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Review

Liposomes as delivery systems for antibiotics

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ABSTRACT

Liposomes are currently in common use as universal drug carriers in the cosmetic and pharmaceutical industries. The manipulation of different physicochemical properties of liposomes enables the design of particular carriers with the desired pharmacokinetic and pharmacodynamic properties. Most studies regarding liposomal antibiotics deal with aminoglycosides, quinolones, polypeptides, and betalactames. Some of the studies focused on improving pharmacokinetics and reducing toxicity, while others involved enhancing antibacterial activity. In an era of an avalanche of increasing bacterial resistance and severe problems in treating bacterial infections, the application of liposomal antibiotic carriers could be useful, but the high cost of liposome preparation and treatment should also be considered.

Contents

1. Introduction

Liposomes are currently in common use as universal drug carriers in the cosmetic and pharmaceutical industries. In healthcare there are antitumor anthracyclines such doxorubicin and antifungal amphotericin B liposomal formulations available (Allen and Martin, 2004; Bakker-Woudenberg et al., 1994). Intensive research is focused on antibiotics entrapped in liposomes to enhance their antibacterial activity and pharmacokinetic properties. Lipid vesicles as drug carriers significantly influence on drug distribution and reduce toxic side effects during antibiotic therapy (Allen, 1998; Bakker-Woudenberg et al., 1993, 1994; Bakker-Woudenberg, 2002; Sapra and Allen, 2003). One of the most serious problems of current medicine is the increase in drug resistance among bacterial pathogens, which limits conventional therapy. Many researchers are making efforts to discover new classes of antibacterial drugs, but some studies are focused on improving currently available antibiotics in a new form (liposomal formulations) (Abeylath and Turos, 2008; Pinto-Alphandary et al., 2000; Schiffelers et al., 2001d; Sihorkar and Vyas, 2001; Swenson et al., 1988).

2. Structure and properties of liposomes

Liposomes are spherical vesicles consisting of one or more phospholipid bilayers surrounding a water space. The diameter of the

Abbreviations: CHOL, cholesterol; Con-A, concanavalin-A; DDAB, dimethyldioctadecylammonium bromide; DOTAP, dioleoyloxytrimethylammoniumpropane; DOPE, dioleoylphosphatidylethanolamine; DP, dihexadecyl hydrogen phosphate; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DPPS, dipalmitoylphosphatidylserine; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; DSPG, distearoylphosphatidylglycerol; O-SAP, O-stearylamylopectin; PC, phosphatidylcholine; PEG, polyethyleneglycol; PI, phosphatidylinositol; PS, phosphatidylserine; SA, stearoylamine.

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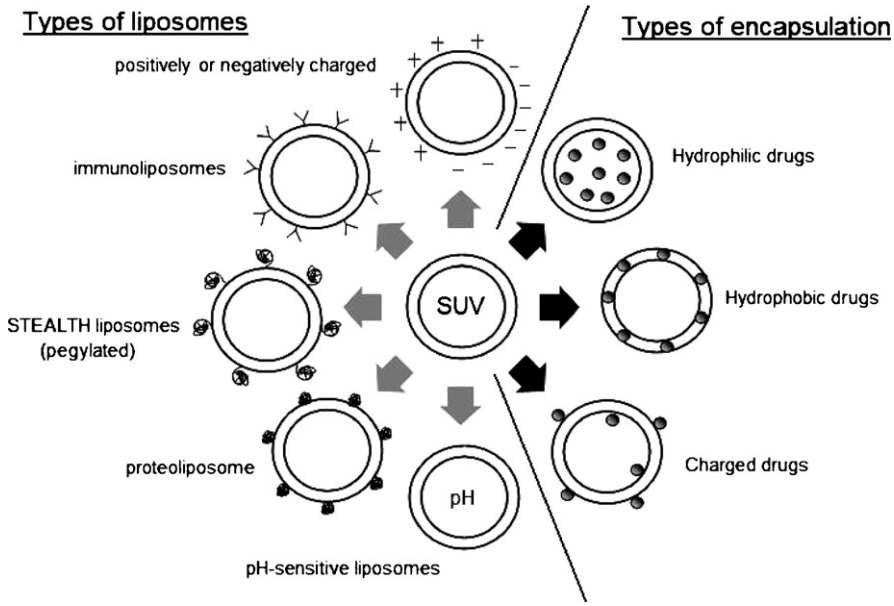

Fig. 1. Properties of liposomes.

liposome varies from 0.02 to 10 μm. Vesicle formulations are usually based on natural and synthetic phospholipids and cholesterol. The structure may also possess lipoproteins (Ulrich, 2002). The physicochemical properties of liposomes (Fig. 1) can be modified by changing:

- the types of lipids;
- the composition and proportions of lipids in the liposomal formulation;
- the size of the liposome;
- the charge of the liposomal surface: positive, negative, or neutral;
- pH sensitivity;
- temperature sensitivity;
- the fluidity of the liposomal membrane: rigid and fluid liposomes.

