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Nanotechnology Tools for Efficient Antibacterial Delivery to *Salmonella*

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1. Introduction

In recent years, an increasing number of salmonellosis outbreaks have been recorded around the world, and probably there should be more cases that were not detected or reported (1). Many different types of *Salmonella* exist, some of which cause illness in both animals and people, and some types cause illness in animals but not in people. The various forms of *Salmonella* that can infect people are referred to as serotypes, which are very closely related microorganisms that share certain structural features. Some serotypes are only present in certain parts of the world (1). *Salmonella* spp are gram negative anaerobic and intracellular bacteria. Salmonellosis, mainly due to *Salmonella typhimurium*, occurs more frequently in HIV-infected patients than in healthy individuals and the frequency of bacteraemia is much higher in such patients (2).

Despite the discovery of new antibiotics, treatment of intracellular infections often fails to eradicate the pathogens completely. One major reason is that many antimicrobials are difficult to transport through cell membranes and have low activity inside the cells, thereby imposing negligible inhibitory or bactericidal effects on the intracellular bacteria (3). In addition, antimicrobial toxicity to healthy tissues poses a significant limitation to their use (3). Therefore, the delivery of the drug to the bacterial cells is currently a big challenge to the clinicians. This is on top of the problems posed by the emerging Multi-Drug Resistant species. Moreover, the reduced membrane permeability of microorganisms has been cited as a key mechanism of resistance to antibiotics (4).

Indeed, the challenge is to design the means of carrying an antibiotic into bacterial cells. The pioneer concept of targeted drugs was developed by Ehrlich in 1906 and defined as the 'magic bullet'. Since then targeted drug delivery has involved design and development of small molecule drugs that can specifically interact with the intended receptors in intended tissues. For example prodrugs can be designed for brain delivery of the active drug (5). Another common example is colon delivery of prodrugs designed to release the drug by taking advantage of the bacterial reductase enzymes in colon (6).

However, the drug development process is inevitably lengthy and breakthroughs are quite scarce which has led to the ever increasing cost of discovery and development of new drugs (7). On the other hand, nanotechnology offers a more convenient method for targeted therapy.

Logistic targeting strategies can be employed to enable the drug to be endocytosed by phagocytic cells and then released into the bacteria. To reach the above goal, a drug carrier is generally needed for a drug to arrive at the target site (8). The first study employing a drug carrier for targeted drug delivery was published approximately 40 years ago, using antibodies as carriers of radioactivity for the specific recognition of tumor cells (9). The ideal drug carrier ensures the timely release of the drug within the therapeutic window at the appropriate site, is neither toxic nor immunogenic, is biodegradable or easily excreted after action, and is preferably cheap and stable upon storage (10). Out of different types of drug carriers that have been investigated, many are soluble macromolecular carriers or liposomes (11-15).

By searching all published work on drug carriers it can be concluded that "the ideal drug carrier" does not exist. The suitability of a drug carrier is determined by the disease that will be targeted, its access to the pathological site, and the carriers' ability to achieve appropriate drug retention and timely drug release (16). When these types of formulations are administered by the intravenous route, phospholipidic, polymeric or metal particles are localized preferentially in organs with high phagocytic activity and in circulating monocytes, ensuring their clearance (8). The ability of circulating carriers to target these cells is highly dependent on tissue characteristics and on the carrier's properties. The liver rather than the spleen or bone marrow captures the submicronic particles (8). Immediately after injection, the foreign particles are subjected to opsonization by plasma proteins. This is the process by which bacteria are altered by opsonins so as to become more readily and more efficiently engulfed by phagocytes. In this way, 'classical' or 'conventional' carriers are recognized by the mononuclear phagocytic system (8).

The approaches for drug carrier to improve the drug's antibacterial efficacy are shown in Figure 1. In most cases, i.v. administration of the formulation is needed particularly for passive and active targeting.

The local administration of drug/carriers will increase the residence time of antibiotics at the site of infection (17-19). These carriers are generally investigated with the intention to treat local infections in body parts with limited blood flow as in bone, joint, skin, and cornea.

In passive targeting after i.v. administration of carriers which tend to be taken by phagocytic cells, drug-carrier complex will target intracellular infections. These infections are often difficult to treat as a result of limited ability of the antimicrobial agent to penetrate into cells. This approach makes use of the recognition of drug carriers (nanoparticles) as foreign material in the bloodstream by the phagocytic cells of the mononuclear phagocyte system, the cell type often infected with microorganisms (20, 21).

Regarding the other two approaches (passive targeting with long-circulation time, and active targeting) the targeting of infectious foci is not restricted to mononuclear phagocyte system tissues. In passive targeting a drug carrier with long duration of circulation is used and this is an area which has extensively been investigated, whereas in active targeting carriers specifically bind to the infectious organism or host cells involved in the inflammatory response.

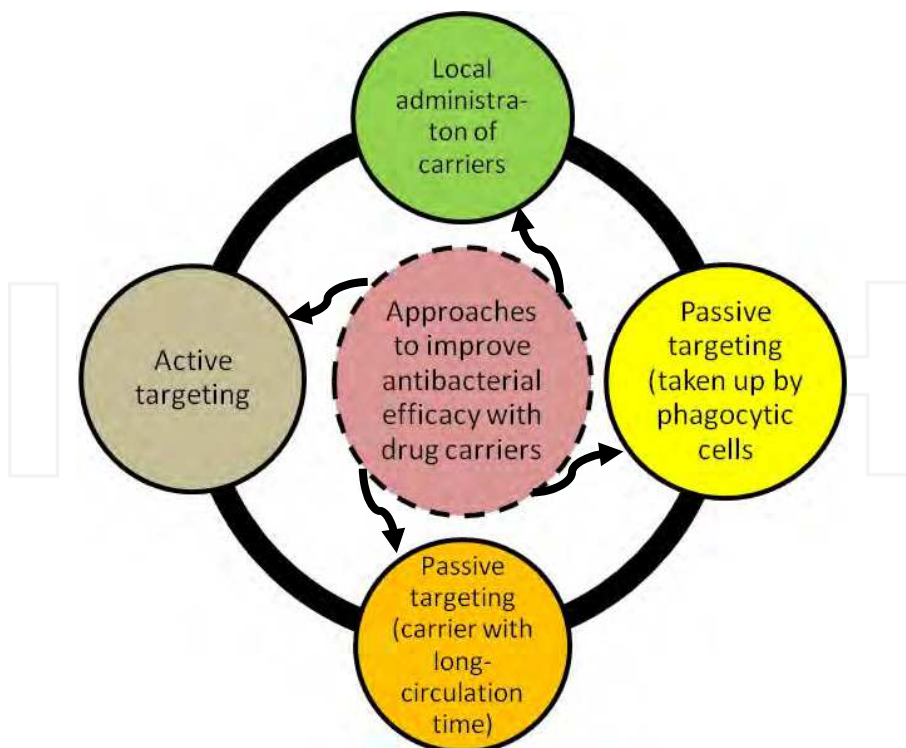


Fig. 1. Drug carrier approaches targeting bacterial infections to improve antibacterial efficacy of drugs.

This chapter focuses mainly on the current research for increasing anti-*salmonella* performance of antibiotics by means of liposomes and nanoparticle systems. Structure, properties, advantages and disadvantages of these drug delivery systems have been discussed. It is clear that such systems may improve the antibiotic efficacy by increasing the drug concentration at the surrounding of the bacteria.

