159], as already proved by the antitubercular drugs (rifampicin, isoniazid, and pyrazinamide) incorporated into various formulations of solid lipid particles ranging from 1.1 to 2.1 μm and nebulized to guinea pigs [160].

4.5. Fullerenes

Fullerenes are a new form of carbon, with hollow sphere, ellipsoid, or tube size. The cationic fullerene derivatives bearing a substituted-quinazolin-4(3H)-one moiety as a side arm were reported to have a very good inhibitory potential on *M. tuberculosis* [161].

4.6. Dendrimers

Mannosylated dendrimers proved to be an efficient drug delivery system for rifampicin in a rat alveolar macrophages, the sustained release taking place in a pH-dependent manner [162].

The mannosylated fifth-generation poly(propyleneimine) dendrimer nanocarriers proved to be very efficient for the intracellular uptake of lamivudine, a nucleoside/nucleotide reverse transcriptase inhibitor, and the reduction of the HIV-1 viral load in the infected MT2 cells [163].

Pegylated lysine-based copolymeric dendrimer improved the proved anti-*P. falciparum* of artemether drug, as revealed by the increased drug stability, enhanced solubility, and prolonged drug circulation half-life [164].

A fourth-generation hydroxyl-terminated poly(amidoamine) (PAMAM) dendrimer was used as the intracellular vehicle of azithromycin for the treatment of chlamydial inclusions, proving to be more efficient than the free drug with a sustained effect lasting for 24–48 h post-infection [165].

4.7. Zeolites

Zeolites are crystalline materials with frameworks comprising Si, Al, and O [166]. The zeolites possess nanochannels and cages of regular dimensions [167]. The nanochannels (pores) of zeolites are open allowing the diffusion of therapeutic agents from the exterior to the interior of the zeolites. These networks exhibit a large specific surface area and a good stability in different environments [167].

Mesoporous silica nanoparticles proved to increase the efficiency of the rifampin and isoniazid against *M. tuberculosis* infecting the THP-1 cells [168].

The capability of porous sol-gel processed silica as a carrier for gentamicin has been demonstrated, showing a significantly higher rate of bacterial clearance from organs than did the free drug [169].

4.8. Erythrocytes

Erythrocytes have a great the potential to provide an effective therapy against intracellular pathogens. Amikacin encapsulation in human carrier erythrocytes demonstrated a slow and sustained release from the loaded carrier till 48 h, suggesting the potential use of the erythrocytes as a slow release system for antibiotics [170].

5. Conclusion

In the last years, there was an important progress in improving the drug delivery systems for fighting intracellular bacterial infections. The proposed solutions led to decreased toxicity, improved bioavailability, and prolonged and sustained release associated with reduced frequency of administration and enhanced antimicrobial activity. However, the design of the optimal drug carrier for the intracellular release of different antibiotics should rely on the elucidation of the intracellular kinetics (accumulation, degradation, and distribution in different intracellular compartments and activities) of the respective drugs.

The most promising results have been obtained by using natural or synthetic polymers and liposomes and other lipid formulation carriers, whose efficiency has been demonstrated by *in vitro* and *in vivo* experimental studies, as well as in clinical trials, and which could therefore represent efficient strategies for fighting severe microbial infections produced by facultative or obligate intracellular microorganisms as well as for viral infections.

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