Regarding the variety of liposomal formulations, the vesicles are universal carriers for both hydrophilic and hydrophobic compounds. Hydrophilic elements are dissolved in the water space inside the vesicles. The most useful for this are LUVs (*Large Unilamellar Vesicles*) because the volume of encapsulated water is relatively high (Gregoriadis, 1995; Sharma and Sharma, 1997). Hydrophobic compounds are located in the lipid bilayer, and MLVs (*Multilamellar Vesicles*) or SUVs (*Small Unilamellar Vesicles*) may be applied. Charged drugs can be associated to the lipid surface (Gregoriadis, 1995; Sharma and Sharma, 1997). The size of the liposomal vesicles significantly influences drug distribution. Large (>1 μm) MLV formulations are usually not used as antibiotic carriers, but SUVs of ~100 nm exhibited high efficacy in the eradication of bacterial pathogens (Krieger et al., 1999).

3. Advantages of liposomal antibiotics

There are some antibiotics of limited application in healthcare because of toxicity or weak biodistribution and pharmacokinetics. Despite very efficient antibacterial activity, these drugs can only be used as last-chance treatment when the risk of severe side effects is high. Encapsulation of the drugs in lipid vesicles is a good solution for designing the required pharmacokinetic and pharmacodynamic properties (Allen, 1998; Bakker-Woudenberg et al., 1993, 1994; Bakker-Woudenberg, 2002; Swenson et al., 1988).

There are many advantages of liposomes as antibiotic carriers:

- improved pharmacokinetics and biodistribution;
- decreased toxicity;
- enhanced activity against intracellular pathogens;
- target selectivity;
- enhanced activity against extracellular pathogens, in particular to overcome bacterial drug resistance.

The variety of liposomal formulations allows the design of effective antibiotic forms and subsequent therapeutic success (Abeylath and Turos, 2008; Schiffelers et al., 2001d; Sharma and Sharma, 1997; Yimei et al., 2008).

3.1. Improved pharmacokinetics and biodistribution, decreased toxicity

There is much evidence of the benefits of liposomes as antibiotic delivery systems. The advantage of liposomal carriers is the possibility of a gradual and sustained release of antibiotics during drug circulation in the body. This allows maintaining the proper drug concentration for a relatively long term. In comparison, administration of the free antibiotic exhibits a quick and short effect and requires several doses per day (Hamidi et al., 2006). Drug encapsulation in liposomal vesicles improves the pharmacokinetics and also protects antibiotics against the hydrolytic activity of enzymes and chemical and immunological deactivation (Allen, 1998; Omri and Ravaoarinoro, 1996a,b; Schiffelers et al., 2001d). Conventional liposomes applied by intravenous administration are recognized as foreign antigens by the immunological system and are opsonised. This activates nonspecific defence mechanisms and the liposomes are taken up by the mononuclear phagocyte system (MPS), which leads to lower blood circulation time and fast blood clearance. Liposomes accumulate in the liver, spleen, lungs, and kidneys (Bakker-Woudenberg et al., 1994; Bakker-Woudenberg, 2002; Schiffelers et al., 2001d). This phenomenon (phagocytosis of liposomes) is desirable for intracellular pathogen eradication, but unfavourable for other kinds of infection (Lasic, 1998; Voinea and Simionescu, 2002). The MPS uptake rate depends on several liposomal properties, such as size, charge, and fluidity. The blood clearance of small vesicles (~100 nm) rises to several hours, in comparison with several minutes for MLV formulations. Rigid and uncharged vesicles circulate longer than fluid and charged ones

Table 1
Liposomal antibiotics designed for tuberculosis treatment.

Antibiotic	Bacteria	Reference
Isoniazid; rifampicin	*Mycobacterium tuberculosis*	Deol and Khuller (1997); Dutt and Khuller (2001); Labana et al. (2002)
Amikacin	*Mycobacterium tuberculosis*	Whitehead et al. (1998); Donald et al. (2001)
Amikacin, gentamicin streptomycin	*Mycobacterium avium* complex (MAC)	Duzgunes et al. (1988, 1991, 1996); Nightingale et al. (1993); Wiley et al. (1994); Brandissou et al. (1997); Sesin et al. (1996); de Steenwinkel et al. (2007)
Clarithromycin azithromycin	*Mycobacterium avium* complex (MAC)	Oh et al. (1995); Salem and Duzgunes (2003)
Ciprofloxacin, sparfloxacin	*Mycobacterium avium* complex (MAC)	Oh et al. (1995); Duzgunes et al. (1996)

(Beaulac et al., 1997; Scherphof et al., 1997). The plasma circulation time of antibiotics can be improved by encapsulation in polyethylene glycol-coated (pegylated) (STEALTH) liposomes. The hydrophilic layer composed of PEG protects the vesicles from the MPS and allows a long liposome circulation in the blood system. Sterically stabilised STEALTH liposomes exhibit sustained release of drug and are able to accumulate selectively at sites of infection (Bakker-Woudenberg et al., 1993; Ceh et al., 1997). The most promising *in vivo* results in improving pharmacokinetics and reducing toxicity were obtained for aminoglycosides, quinolones, and polymyxin B (Bakker-Woudenberg, 2002; Marier et al., 2002, 2003; Omri et al., 2002; Schiffelers et al., 2001d; Xiong et al., 1999).