2. Liposomes for antisalmonellosis drug delivery

2.1 Introduction

Liposomes are composed of small vesicles of a bilayer of phospholipid, encapsulating an aqueous space ranging from about 30 to 10000 nm in diameter (Figure 2). They are composed of one or several lipid membranes enclosing discrete aqueous compartments. The enclosed vesicles can encapsulate water-soluble drugs in the aqueous spaces, and lipid soluble drugs can be incorporated into the membranes. They are used as drug carriers in the cosmetic and pharmaceutical industry. The main routes of liposome administration are parenteral, topical and inhalation, and, in a few occasions, possibly other routes of administration can be used. Majority of current products are administered parenterally (22).

Liposome structure was first described in 1965, and they were proposed as a drug delivery nanoparticle platform in 1970s. In 1995, Doxil (doxorubicin liposomes) became the first liposomal delivery system approved by the Food and Drug Administration (FDA) to treat AIDS associated Kaposi's sarcoma (23). Liposomal drug delivery systems can be made of either natural or synthetic lipids. The main building blocks of some liposomal formulations are phospholipids (22). These are natural biomacromolecules that play a central role in human physiology as they are structural components of biological membranes and support organisms with the energy (24). They are amphiphilic molecules, poorly soluble in water, consisting of a hydrophilic part containing hydroxyl groups (the polar head), a glycerol backbone and two fatty acid chains, which form the hydrophobic part. One of the most commonly used lipids in liposome preparation is phosphatidylcholine, which is an electrically neutral phospholipid that contains fatty acyl chains of varying degrees of saturation and length. Cholesterol is normally incorporated into the formulation to adjust membrane rigidity and stability (8). Liposomes can be characterized in terms of size and lamellarity as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and multi lamellar vesicles (MLV). MLVs are usually considered large vesicles and aqueous regions exist in the core and in the spaces between their bilayers. The structure of these liposomes is shown in Figure 2.

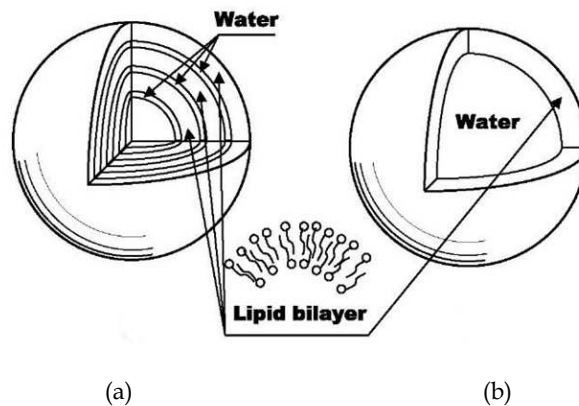


Fig. 2. Schematic structures of (a) multilamellar and (b) unilamellar liposomes (the picture was taken from <http://what-when-how.com/nanoscience-and-nanotechnology/nanoencapsulation-of-bioactive-substances-part-1-nanotechnology>).

The main advantages of liposomes as drug delivery systems can be in their versatile structure that can be easily modified according to experimental needs; they can also encapsulate hydrophilic drugs in their aqueous compartments and hydrophobic drugs in their bilayers, while amphiphilic drugs will be partitioned between the two. Moreover, being mainly made of phospholipid, they are non-toxic, non-immunogenic and fully biodegradable. Methods for preparing liposomes can take into consideration parameters such as the physicochemical characteristics of the liposomal ingredients, materials to be contained within the liposomes, particle size, polydispersity, surface zeta potential, shelf time, batch-to-batch reproducibility, and the possibility for large-scale production of safe and efficient products (23).

2.2 Preparation of liposoms

Liposome formation happens spontaneously when phospholipids are dispersed in water. However, in order to obtain the desired formulation with particular size and structure, various methods such as thin film method (24), sonication (25), extrusion (26), injection methods (27), dehydrated-rehydrated vesicles (28), reverse phase evaporation (29) and one step method (30) have to be used.

Each technique is briefly described below, but for more details, it is recommended to refer to the cited references. In brief, in thin film method liquids are dissolved in organic solvents and the solvent is removed under vacuum or nitrogen stream to form a thin film on the wall of a flask or test tube. In order to complete the formation of liposomes aqueous phase is added to the lipid film at a temperature above the phase transition of the lipid (24).

The sonication method is usually used to reduce the particle size and lamellarity of MLVs. In case of using the probe sonicator, the reduction in size of the liposomes can be guaranteed (25).

In order to get very homogeneous vesicles with a predetermined size, the extrusion technique is used. MLVs are extruded under pressure through particular filter with well-defined pore sizes from 30 nm to several micrometers. If the extrusion is repeated several times unilamellar liposomes can be formed (26).

Very small unilamellar vesicles with a particle size of 30 nm can be prepared using the ethanol injection method. Generally, lipids are dissolved in ethanol and injected rapidly into the aqueous solution, under stirring. At the end, the injected ethanol has to be removed from the system (27).

As dehydrated-rehydrated vesicles are able to hold high amounts of hydrophilic drugs under mild conditions, therefore this method is suitable for the drugs that are losing their activity under harsh conditions (28). Empty liposomes, usually unilamellar vesicles, are disrupted during a freeze drying step in the presence of the drug meant to be encapsulated. A controlled rehydration is obtained in the presence of concentrated solution of the drug. This technique can produce large oligolamellar liposomes of a size around 400 nm to several micrometers. It has been shown that in case of producing smaller liposomes (100-200 nm) sucrose can be added (31).

In the reverse phase evaporation technique which is similar to thin film technique, lipids are dissolved in organic solvent and the solvent is removed by evaporation (29). The thin film is resuspended in diethyl ether followed by the addition of third of water and the suspension is sonicated in a bath sonicator. The emulsion is evaporated until a gel is formed and finally the gel is broken by the addition of water under agitation. The traces of organic solvent should be removed by evaporation (29).

Finally, in the one-step method, lipid dispersion should be hydrated at high temperatures under nitrogen gas stream. This method has the capability to produce liposomes in the range of 200-500 nm (30).

2.3 Targeted delivery by liposomes

The main methods of delivery from liposome to cytoplasm include the exchange of membrane and lipids, contact release, adsorption, fusion and endocytosis. Through these

processes, drugs can be released into the bacterial or eukaryotic cells. Liposomal formulations have been used for the delivery of antitumor anthracyclines such as doxorubicin (23) and antifungal agent amphotericin B. Targeted delivery of liposomes to tumor cells has been explored through arsenoliposomes (32). Liposomes for antibacterial chemotherapy are under intensive research to enhance the antibacterial activity and improve pharmacokinetic properties. Advantages of liposomal antibiotics include improved pharmacokinetics, decreased toxicity, enhanced activity against intracellular pathogens, target selectivity and as a tool to overcome bacterial drug resistance (3).

Some liposomes are unique because they can be selectively absorbed by tissues rich in reticuloendothelial cells, such as the liver, spleen and bone marrow. This can serve as a targeting mechanism, but it also removes liposomes from the circulation rather rapidly. Although the poor stability of liposomes, particularly the rapid uptake from the body is not desirable, it could be useful for eradicating the infection by 'passive targeting' through macrophage activation and killing or elimination of parasitic infections.

On the other hand, surface charge and phospholipid composition can affect the interactions of liposomes with bacterial cell surface. For example it has been shown that cationic liposome formulations are more efficient in binding to skin bacterial cells (33).