The Dutch group thoroughly investigated the activity of aminoglycosides and ciprofloxacin encapsulated in STEALTH liposomes on an infected rat lung tissue model (Bakker-Woudenberg et al., 1986, 1989, 1993, 1994, 1995, 2001, 2002; Bakker-Woudenberg, 2002; Schiffelers et al., 1999, 2001a,b,c,d). In *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* pneumonia in rats, once daily administration of ciprofloxacin in liposomal form produced the same results as the free drug given twice daily. Delayed clearance and increased and prolonged concentrations in the blood and tissues were observed for liposomal ciprofloxacin. PEG-coated liposomal ciprofloxacin was nontoxic at relatively high doses (Bakker-Woudenberg et al., 1995, 2001). The investigators examined the influence of PEG density, particle size, bilayer fluidity, and surface charge on liposome localization, blood clearance kinetics, and biodistribution (Schiffelers et al., 1999). A reduction in PEG density or rise in particle size induced higher MPS uptake and reduced the drug concentration in lung tissue. A negative charge on the vesicle surface led to reduced localization of this liposome preparation at the site of lung infection, although the blood clearance kinetics remain the same. The fluidity of the formulation did not change the biodistribution of the PEG liposomes. Evidence of the superior efficacy of antibiotics encapsulated in STEALTH liposomes compared with the free drug was also described by Schiffelers et al. (2001b). It was demonstrated that increased capillary permeability at the infection site caused a high local concentration of liposomes. Additionally, the presence of bacterial antigens induced an inflammatory response, which led to a further increase in liposome extravasation. These mechanisms significantly improved the antibacterial activity of the encapsulated antibiotics and allowed the eradication of pathogen from the infected tissue. The application of gentamicin encapsulated in PEG liposomes in a *K. pneumoniae* pneumonia rat model showed positive therapeutic results in eradicating gentamicin-sensitive and -resistant strains (Schiffelers et al., 2001a,c). Similar results were obtained by Ellbogen et al. (2003) with regard to the application of ciprofloxacin encapsulated in sterically stabilised liposomes in treating *Streptococcus pneumonia* infections in a rat model. Use of STEALTH liposomes resulted in a prolonged circulation time and improved pharmacokinetics of ciprofloxacin, leading to a higher concentration of the drug in serum and lung lavage fluid. Nevertheless, the survival rates of the infected animals were similar in the groups treated with equivalent doses of liposomal ciprofloxacin versus free ciprofloxacin.

Xiong et al. (1999) investigated the efficiency of amikacin in the MiKasome® preparation composed of PC/CHOL/DSPG (NeXstar Pharmaceuticals, USA) in combined treatment with oxacillin in a rabbit model of experimental endocarditis caused by *Staphylococcus aureus*. No significant differences in *S. aureus* eradication were shown compared with conventional amikacin application. The combination of a b-lactam plus both conventional and liposomal aminoglycoside acted synergistically. Despite the short circulation time in the blood system, fluid vesicles may offer advantages in local applications such as intratracheal administration in treating lung infection. Beaulac et al. (1996, 1998) evaluated the *in vivo* and *in vitro* efficacy of Fluidosomes® fluid vesicles (DPPC/DMPG 18:1) as a carrier for tobramycin in the eradication of *P. aeruginosa*, causing chronic lung infection. *In vitro* experiments performed on a wider bacterial group (*P. aeruginosa*, *Burkholderia cepacia*, *Escherichia coli*, *Stenotrophomonas maltophilia*, and *S. aureus*) showed that tobramycin encapsulated in the fluid formulation exhibited antibacterial activity in a sub-MIC concentration. Fluidosomes® were more effective than rigid vesicles bearing tobramycin and free tobramycin in eliminating bacteria from lung tissue when the same doses were administered. A high concentration of the drug at the infection site was maintained for a long period of time and a 10-times lower tobramycin concentration was simultaneously noted in the kidneys compared with the free drug (lower toxicity). Further studies concerning the systemic and local immunogenicity of Fluidosomes® performed by intraperitoneal and intratracheal mouse immunization showed that no significant immune response developed (Sachetelli et al., 1999). Similar results were obtained by Marier et al. (2002, 2003) and Omri et al. (2002) for fluid liposomal formulations of DPPC/DMPG (10:1) and DPPC/Chol (2:1), respectively. Animal chronic lung infections caused by *P. aeruginosa* and *B. cepacia* were successfully treated by liposomal tobramycin and polymyxin B. The investigators suggested that the application of fluid liposomes could be very promising for local administration because besides the sustained concentration of the antibiotic, minimal systemic absorption, and reduced toxicity, enhancement of antibacterial activity was described.

3.2. Enhanced activity against intracellular pathogens

The application of liposomes as a drug delivery system was very successful in eradicating intracellular pathogens. Liposomes were applied to various types of infections. In the treatment of diseases caused by intracellular bacteria, rigid conventional liposomal vesicles and PEG-coated ones improved drug retention in the proper tissues, provided sustained release, decreased toxicity, and enhanced the concentration at the site of infection. Some experiments focused on tuberculosis, a severe and difficult to treat infection (Table 1). It was shown that the application of liposomal forms of isoniazid, rifampin, and clarithromycin sig-

Table 2
Liposomal antibiotics used for intracellular bacteria eradication.

Antibiotic	Bacteria	Reference
Ampicillin	*Listeria monocytogenes*	Bakker-Woudenberg et al. (1986, 1989)
Gentamicin	*Brucella melitensis; Brucella abortus*	Hernandez-Caselles et al. (1989); Vitas et al. (1997)
	Salmonella enterica serovar Typhimurium	Lutwyche et al. (1998); Cordeiro et al. (2000)
	Listeria monocytogenes	Lutwyche et al. (1998)
Ciprofloxacin	*Salmonella enterica* serovar Typhimurium	Webb et al. (1998)
	Francisella tularensis	Wong et al. (2003)

nificantly enhanced antibacterial efficacy compared with the free drugs (Labana et al., 2002; Salem and Duzgunes, 2003). The design of different vesicle formulations made it possible to determine the best antitubercular drug delivery system. Deol and Khuller found that tagging O-SAP (O-stearylamylopectin) to STEALTH liposomes increased their affinity to lung tissue. Furthermore, the *in vivo* stability of these formulation exhibited sustained release of drugs and low toxicity to peritoneal macrophages and tissues (Deol and Khuller, 1997). Antibiotics encapsulated in long-circulating and lung-specific STEALTH liposomes allowed reducing the dose (one-third of that recommended) and administration (once weekly) with no changes in the drug concentration profile (Labana et al., 2002). Hepatotoxicity was reduced and sustained release was prolonged when isoniazid and rifampin were encapsulated in liposomes or poly(lactic/glycolic acid) PLG microspheres (Dutt and Khuller, 2001). Tuberculosis required multidrug treatment consisting, for instance, of isoniazid, rifampin, and clarithromycin. Investigators showed that additional application of liposomal aminoglycosides significantly decreased *Mycobacterium avium* load followed by complete killing, including that of persistent mycobacteria (Duzgunes et al., 1988, 1991, 1996). Amikacin encapsulated in PC/CHOL//DSPE-PEG vesicles prevented relapse of infection and reduced the total treatment duration (de Steenwinkel et al., 2007).

Liposomal formulations of antibiotics were also investigated in the eradication of other obligatory and non-obligatory intracellular pathogens (Table 2). One of the first experiments involving liposomal systems for intracellular pathogen eradication was developed by Bakker-Woudenberg et al. (1986, 1989). The experiment focused on *Listeria monocytogenes* eradication. Ampicillin encapsulated in fluid formulations of CHOL/PC/PS and CHOL/DSPC/DPPG demonstrated a rapid uptake by macrophages. The delayed intracellular release of the encapsulated ampicillin, resulting in a lower rate of *L. monocytogenes* killing efficacy, was noted for the less fluid formulation. Conventional liposomes bearing gentamicin were also used by Vitas et al. (1997) and Hernandez-Caselles et al. (1989) for the treatment of brucellosis in an animal model. Accumulation of the drug in the liver and spleen was obtained as a consequence of MPS uptake of liposome vesicles, which led to a decrease in bacterial number in these tissues.

There is also the possibility of controlled drug release by using pH-sensitive liposomes. Their structure is stabile in the blood circulation, but in an altered pH environment (in phagolysosomes), an unstable membrane allows leakage of drug content. The pH-sensitive PEG-coated vesicles composed of DOPE lipids were applied to intracellular pathogens such as *Salmonella* sp. and *L. monocytogenes* (Cordeiro et al., 2000; Lutwyche et al., 1998). Gentamicin encapsulated in the pH-sensitive formulation was more effective against vacuole-resident wild-type *Salmonella* Typhimurium than were the DPPC control formulations. These pH-sensitive formulations also efficiently eliminated *S.* Typhimurium and *L. monocytogenes* strains residing in the cytoplasm.

Other investigators focused on fluoroquinolones as potentially effective drugs for treating intracellular infections. Ciprofloxacin loaded into large unilamellar vesicles (LUV) of SM/CHOL, DSPC/CHOL, or DPPC/CHOL formulations demonstrated increased circulation lifetime, which resulted in enhanced delivery of the drug to the liver, spleen, kidneys, and lung after intravenous administration. Increases in longevity compared with the free drug were also noted in the cases of intraperitoneal and intratracheal administration. The antibacterial activity of liposomal ciprofloxacin was one hundred times greater than that of the free drug against *Salmonella* cells located in the liver and spleen (Webb et al., 1998). Similar results were obtained by Wong et al. (2003). The application of ciprofloxacin encapsulated in SUVs composed of PC/CHOL significantly improved antibacterial activity against *Francisella tularensis* infections. Aerosol and intravenous administration demonstrated complete eradication of the pathogen, in contrast to the ineffective free ciprofloxacin.