Moreover, by attaching targeting ligands such as immunoglobulines (34), antibody segments, aptamer (35), peptides and small molecule ligands, and oligosaccharide chains (36), to the surface of the liposomes, they can selectively bind to microorganisms or infected cells and then release the drug payloads to kill or inhibit the growth of the microorganisms (23). The highly specific liposomes are those containing antibodies or immunoglobulin fragments which have affinity to specific receptors on the surface of the infected tissue cells or pathogens (3).

Biofilm surface characteristics have also been used for targeted delivery. Biofilms are microbial aggregations that are covered in an extracellular matrix of polymeric substances. The matrix is usually composed of complex mixture of oligomeric and polymeric molecules such as proteins, lipids and polysaccharides which, as Microbial Associated Molecular Patterns (MAMPs), elicit host defenses (37). Pathogens are much more difficult to control when living in biofilms. This is partly due to the matrix preventing drug transport to the microbial cells. Moreover, bacteria in biofilms grow slower and have reduced metabolic activity, and therefore they are expected to be less susceptible to the antibiotics (38). Currently a great deal of research is focused on exploring new chemotherapeutic targets in biofilms (37). On the other hand liposomes have proven efficient in targeting and eradication of various types of biofilms. Examples are immunoliposomes with high affinity to various oral bacteria including *Streptococcus oralis* (34) and polysaccharide-coated liposomes for the efficient delivery of metronidazol to periodontal pocket biofilm (39).

pH-sensitive liposomes offer another method for targeting and efficiently delivering the liposomal content into cytoplasm. Such liposomes are stable at physiological pH but undergo destabilization under acidic conditions. Therefore, they are able to promote fusion of target plasma or endosomal membranes, the so called 'fusogenic' properties, at acidic pH (40). Several mechanisms can trigger pH-sensitivity in liposomes. One of the most widely used methods is the use of a combination of phosphatidylethanolamine (PE) or its derivatives with compounds containing an acidic group that act as a stabilizer at neutral pH (41). Other more recent methods include the use of novel pH-sensitive lipids, synthetic

fusogenic peptides/proteins (42) and association of pH-sensitive polymers with liposomes (43). pH-sensitive liposomes have found applications in many therapeutic area including the antibiotic delivery to intracellular infections (44).

2.4 Pharmacokinetics consideration of liposomal drug delivery

Liposomal carriers can lead to sustained release of antibiotics during drug circulation in the body. Thus, appropriate levels of drug will be available for a longer duration in comparison with the conventional antibiotic formulations where the outcome is a quick and short effect (45). However, conventional liposomes are quickly opsonized after intravenous administration and therefore they are taken up by the mononuclear phagocyte as foreign antigens. As a consequence blood circulation time is lowered. By controlling the physicochemical properties of the vesicles (size and charge distribution, membrane permeability, tendency for aggregation or fusion, drug encapsulation efficiency, membrane rigidity) and therefore their interaction with the biological environment, many different types of liposomes with the aim of obtaining longer circulation half-lives can be developed (8).

The plasma circulation time of antibiotics can be improved by encapsulation in polyethylene glycol-coated (pegylated) (STEALTH) liposomes. The PEG coating forms a hydration layer that retards the reticuloendothelial system recognitions of liposomes through sterically inhibiting hydrophobic and electrostatic interactions with plasma proteins (46). Other methods that can confer hydrophilicity or steric repulsion are by the use of compounds having sialic residues, or through MLVs containing phospholipids with long saturated chains and negative surface charge (47). The increased half lives of stealth liposomes increase their ability to leave the vascular system into some extravascular regions.

2.5 Antibiotic loaded liposomes against *Salmonella* spp

One of the distinguishing features of liposomes is their lipid bilayer structure, which mimics cell membranes and can readily fuse with the cell membrane and deliver the antibiotic contents into the cellular cytoplasm. As a result, drug delivery may be improved to bacterial and eukaryotic cells alike. By directly fusing with bacterial membranes, the drug payloads of liposomes can be released into the cell membranes or to the interior of the bacteria. In terms of extracellular pathogens, improved antibiotic delivery into the bacterial cells is of particular importance especially since it can interfere with some of the bacterial drug-resistance mechanisms which involve low permeability of the outer membrane or efflux systems (48).

Liposomes are particularly successful in eradicating intracellular pathogens. Examples of these include liposomal formulations of antituberculosis agents isoniazid and rifampin (49), and ampicillin loaded liposomes for eradication of *Listeria monocytogenes* (50). This is partly due to improved drug retention in the infected tissue and the decreased toxicity as a result of sustained release of drug from liposomes. Moreover, liposomal formulations often have improved antibiotic pharmacokinetics with extended circulation time and prolonged tissue retention.

Liposomal chemotherapeutics for the treatment of salmonellosis may employ some of the conventional antibiotics with proven inhibitory or cidal activity *in vitro*. Bacterial gastrointestinal infections with *Salmonella typhi* may be treated with chloramphenicol. Alternatives to

chloramphenicol include amoxicillin, co-trimoxazole and trimethoprim (51). Recently treatment with cephalosporins and fluoroquinolones has become popular, as several members of these antibiotic families have been shown to be effective. The treatment of paratyphoid fever is the same as that for typhoid (51). *Salmonella* food-poisoning is self-limiting and does not require antibiotic therapy, unless the patient is severely ill or blood cultures indicate systemic infection. In this case, third generation cephalosporins or fluoroquinolones are the most reliable agents (51). Ceftriaxone or a first generation fluoroquinolone such as ciprofloxacin, ofloxacin or pefloxacin but not norfloxacin have been recommended as the first choice in typhoid and paratyphoid by The Sanford Guide to Antimicrobial Therapy (52). The improved efficiency of liposome formulations of antibiotics has been shown *in vitro* and *in vivo*. The *in vitro* infection models utilize macrophages infected with *salmonella*.

2.5.1 Penicillin loaded liposomes

The tissue distribution of ampicillin loaded liposomes was studied in normal noninfected mice and showed that ampicillin concentrated mostly in the liver and spleen (53). The Liposome formulation of ampicillin was significantly more effective than free ampicillin in reducing mortality in acutely infected mice with *Salmonella typhimurium* C5. These liposomes were quite efficient in targeting ampicillin to the spleen but were less effective in targeting ampicillin to the liver and reducing mortality in acute salmonellosis (53).

2.5.2 Cephalosporine loaded liposomes

Third generation cephalosporines have been indicated as suitable candidates for the treatment of *Salmonella* infections (52). Liposome formulations of these antibiotics may improve pharmacokinetics and also the targeted delivery to the intracellular infections. In a study with cephalotin, treatment of infected macrophages with multilamellar liposome-encapsulated cephalothin enhanced the intraphagocytic killing of *Salmonella typhimurium* over that by macrophages treated with free cephalothin (54). Resident murine peritoneal macrophages were shown to be capable of interiorizing the liposome-antibiotic complex leading to a relatively high intracellular concentration of cephalothin. The intracellular killing of the bacteria was maximal at 60 min of incubation; at this time, 60% of the interiorized organisms had been killed (54).

Desiderio & Campbell infected mice with *Salmonella typhimurium* to investigate the effectiveness of liposome-encapsulated cephalothin treatment (55). In the study they also compared the results with formulations containing free cephalothin. They showed that following intravenous administration, liposome-encapsulated cephalothin was cleared from the circulation more rapidly and concentrated in the liver and spleen. Treatment of infected mice with the liposome antibiotic complex was more efficacious in terms of reducing the number of *Salmonella typhimurium* in these organs compared to the injection of free antibiotic, although treatment did not completely eliminate the bacteria from this site (55).

Another study showed that egg phosphatidylcholine liposomes containing cephalpirin were relatively stable in serum, and provided acceptable serum levels of cephalpirin for 24 hr after i.v. administration while free drug at a similar dosage was undetectable in 3-5 hr. Moreover, the liposome formulation, as opposed to the free drug, could be used successfully for prophylaxis. Cephalpirin activity in the spleen and liver was greatly increased and persisted

for at least 24 hr when iv injections of the liposome formulation was used. This formulation of liposome, in contrast with the other liposome formulation containing tris salt of cholesterol hemisuccinate, could prolong survival in mice infected with *Salmonella typhimurium* (56).

Ceftiofur sodium is a third generation broad spectrum cephalosporin widely used clinically to treat respiratory diseases and mastitis. Its spectrum also covers *Salmonella* spp. The liposome formulations of ceftiofur were prepared in order to increase drug half life *in vivo* for veterinary purposes (57). The pharmacokinetic study in healthy cows showed that liposome preparations provided therapeutically effective plasma concentrations for a longer duration (elimination half life of more than double) than with the drug alone. These liposomes were stable and the minimum inhibitory concentrations against *Salmonella enteritidis* were 1/4th that of free ceftiofur sodium (57).

2.5.3 Aminoglycoside loaded liposomes

Despite the susceptibility of *Salmonella* spp to aminoglycosides, their use against many important intracellular bacterial infections has been limited due to the cell membrane permeability problems. Lutwyche et al. prepared several liposomal encapsulation formulations including pH-sensitive DOPE-based carrier systems containing gentamicin in order to achieve intracellular antibiotic delivery and therefore increase the drug's therapeutic activity against intracellular pathogens (58). They reported the superiority of some of the pH-sensitive liposomes over conventional liposome formulations, which was associated with the intracellular delivery of the antibiotic and was dependent on endosomal acidification. This liposomal carrier demonstrated pH-sensitive fusion that was dependent on the presence of unsaturated phosphatidylethanolamine (PE) and the pH-sensitive lipid N-succinylidoleoyl-PE. These formulations also efficiently eliminated intracellular infections caused by a recombinant hemolysin-expressing *Salmonella typhimurium* strain which escape the vacuole and reside in the cytoplasm. Moreover, *in vivo* pharmacokinetics and biodistribution tests confirmed that encapsulation of gentamicin in pH-sensitive liposomes significantly increased the concentrations of the drug in plasma compared to those of free gentamicin. Furthermore, liposomal encapsulation increased the levels of accumulation of drug in the infected liver and spleen by 153- and 437-folds, respectively (59).

Other investigations have indicated that even with conventional liposomes, liposome encapsulated gentamicin is less toxic in mice than is free gentamicin and is extremely effective-therapy for disseminated *Salmonella* infections in mice. For example when gentamicin sulfate was encapsulated in liposomes composed solely of egg phosphatidylcholine, the mean half-lives of the encapsulated drug in serum were around four times that of free (nonencapsulated) gentamicin in mice and rats following i.v. administration. Moreover, liposome encapsulation led to higher and more prolonged activity in organs rich in reticuloendothelial cells especially in spleen and liver. In acute septicemia infections in mice, the liposomal formulation showed enhanced prophylactic activity when compared with the free drug. In a model of murine salmonellosis, liposomal gentamicin greatly enhanced the survival rate (60). Similarly, a single iv injection of low dose gentamicin loaded multilamellar liposomes (composed of egg phosphatidylcholine, egg phosphatidylglycerol, cholesterol and alpha-tocopherol) resulted in 80% survival of mice infected with *Salmonella Dublin*, while zero survival was observed when treated with the same amount of free gentamicin. Higher concentrations of free gentamicin led to

neuromuscular paralysis, while the slow release of this dose from liposomes increased the survival rate to 100%. After the single dose treatments with liposomes, high concentrations of the drug were detectable for 10 days (61). The liposome-encapsulated gentamicin has also been proven successful in the treatment of Mycobacterium Avium-M intracellular complex (MAC) bacteremia in AIDS patients. In this case, MAC colony counts in blood fell by 75% or more when given intravenously twice weekly for 4 weeks (62).

Another effective antibiotic for liposomal formulation which attracted the interest of researchers is streptomycin. Conventional liposomal formulation of streptomycin made with egg yolk phosphatidylcholine was investigated using *in vivo* model of *Salmonella* infection in mice. Liposome-entrapped streptomycin prolonged the survival to more than 15 days for all mice infected with the virulent strain of *Salmonella enteritidis*, while treatment with the same dose of free streptomycin resulted in all of the mice dying between days 5 and 7. The prolongation of survival was due to suppression of the multiplication of *S. enteritidis*. Furthermore, the liposome-entrapped drug was less toxic than the free drug when applied at high doses. A tissue distribution study in various organs demonstrated that liposomal streptomycin was selectively accumulated in the spleen and liver with concentrations in these organs about 100 times higher than those in mice receiving the free drug (63).

In contrast to this, another investigation on *S. enteritidis* indicated a less concentration of streptomycin administered using some of the liposome formulations in the liver and spleen in comparison with the free drug (9). In this study, several formulations of streptomycin sulfate liposomes, prepared from a mixture of L- α -dipalmitoyl phosphatidyl choline (DPPC) and cholesterol with or without a charge inducing agent, were used in drug targeting experiments using Swiss mice. The biodistribution results indicated that although, in comparison with the free drug, some of the liposome formulations exhibited 2-3 times higher concentration of streptomycin in the liver and spleen, this effect decreased over time from one to seven days. Despite this, the survival rate experiments indicated a definite protection against *Salmonella enteritidis* exhibited by the liposome-encapsulated streptomycin compared to the free drug (64). Therefore, it seems that the liposome formulation plays the major role in the targeting effect and the delivery efficiency of the liposomes for intracellular infections.

2.5.4 Fluoroquinolone loaded liposomes

Ciprofloxacin is a synthetic bactericidal fluoroquinolone which inhibits the activity of bacterial DNA gyrase, resulting in the degradation of bacterial DNA by exonuclease activity. Consequently, ciprofloxacin has broad-spectrum efficacy against a wide variety of bacteria, including the family *Enterobacteriaceae* of which *Salmonella* spp is a member of (65). It has been used in the treatment of individuals with *Salmonella* infections, including those with typhoid fever and chronic typhoid carriers (52). Despite the enormous success with ciprofloxacin, there are some factors which limit the drug's clinical utility, such as its poor solubility at physiological pH and rapid renal clearance. Several investigations have focused on the formulation of this drug as liposomes, in order to improve the drug delivery.

Ciprofloxacin loaded liposomes, consisting of dipalmitoyl-phosphatidylcholine, dipalmitoyl-phosphatidylglycerol and cholesterol, were used to treat *Salmonella Dublin* infected mice (66). It has been reported that a single injection of liposome formulation was 10 times more effective than a single injection of free drug at preventing mortality. Treatment with liposomal ciprofloxacin produced dose-dependent decreases in bacterial

counts in spleen, stool, and Peyer's patches, indicating that the drug had distributed to all areas of inflammation, not just to the major reticuloendothelial system organs. Although liposome formulation was cleared rapidly from the blood, drug persisted in the liver and spleen for at least 48 h after administration of a dose (66).