3.3. Target selectivity

Intensive research on drug carriers demonstrated the possibility to target liposomes to particular tissues, organs, and even microorganisms (Allen, 1998; Deol and Khuller, 1997). Target selectivity of liposomal drug formulations may be achieved by (Fig. 1):

- addition of specific immunoglobulins (Forssen and Willis, 1998; Maruyama et al., 1997; Robinson et al., 1998);
- addition of proteins (Forssen and Willis, 1998; Jones et al., 1993);
- addition of specific oligosaccharide chains (Forssen and Willis, 1998);
- construction of pH-sensitive vesicles (Cordeiro et al., 2000; Gerasimov et al., 1999; Ulrich, 2002);
- construction of thermo-sensitive vesicles (Voinea and Simionescu, 2002).

The composition of the vesicle surface conditions the type of specific and nonspecific interaction with the target. In the case of nonspecific action, the charge of the membrane plays the main role. Eukaryotic and bacterial cells possess negatively charged surfaces, which is why positively charged liposomal vesicles exhibited the strongest vesicle–cell interactions. Specifically targeted liposomes are equipped with proteins, antibodies, or immunoglobulin fragments which have affinity to specific receptors located on the target surface (infected cells or pathogens). Specifically coated vesicles could be directed toward particular infected tissue or to strictly defined pathogens.

Liposomes as drug carriers are very promising in preventing biofilm formation and treatment (Kaszuba et al., 1997; Sihorkar and Vyas, 2001). The main problem with biofilm-producing bacteria involves the local concentration of bacterial colonies covered by an extracellular matrix of polymeric substances which prevent drug transport to the hidden microbial cells. The main aim of liposomal drug application was to target matrix or biofilm bacteria by specific attachment, allowing the drug to be released in the vicinity of the microorganisms. This would significantly increase the local drug concentration and simplify targeted delivery. This targeted transport was realized using site-specific ligands such as immunoglobulins, oligosaccharides, and proteins. On the other hand, even surface charge and specific phospholipid composition

influences the direct interactions with the biofilm matrix and bacterial cell surface. Vesicular systems, especially liposomes, were widely and thoroughly investigated by several authors as targeting devices for bacterial biofilm prevention and treatment (Table 4). Most of the studies were dedicated to plaque-forming bacteria (oral flora) or skin bacteria producing a biofilm on artificial elements such as catheters. The first trials to exploit biofilm-associated surface determinants (antigens) for target selectivity were done by Robinson et al. (1998). They tested the specificity and affinity of immunoliposomes to *Streptococcus oralis* biofilms. The anti-oralis immunoliposomes exhibited the greatest affinity to various oral residents, i.e. *S. oralis*, *Streptococcus sanguis*, *Streptococcus gordonii*, *Streptococcus salivarius*, and *Streptococcus mutans*, but not so well to *S. oralis*. The immunovesicles showed nonspecific interaction of the liposomes with other bacteria of typically multi-species biofilm. Robinson et al. (2001) also prepared simple formulations containing DMPC, CHOL, DDAB, DPPC, SA, and PI, yielding cationic and anionic vesicles. The affinity of the liposomes to *S. salivarius*, *S. sanguis*, and skin-associated bacteria (*Staphylococcus epidermidis* and *Proteus vulgaris*) was tested. It was found that the adsorption of the liposomes to the biofilms strictly depended on the bacterial species and strain. In a mixed biofilm structure, each bacterium adsorbed the liposomal vesicles independently. Both cationic and anionic formulations interacted with the biofilm bacteria, but with different species. It was found that cationic liposomes were more efficient than anionic in adhering to skin bacteria.

Another group investigated the affinity of lectinized liposomes to biofilm formulations composed of DPPC (or DPPG) and PI succinylated by Con A (sCon-A) (Jones et al., 1993). They were tested as triclosan carriers against oral and skin biofilm-forming pathogens (*S. sanguis*, *S. epidermidis*, and *P. vulgaris*). These proteoliposomes proved to be more effective in eliminating periodontal pocket bacteria than free triclosan because of their targeted properties when the drug was delivered directly into the cellular interiors of the biofilms. The same group of scientists tested standard nonlectinised liposomal formulations as an efficient drug delivery system (Jones et al., 1994). It was found that DPPC/PI and DPPC/DPPG formulations bearing triclosan were successfully adsorbed by the biofilm-associated bacteria *S. epidermidis* and *P. vulgaris* and the oral bacterium *S. sanguis*. The antibiotic in all the applied liposome formulations inhibited bacterial growth in the biofilms after a very short period of exposure, even 2 min being sufficient to obtain desirable effects. These experiments suggest that liposomes of appropriately chosen lipid composition can be used as effective systems for targeting and delivering bactericide to biofilm-forming bacteria. The potential use of ligand-anchored vesicles against the oral bacteria *S. mutans* was tested by Vyas et al. (2000, 2001). Several liposomal systems bearing metronidazole were designed: mannan (polysaccharide) coated, sialo-mannan coated, and lectinised (Con-A) PC/CHOL/SA liposomes. The researchers proposed that lectin–carbohydrate interactions are the principle mechanism for drug delivery to plaque-forming bacteria. The interactions between vesicles and epitopes expressed on the bacterial cell surface, such as glycocalyx, were studied and it was shown that polysaccharide-coated vesicles were an efficient system of metronidazol delivery to periodontal pocket biofilm and inhibition of pathogenic bacteria. Catuogno and Jones (2003) designed anionic PI/DPPC and cationic DDAB/DPPC/CHOL vesicles with zinc citrate particles adsorbed on the surface. Liposomes containing triclosan and penicillin G were tested on the oral flora bacterium *S. oralis*. The addition of zinc particles significantly increased the inhibitive effect on microbial growth and a synergic effect between the applied antimicrobials and zinc was noted.