In a similar study, Webb et al. encapsulated ciprofloxacin into large unilamellar liposomes. The LUVs composed of dipalmitoylphosphatidylcholine-cholesterol, distearoylphosphatidylcholine-cholesterol, or sphingomyelin-cholesterol. In comparison with the free drug, the liposomal formulations increased the circulation lifetime of the drug by >15 fold and resulted in 10^3 to 10^4 fold fewer viable *Salmonella typhimurium* in the livers and spleens after intravenous administration (67). These results show the utility of liposomal encapsulation in improving the pharmacokinetics, biodistribution, and antibacterial efficacy of ciprofloxacin.

3. Polymeric nanoparticles for antisalmonellosis drug delivery

3.1 Introduction

Nanoparticles (NP) are solid colloidal particles with particle sizes smaller than 1000 nm. However, most nanoparticles utilized in drug delivery are in the size range of 100–200 nm. Nanoparticles can be classified into two main subgroups: nanospheres and nanocapsules. Nanospheres have a matrix-type structure, and drug molecules can be adsorbed on their surface or entrapped inside their matrix. Nanocapsules have a capsule-like structure and possess the capability of encapsulating the drug molecules inside the capsule or adsorbed to them externally. Because these systems have unique characteristics, such as very small particle size, high surface area, and possibility of surface modification, they have been attracting much interest for drug-delivery purposes during recent years. Nanoparticles are able to adsorb and/or encapsulate a drug, thus protecting it against chemical and enzymatic degradation. Generally, the drug is dissolved, entrapped, encapsulated or attached to a NP matrix and depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Owing to their polymeric nature, nanoparticles (Figure 3) may be more stable than liposomes in biological fluids and during storage.

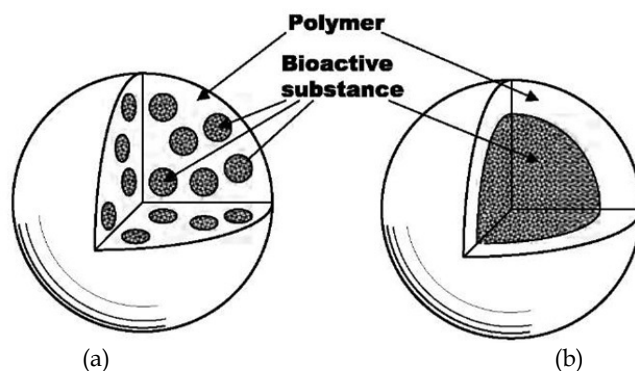


Fig. 3. Schematic structures of (a) nanosphere and (b) nanocapsule type nanoparticles (the picture was taken from <http://what-when-how.com/nanoscience-and-nanotechnology/nanoencapsulation-of-bioactive-substances-part-1-nanotechnology>).

Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. In order for nanoparticles to minimize the side effects, the polymers associated with nanoparticles must be degraded *in vivo* due to intracellular polymeric overloading. Thus in recent years, biodegradable polymeric nanoparticles have attracted considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs, their ability to target particular organs, as carriers for DNA in gene therapy, and their ability to deliver proteins, peptides and genes through a peroral route of administration (68). Most polymers PLGA, chitosan, gelatin, alginate, and poly cyanoacrylate can be used in the formulation of nanoparticles.

It is believed that nanoparticles could be effective in increasing drug accumulation at the site of infection with reduced toxicity and side effects after parenteral or oral administration (69, 70). Polymeric nanoparticles have been explored to deliver a variety of antimicrobial agents to treat various infectious diseases and have shown great therapeutic efficacy (71).

3.2 Antibiotic loaded cyanoacrylate nanoparticles

The polymers involved in nanoparticle structure should be degraded in order to release the drug, therefore, there should be a direct correlation between the rate of degradation and the drug release rate. If degradation happens in the presence of esterase, it was shown that the degradation of the polymer in esterase-free medium is low, therefore, the drug release rate is low accordingly. The drug release was increased when the medium contained carboxyesterase (72).

The *in vitro* interaction between [³H]ampicillin-loaded polyisohexylcyanoacrylate nanoparticles and murine macrophages infected with *Salmonella typhimurium* was investigated and the results showed that the uptake of nanoparticle-bound [³H]ampicillin by non-infected macrophages was six- and 24-fold greater respectively compared to free [³H]ampicillin. However, there was no difference between nanoparticle-bound ampicillin and free ampicillin in terms of bactericidal activity against intracellular *Salmonella typhimurium*. This unexpected observation might be accounted for by bacterium-induced inhibition of phagosome-lysosome fusion within the macrophages, thereby preventing contact between the bacteria in the phagosomes and the nanoparticles in the secondary lysosomes (73).

In another study the intracellular distribution of (³H)ampicillin-loaded polyisohexylcyanoacrylate nanoparticles in the same cells using ultrastructural autoradiography was investigated by the same authors (74). Ampicillin penetration and retention into the cells obviously increased by means of nanoparticles. After 2-4 h treatment with the nanoparticle formulation, numerous intracellular bacteria were seen to be in the process of destruction. After 12 h treatment, numerous spherical bodies and larger forms were seen in the vacuoles and it was an indication of marked damaging action of the ampicillin on the bacterial walls. The targeting of ampicillin therefore allowed its penetration into the macrophages and vacuoles infected with *Salmonella typhimurium* (74).

Pinto-Alphandary et al. used transmission electron microscopy to prove that ampicillin which usually penetrates into cells at a low level is directly carried in when loaded on nanoparticles, and brought into contact with intracellular bacteria (75). They concluded that ampicillin loaded polyisohexylcyanoacrylate nanoparticles is an ideal formulation when an intracellular targeting for ampicillin is needed.

Page-Clisson et al. (76) investigated the antibacterial efficiency of polyalkylcyanoacrylate nanoparticles loaded with ciprofloxacin and ampicillin against *Salmonella typhimurium*. It was shown that *in vivo* treatment with ciprofloxacin led to 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 (76).

Ampicillin was also attached to nanoparticles of polyisohexylcyanoacrylate (PIHCA) for the treatment of C57BL/6 mice experimentally infected with *Salmonella typhimurium* C5. The injection of the nanoparticles containing ampicillin treated all mice, whereas by the injection of non-loaded nanoparticles all mice died within 10 days (77).

3.3 Antibiotic loaded PLGA nanoparticles

Some polymeric nanoparticles may be more effective than liposomes in acute salmonellosis model due to better stability of nanoparticles in serum compared to liposomes. Therefore it is believed that antibiotic loaded nanoparticles can improve the targeting, particularly in the case of intracellular bacteria. For example, gentamicin (78), azithromycin and clarithromycin loaded nanoparticles using poly(lactide-co-glycolide) [PLGA] (79, 80) were more effective than corresponding intact drug against *Salmonella typhimurium*.

As mentioned before, nanoparticles should be degraded *in vivo* to avoid side effects and it has been shown that PLGA nanoparticles fulfill such requirements. Therefore, in most cases for antibiotics such as rifampicin (81), amphotericin (82), azithromycin (79) and clarithromycin (80) PLGA nanoparticle preparations have been recommended.