As mentioned previously, cationic formulations of liposomes exhibited significant adherence to the skin-associated bacteria *S. epidermidis*. Sanderson et al. (1996) and Sanderson and Jones (1996) prepared standard cationic vesicles of a DPPC/CHOL/DDAB mixture of entrapped vancomycin or gentamicin. The enhancement of bactericidal activity in comparison with free drug was evident for vancomycin, but less so for gentamicin. The activity of the preparation mostly depended on the fluidity of the liposomal membrane and the drug encapsulation efficacy. Kim et al. (1999) focused on liposomal composition and lipid proportions and their impact on adsorption to bacterial biofilm. Cationic formulations of DPPC/CHOL/SA and DPPC/CHOL/DDAB were prepared in different proportions. The adsorption of the liposomes to *S. aureus* biofilms was measured as a function of liposome composition and liposomal lipid concentration. It was shown that the most biofilm-adhesive liposome composition had 20–25 mol% of SA and DDAB cationic phospholipids. The DPPC/CHOL/DDAB vesicles carrying vancomycin exhibited better antimicrobial effect than the free drug even after a short (30 min) exposure time.

Severe problems regarding biofilm-producing bacteria are connected with the attachment preferences of these microbes to artificial component such as catheters and implants widely used in medical procedures. *P. aeruginosa* is a famous pathogen involved in biofilm formation. DiTizio et al. (1998) suggested a liposomal drug system to bacterial biofilm prevention on urinary catheters. The system consisted of a PEG-gelatine hydrogel in which PEGDSPE liposomes were located. The hydrogel system bearing ciprofloxacin was applied to the surface of a catheter and then the intensity of *P. aeruginosa* biofilm formation was measured. The hydrogel system significantly reduced bacterial growth and completely inhibited pseudomonal adhesion to the catheter surface during 7 days of treatment. The authors suggested that liposomal antibiotics may be successfully applied in the prevention of biofilm formation on artificial elements used in medical procedures. Other authors also focused on the easy adhesion of *P. aeruginosa* to different surfaces, which allows colonization and disease development. Trafny et al. (1995) tested amikacin and polymyxin B encapsulated in PC/CHOL liposomes as protection against *P. aeruginosa* adhesion to collagen type I. Unfortunately, only the liposomal form of amikacin showed a satisfactory antimicrobial effect compared with the free drug. It was concluded that the application of liposome-encapsulated amikacin may be advantageous in injured tissues in which extracellular matrix structures become exposed.

Although these developed liposomes are functional *in vitro* against bacterial biofilms, there are some problems associated with specific liposome binding to the bacterial matrix surface. It was discovered that direct interactions between lipid vesicles and biofilm depend on liposome fluidity and composition. It was also shown that some formulations are species specific.

3.4. Enhanced activity against extracellular pathogens, in particular in overcoming bacterial drug resistance

Liposomal formulations significantly improved antibiotic pharmacokinetics, prolonging circulation time and tissue retention. The vesicles may be targeted by anchored ligands to particular bacteria or bacterial structures such as biofilm. This delivery system was successfully applied in the eradication of intracellular pathogens by accumulation in the mononuclear phagocyte system. Furthermore, liposomes were investigated as antibiotic carrying systems against extracellularly multiplying pathogens. There are a number of publications describing the proper lipid formulations, drug distribution, and vesicle–bacterium interactions leading to enhancement of antimicrobial drug activity against most common extracellular bacteria, such as *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *Acinetobacter* sp., and *S. aureus*. The antibiotics chosen for encapsulation were mostly fluoroquinolones and aminoglycosides (Table 3). Some of the researchers tested the antimicrobial efficacy of liposomal polymyxin B or meropenem (betalactam) (Table 4).

Table 3
Liposomal aminoglycosides and quinolones used for extracellular bacteria eradication.

Antibiotic	Bacteria	Reference
Amikacin	*Pseudomonas aeruginosa*	Mugabe et al. (2006); Trafny et al. (1995); Dupont et al. (2008); Dubus and Ravilly (2008); Okusanya et al. (2009)
	Burkholderia cenocepacia	Halwani et al. (2007)
	Staphylococcus aureus	Xiong et al. (1999)
Gentamicin	*Pseudomonas aeruginosa*	Drulis-Kawa et al. (2006b); Rukholm et al. (2006); Mugabe et al. (2006); Gubernator et al. (2007)
	Escherichia coli	Drulis-Kawa et al. (2006b); Gubernator et al. (2007)
	Klebsiella pneumoniae	Schiffelers et al. (2001a,c); Drulis-Kawa et al. (2006b); Gubernator et al. (2007)
Tobramycin	*Pseudomonas aeruginosa*	Beaulac et al. (1996, 1998); Sachetelli et al. (1999, 2000); Marier et al. (2002, 2003); Mugabe et al. (2006)
	Stenotrophomonas maltophilia, *Escherichia coli*; *Staphylococcus aureus*	Beaulac et al. (1998)
	Burkholderia cepacia; *Burkholderia cenocepacia*	Beaulac et al. (1998); Marier et al. (2002, 2003); Halwani et al. (2007)
Ciprofloxacin	*Pseudomonas aeruginosa*	DiTizio et al. (1998); Bakker-Woudenberg et al. (2002); Gubernator et al. (2007); Bruinenberg et al. (2008)
	Escherichia coli	Gubernator et al. (2007)
	Klebsiella pneumoniae	Bakker-Woudenberg et al. (2001); Gubernator et al. (2007)
	Streptococcus pneumoniae	Ellbogen et al. (2003)
Ofloxacin	*Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*	Furneri et al. (2000)