Mohammadi *et al.*, showed that azithromycin and clarithromycin-loaded (PLGA) nanoparticles (NPs) prepared with three different ratios of drug to polymer have better antibacterial activity against *Salmonella typhi* (79). In other words, the nanoparticles were more effective than pure azithromycin and clarithromycin against *Salmonella typhi* and *S. aureus*, respectively, with the nanoparticles showing equal antibacterial effect at 1/8 concentration of the intact drug. Both studies on azithromycin and clarithromycin proved that the antibacterial activity of nanoparticles were about 8-fold more than the free azithromycin and clarithromycin (Figure 4). The higher antibacterial effect of clarithromycin and azithromycin may have resulted from higher bacterial adhesion of the nanoparticles. For example, an adhesion of Eudragit nanoparticles containing PLGA to the *S. aureus* bacteria was reported (83). Although, Figure 4 shows that the ratio of drug:PLGA has no significant effect on antibacterial activity of azithromycin and clarithromycin, Table 1 shows that the particle size of nanoparticles, their zeta potential and the encapsulation efficiency are remarkably dependent on the ratio of drug:polymer used in the formulations. This indicates that by controlling the ratio of drug:carrier the desirable particle size and zeta potential could be achieved. As it is shown in Figure 5 all nanoparticles were spherical in appearance.

Several investigations have shown that nanoparticles could not be very effective on all different types of bacteria and that the antibacterial effect depends on bacterial type (84). For example, recently Martins et al. evaluated the antibacterial activity of PLGA nanoparticles containing violacein against different bacteria (84). Although, they showed that the MIC with nanoparticles is 2-5 times lower than free violacein against *Staphylococcus aureus*, the results failed to show any significant activity against *Escherichia coli* and *Salmonella enterica*.

Formulations	Encapsulation efficiency (%)	Mean particle size (nm)	Zeta potential (mV)
AZI:PLGA (1:1)	50.5 ± 3.4	252 ± 5	-5.6 ± 2.15
AZI:PLGA (1:2)	66.8 ± 2.8	230 ± 7	-11.10 ± 1.87
AZI:PLGA (1:3)	78.5 ± 4.2	212 ± 4	-15.56 ± 2.53
CLR:PLGA (1:1)	57.4 ± 4.3	280 ± 15	-6.3 ± 1.70
CLR:PLGA (1:2)	72.9 ± 3.2	223 ± 12	-10.08 ± 1.63
CLR:PLGA (1:3)	80.2 ± 4.0	189 ± 10	-14.26 ± 1.92

Table 1. Encapsulation efficiency, mean particle size and zeta potential of various formulations containing Azithromycin and clarithromycin (data taken from references 79, 80)

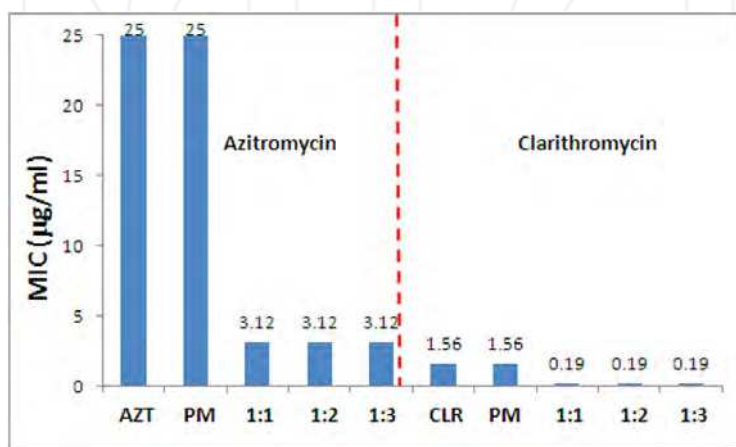


Fig. 4. Minimum inhibitory concentrations (MICs) of the intact AZT, CLR, physical mixtures (PM) and drug-loaded nanoparticles suspensions with different drug:PLGA ratios (data are reproduced from references 79, 80).

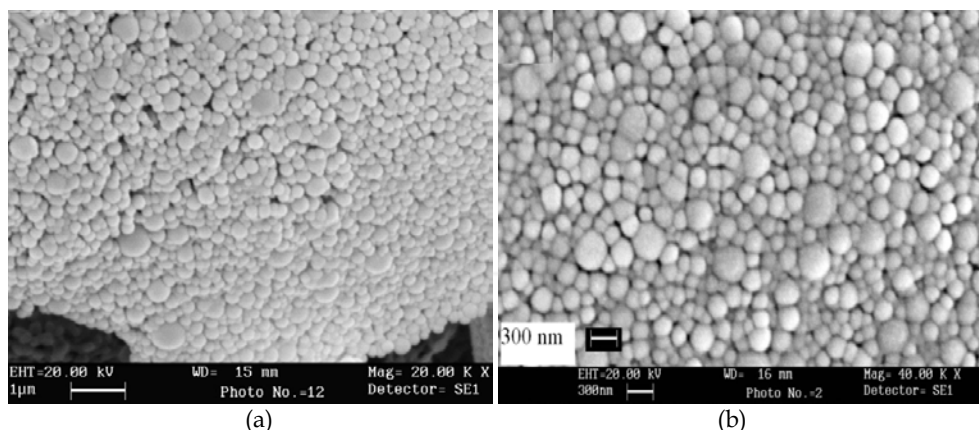


Fig. 5. SEM images of clarithromycin and azithromycin-loaded nanoparticles with the ratio of drug:PLGA 1:2 (SEM taken from ref. 80).

3.4 High loading antibiotic nanoparticles

One of the problems with antibiotic loaded nanoparticles is that in some cases the capacity of a polymeric drug carrier should be engineered to incorporate high concentrations of antibiotics to achieve the required dosage, yet avoid side effects that may be associated with higher amounts of carriers. This seems a difficult task, however, Ranjan *et al* introduced two novel technologies by which high concentrations of gentamicin could be incorporated into the formulations (85).

In the first technology, Ranjan *et al.*, made an attempt to enhance antibacterial efficacy of gentamicin using a new technology called core-shell nanostructures (78). In this research pluronic based core-shell nanostructures encapsulating gentamicin were prepared. The maximum antibiotic loading was 20% in their formulation with a zeta potential of -0.7. It was shown that when using core-shell nanostructures containing gentamicin, not only that significant reduction in toxicity and side effects was evident, but also the percentage of viable bacteria in the liver and spleen was significantly reduced (78).

In the second technology, Ranjan *et al* (85) incorporated gentamicin into macromolecular complexes with anionic homo- and block-copolymers via cooperative electrostatic interactions between cationic drugs and anionic polymers (Figure 6). They showed the possibility of incorporating 26% by weight of gentamicin in the nanoplexes with average diameter of 120 nm and zeta potential of -17 (85). This was 6% more drug loading compared to their previous study. Their study showed that in addition to the high loading of drug carried by these polymeric nanoplexes, the nanoplexes can potentially improve targeting of intercellular pathogens such as *salmonella*.