Beaulac et al. (1998) and Sachetelli et al. (2000) stated that Fluidosomes® fluid vesicles (DPPC/DMPG 18:1) fused with the bacterial membrane of *P. aeruginosa*, releasing their contents (tobramycin) directly to the periplasmic space. This allowed achieving an antibacterial effect with a sub-MIC concentration of the drug. Direct interaction/fusion between liposomes and bacterial cells has become very promising in the eradication of drug-resistant *P. aeruginosa* strains. It is well known that *P. aeruginosa* resistance is mostly related to the low permeability of the outer membrane or to an efficient efflux systems (Hancock and Brinkman, 2002; Livermore, 2001). The application of liposomal antibiotics could thus overcome bacterial resistance mechanisms (Beaulac et al., 1998; Omri and Ravaoarinoro, 1996a,b). A Canadian group (Halwani et al., 2007, 2008; Mugabe et al., 2006; Omri and Ravaoarinoro, 1996a,b; Rukholm et al., 2006) also observed fusion mechanisms in other liposomal formulations. They focused on the *in vitro* activity of aminoglycosides encapsulated in several cholesterol liposomal formulations. Liposomes of DMPC/CHOL (molar ratio 2:1) containing gentamicin showed better antipseudomonal activity than the free drug (Rukholm et al., 2006). Furthermore, even for a highly resistant *P. aeruginosa* strain a 16-fold reduction in MIC was noted (512 μg/ml for the free drug versus 32 μg/ml for liposomal gentamicin). A DMPC/CHOL (molar ratio 2:1) gentamicin formulation improved killing time and prolonged antimicrobial activity. Similar results in MIC reduction were obtained for liposomes of DPPC/CHOL (molar ratio 2:1) containing amikacin, gentamicin, and tobramicin (Mugabe et al., 2006). The authors defined the mechanism of liposome–bacterium interaction by applying transmission electron microscopy (TEM), flow cytometry, lipid mixing assay, and immunocytochemistry. They demonstrated liposome–bacterial membrane fusion, observing intimate interaction of the liposomal vesicles with the outer membrane of *P. aeruginosa*, leading to membrane deformation. The maximal fusion rate was achieved after 1 h of incubation for an antibiotic-sensitive strain of *P. aeruginosa*, but not until 6 h for a drug-resistant strain. DPPC/CHOL (molar ratio 2:1) vesicles bearing aminoglycosides significantly enhanced their antimicrobial efficacy by overcoming the pseudomonal outer-membrane low-permeability barrier. Parallel experiments were done with a DSPC/CHOL (molar ratio 2:1) formulation for high-resistant strains of *B. cenocepacia* (Halwani et al., 2007). Liposomal formulations reduced MICs by 4–16 times for highly antibiotic-resistant strains. Electron microscopy (TEM), flow cytometry, lipid mixing assay, and immunocytochemistry showed evidence of fusion between lipid vesicles and bacterial outer membrane, enhancing antibiotic penetration into the bacterial cells. This Canadian group also performed experiments with other antibiotics entrapped in the same system of cholesterol vesicles (Alipour et al., 2008). Formulations of DPPC/CHOL and POPC/CHOL containing polymyxin B proved to be more effective against various bacterial species than the free drug. *In vitro* activity was tested on *Bordetella bronchiseptica*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *Acinetobacter lwoffii*, and *Acinetobacter baumannii* strains. Liposomal polymyxin

Table 4
Liposomal antibiotics from other chemical groups used for extracellular bacteria eradication.

Antibiotic	Bacteria	Reference
Meropenem	*Pseudomonas aeruginosa*	Drulis-Kawa et al. (2006a,b); Gubernator et al. (2007)
	Escherichia coli; *Klebsiella pneumoniae*	Drulis-Kawa et al. (2006b); Gubernator et al. (2007)
	Staphylococcus aureus	Drulis-Kawa et al. (2006b)
Metronidazol	*Streptococcus mutans*	Vyas et al. (2001)
Polymyxin B	*Pseudomonas aeruginosa*	Trafny et al. (1995); Omri et al. (2002); Alipour et al. (2008)
	Bordetella bronchiseptica; *Escherichia coli*; *Klebsiella pneumoniae*; *Acinetobacter lwoffii*; *Acinetobacter baumani*	Alipour et al. (2008)
Triclosan	oral streptococci: *Streptococcus salivarius*;*Streptococcus sanguis*; *Streptococcus mutans*; *Streptococcus oralis*	Jones et al. (1993); Robinson et al. (2001); Catuogno and Jones (2003)
	Staphyloccus epidermidis; *Proteus vulgaris*;	Jones et al. (1993); Robinson et al. (2001)
Vancomycin	*Staphylococcus epidermidis*; *Staphylococcus aureus*	Sanderson et al. (1996); Kim et al. (1999)