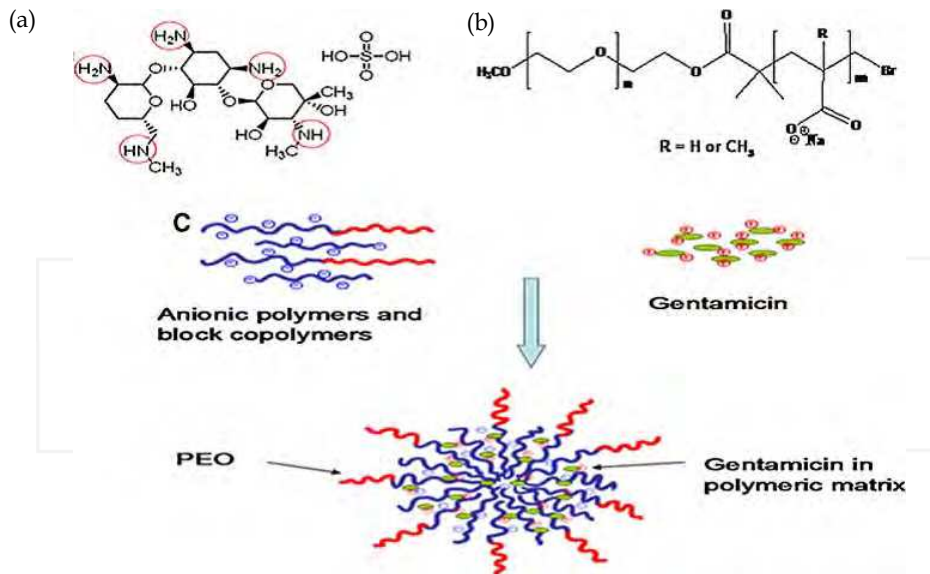


Fig. 6. (a) Gentamicin is cationic aminoglycoside antibiotic with five amino groups, (b) anionic block copolymers for electrostatic complexation to gentamicin, (c) strategy to incorporate gentamicin within polymeric nanoplex (Figure was taken from ref 85).

3.5 Xerogel systems containing antibiotic

During the last fifteen years, a special attention has been dedicated on silica xerogel system to treat diseases due to intracellular pathogens (86-92). The properties of silica xerogel systems such as size, zeta potential, pore structure, and the surface characteristics make them suitable carriers for therapeutics to target the replicative niche of intracellular pathogen. These are ideal systems for the delivery of gentamicin as this antibiotic does not kill intracellular *Salmonella* due to the polar nature of the drug which is associated with low level of intracellular penetration. A study showed that when gentamicin was incorporated into silica xerogel formulations, 31% of the drug entrapped in the matrix system remained biologically active and the bactericidal effect was retained after drug release. The results showed that by incorporation of PEG the drug release can be modulated. Administration of two doses of the xerogel formulations showed a remarkable reduction in the load of *Salmonella enterica* in the spleen and liver of the infected mice (86). A similar study was performed by another group on gentamicin silica xerogel systems showing that the silica xerogel was more effective in clearing the infection in the liver compared to the same dose of the free drugs (87).

3.6 Vaccine delivery by polymeric nanoparticles

Ochoa et al (93) made an attempt to use nanoparticle for the delivery of vaccines. An immunogenic subcellular extract obtained from whole *Salmonella Enteritidis* cells (HE) was encapsulated in nanoparticles made with the polymer Gantrez (HE-NP). When they studied the immunogenicity and protection of HE-loaded nanoparticles against lethal *Salmonella Enteritidis* in mice, an increase in survival was observed compared to a control group (80% of the mice immunized with the HE-loaded nanoparticle formulation survived even when administered 49 days before the lethal challenge). They noticed that the cytokines released from *in vitro*-stimulated spleens showed a strong gamma interferon response in all immunized groups at day 10 post-immunization. However, the immunity induced by HE-loaded nanoparticles at day 49 post-immunization suggests the involvement of a TH2 subclass in the protective effect. It can be concluded from their study that, HE-nanoparticles may represent an important alternative to the conventional attenuated vaccines against *Salmonella Enteritidis* (93).

4. Metal nanoparticles as antisalmonellosis agents

In the fast-developing field of nanotechnology, metal nanoparticles are of great interest due to their multiple applications as chemical catalysts, adsorbents, biological stains, and building blocks of novel nanometer scale optical, electronic, and magnetic devices. Metal nanoparticles are pure metal nano sized material (Figure 7) with the size of usually up to 200 nm. They have been suggested to be suitable for biological applications. It was shown that if the size of these nanoparticles is less than 50 nm they are the most suitable particles as therapeutic agents as the biosystem fails to detect them (94).

Different types of nanometals including copper, magnesium, zinc, titanium, gold, and silver have been investigated but silver nanoparticles have been employed and investigated most extensively compared to the other metals since ancient times to fight infections and control spoilage (95-97). A large number of successful *in vitro* studies were performed for the

evaluation of the antisalmonella effect of metal nanoparticles. These nanoparticles are usually nonspecific and are broad spectrum antibacterial. It is also reported that silver can cause argyrosis and argyria and is toxic to mammalian cells (98). As silver attacks a broad range of targets in the microbes, therefore it is difficult for microbes to develop resistance against silver (99). This property of silver makes it an excellent candidate for antimicrobial effect.

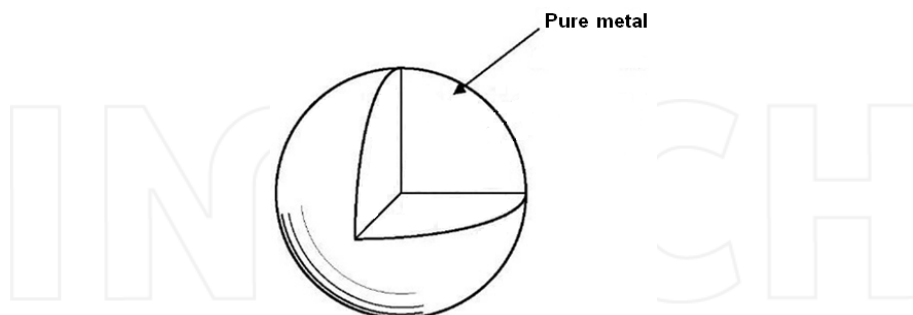


Fig. 7. Schematic structure of a metal nanoparticle

In terms of production, it is suggested that monodispersed particles (very narrow particle size distribution) rather than polydispersed nanoparticles (broad particle size distribution) are preferred. This is because the former distribution is believed to be more effective against microbes due to the high surface/volume fraction so that a large proportion of silver atoms can be in direct contact with their environment (100).

Recently, the potential use of silver nanoparticles on pathogenic bacteria was reviewed (101). There are various physical, chemical or biological methods which can be used to produce metallic nanoparticles. Among these, it seems, the biological method is popular due to the reliability and being eco-friendly. This method has attracted the attention of researchers in the field (102-108). In fact, a number of different species of bacteria and fungi are able to reduce metal ions producing metallic nanoparticles with antimicrobial properties. Recently, it has been shown that silver nanoparticles produced by the fungus *F. acuminatum* have efficient antibacterial activity against multidrug resistant and highly pathogenic, *Salmonella typhi* (109). Additionally, plant extracts can also be used to obtain metallic nanoparticles (110). Metal nanoparticles were also modified to be used in the prevention of biofilm formation on the implanted devices (111-114), however, care must be taken when this type of metal nanoparticles are used due to potential risk on patient's health (115-117).

Researchers suggested that to achieve a better utilization of the antimicrobial activity, metal nanoparticles may be combined with nontoxic and biocompatible polymers. For example, in an attempt NaPGA- (poly (g-glutamic acid)) and CaPGA-coated magnetite nanoparticles were synthesized (118) and their antibacterial activity against *Salmonella enteritidis*, *Staphylococcus aureus* and *Escherichia coli* were tested. The results showed that both produced nanoparticles were more effective against *Salmonella enteritidis* compared to commercial antibiotics, linezolid and cefaclor. In addition, these nanoparticles showed no toxicity toward human skin fibroblast cells.

In few cases polymers such as PVP have been used as steric stabilizers to obtain monodispersed silver nanoparticles (119, 120). Although silver nanoparticles have the

capability to remain dispersed in liquids without major signs of agglomeration, in case of the appearance of aggregation hydrophilic surfactants, proteins, amino acids and PVA (poly vinyl alcohol) can be used (121-125). Metal nanoparticles have also found application in various other fields, i.e. catalysis and sensors as mentioned before (126-128). However, their undesirable and unforeseen effects on the environment and in the ecosystem should not be ignored (129, 130). The antibacterial effect of silver and copper nanoparticles was also investigated on *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (131). The results showed that the efficiency of silver and copper nanoparticles were different on different bacteria. Among the bacteria used, *B. subtilis* showed the highest sensitivity to copper nanoparticles compared to silver, whereas silver nanoparticles were more effective on the other two bacteria compared to copper nanoparticles (133).

Interesting results were reported by Patil *et al* when they synthesized and tested chloramphenicol loaded nano-silver particles against *Salmonella typhi* (97). For the first time they used PVP in their formulations containing silver as a carrier for chloramphenicol. In the formulation, PVP played a dual role. It acts as a stabilizer and linker for binding chloramphenicol to the silver nanoparticles (Figure 8). The nanoparticles showed considerably enhanced activity against clinically isolated *Salmonella typhi*.

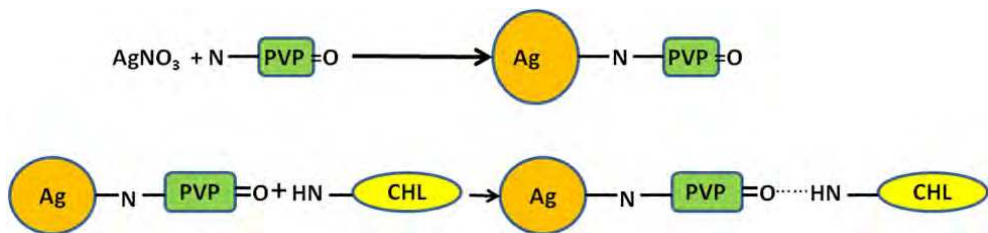


Fig. 8. Top: schematic representation of the synthesis of silver nanoparticles (PVP as a stabilizer); bottom: schematic representation of the synthesis of chloramphenicol loaded silver nanoparticles (PVP as a linker) (figure was reproduced from ref 97).

The summary of some of metal nanoformulations are listed in Table 2.

Gold and platinum nanoparticles have also attracted the attention of researchers due to their antibacterial activity (132, 133). Several research groups studied the cytotoxicity of gold nanoparticles in different cell types (134, 135). It was shown that citrate-capped gold nanoparticles were not cytotoxic to baby hamster kidney cells and human hepatocellular liver carcinoma cells, but cytotoxic to human carcinoma cells at certain concentrations (135).

Despite all research data about the toxicity of gold nanoparticles, still more research for better understanding of gold nanoparticles toxicity is required. Recently, Wang *et al* prepared 16 nm gold nanospheres stabilized with citrate ions and their antimicrobial activity was tested against *Salmonella typhi* bacteria strain TA 102 (133). The results showed that gold nanoparticles are not mutagenic or toxic in *Salmonella*, but is photomutagenic to the bacteria. The photomutagenicity was due to the presence of citrate and Au^{3+} ions used during the preparation of gold nanoparticles. Their final results showed that although there was a good surface interaction between gold nanoparticles and the bacteria, the gold nanoparticles were not able to penetrate into the bacteria.

Type of nanoparticle	Type of <i>Salmonella</i>	Reference
ASAP Nano-silver Solution	<i>Salmonella typhi</i>	(136)
silver colloid nanoparticles	<i>Salmonella enteric</i>	(137)
silver-silicon dioxide hybrid	<i>Salmonella enteric</i>	(137)
ZnO nanoparticles	<i>Salmonella typhimurium</i>	(138)
Spherical silver nanoparticles	<i>Salmonella typhimurium</i>	(139)
Zinc oxide QuantumDots	<i>Salmonella Enteritidis</i>	(140)
Silver nanoparticles	<i>Salmonella typhi</i>	(141)
Silver nanoparticles	<i>Salmonella typhimurium</i>	(142)
Silver bionanoparticles	<i>Salmonella typhi</i>	(143)
Silver bionanoparticles	<i>Salmonella paratyphi</i>	(144)
TiO ₂ nanoparticles	<i>Salmonella typhimurium</i>	(145)
ZnO nanoparticles	<i>Salmonella typhimurium</i>	(145)
Silver nanoparticles	<i>Salmonella typhus</i>	(146)
Iron nanoparticles	<i>Salmonella paratyphi</i>	(147)
silver nanoparticles	Not specified	(148)
Silver Nanoparticles	<i>Salmonella typhimurium</i>	(149)
Silver bionanoparticles	<i>Salmonella typhi</i>	(150)
Ag-SiO ₂ anoparticles	<i>Salmonella typhimurium</i>	(151)
Zn _{1-x} Ti _x O (x = 0, 0.01, 0.03 and 0.05) nanoparticles	<i>Salmonella typhi</i>	(152)
platinum nanoparticles	<i>Salmonella Enteritidis</i>	(153)
CuO nanoparticles	<i>Salmonella paratyphi</i>	(154)

Table 2. Various metal nanoparticles used against different microbes

Similar study was carried out on gold and platinum nanoparticles (132) and the results showed that gold nanoparticles can interact with *Salmonella Enteritidis* but did not penetrate the bacterial cell, whereas platinum nanoparticles were observed inside bacterial cells due to binding to DNA. They concluded that gold nanoparticles can be used alongside with bacteria to deliver the nanoparticles to specific points in the body for targeted delivery.

A major controversy with metal nanoparticles is that whether they are toxic to bacteria or bacteria develops resistance mechanism against these nanoparticles. If the former is true, there might be a devastating effect to the ecosystem which will lead to a global destabilization. Nanoparticles have a greater potential to travel through an organism and could be more toxic due to their larger surface area and specific structural/chemical properties.

Although the evolution of nanotechnology is about to bring various advantages to our lives over conventional formulations but the lung toxicity of metal nanoparticles (155)

should be carefully considered as these nanoparticles are very small and light, and they have larger surface area with a greater potential to travel through an organism or tissues (156). These small particles can travel via nasal nerves to the brain (156, 157). It has been shown that most of metallic nanoparticles such as TiO₂, Ag, Al, Zn, Ni exhibit cellular toxicity on human alveolar epithelial cells (158). The results reported by Park et al (158) showed that these metal nanoparticles could damage the cell directly or indirectly. The cell damage is probably dependent on the size, structure, and composition of the nanoparticles, yet more studies are needed for better understanding of the toxicity mechanism of the metal nanoparticles.

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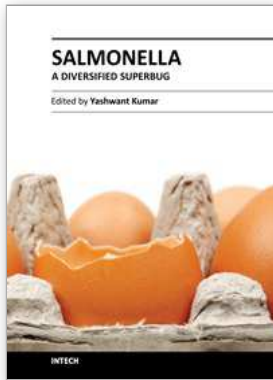
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Salmonella - A Diversified Superbug

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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