B reduced MIC level 4–16 times compared with the free antibiotic. The authors concluded that the application of lipid vesicles as a polymyxin B carrier could reduce the limitations of systemic use of this drug, such as nephrotoxicity, ototoxicity, and neuromuscular blockade, and the liposomal form of this drug would also exhibit higher antimicrobial activity.

Furneri et al. (2000) examined the antimicrobial activities of several liposomal formulations of ofloxacin on standard and wild-type Gram-positive (*Enterococcus faecalis*, *S. aureus*) and Gram-negative strains (*E. coli*, *P. aeruginosa*). The *in vitro* MICs of both antibiotic forms were determined and compared. It was noted that the MIC for the encapsulated ofloxacin was at most half that of the free drug. Liposomes composed of DMPC/CHOL/DP and DMPC/CHOL/DPPS in a molar ratio of 4:3:4 provided the best enhancement in bactericidal activity against the various bacterial strains. In these experiments regarding liposome–bacterial cell interactions it was shown that vesicles reacted with the outer-membrane proteins and LPS (lipopolysaccharides) of Gram-negative bacteria. In the case of Gram-positives, the liposomal vesicles interacted with peptidoglycan elements, leading to the release of liposomal content close to the cytoplasmic membrane. Both mechanisms significantly increased the amount of antibiotic entering the bacterial cell (Furneri et al., 2000). Gubernator et al. (2007) obtained similar results for ciprofloxacin encapsulated in PC/DOPE/DOTAP; PC/CHOL/DOTAP lipid vesicles in a 4:3:4 molar ratio. The *in vitro* antimicrobial activity was evaluated by determining MIC in a group of Gram-negative clinical and standard strains of *P. aeruginosa*, *K. pneumoniae*, and *E. coli.* Ciprofloxacin loaded in these two cationic formulations exhibited an effect at 2–4 times lower concentrations than the free drug. Drulis-Kawa et al. (2006a) tested *in vitro* twelve lipid formulations of liposomal meropenem against *P. aeruginosa* strains. Both meropenem-sensitive and -resistant isolates were used in the study. Two cationic formulations (PC/DOPE/SA 4:4:2 and PC/DOTAP/Chol 5:2:3) improved the activity of encapsulated meropenem, as 2–4 times lower MICs than those of the free drug were noted, but only in the case of the sensitive isolates. None of the studied liposomal forms of meropenem exhibited bactericidal activity against isolates which were drug resistant due to low permeability. The investigators also tested Fluidosomes® (liposomes made of DPPC/DMPG 18:1), which demonstrated fusion with *P. aeruginosa* membranes (Beaulac et al., 1998). Surprisingly, meropenem encapsulated in Fluidosomes® showed 4–16 times higher MICs for the sensitive and resistant strains than did the free meropenem. Similar results were obtained in subsequent studies (Drulis-Kawa et al., 2006b; Gubernator et al., 2007) on *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *S. aureus* strains. The most effective of the various positively charged, negatively charged, and neutral formulations were cationic PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3. The cationic formulations containing meropenem or gentamicin showed better antibacterial efficacy against both Gram-positive and Gram-negative bacteria than the anionic and neutral ones, regardless of the encapsulated drug. The least effective were liposomes containing gentamicin. The authors concluded that sub-MIC antimicrobial efficacy does not correlate with rapid drug release from liposomal formulations, but rather with the electrostatic interaction between the liposomes and the bacterial cell. The direct interactions strongly depended on the bacterial outer-membrane structure. They obtained different results in clinical *P. aeruginosa* strain sensitivities to the liposomal form of the antibiotics because these bacteria exhibit a wide variation in bacterial surface structure (Rivera et al., 1988; Poole, 2002). For *K. pneumoniae* and *E. coli* isolates, the susceptibilities to free and encapsulated antibiotics were more uniform than those of *P. aeruginosa* rods. The enteric rods most often develop enzymatic mechanisms of drug resistance, so the structure of the outer membrane is less variable (Poole, 2001). The authors concluded that besides the importance of bacterial cell-wall structure, other aspects should be taken into account in interpreting liposomal drug activity.

One of these aspects is the physicochemical properties of the antibiotic and another is the location of the drug target in the bacterial cell. Meropenem exhibits slightly amphipathic properties that allow it to penetrate the outer-membrane structures easily. In addition, the target is located in the periplasm (Piddock, 1998; Yang et al., 1995). If meropenem is transported by liposomal vesicles, the drug enters the periplasm at a high concentration due to the interaction between the liposomes and the outer membrane. In such a situation a decrease in MIC, affecting bacterial growth, could be observed. A similar situation takes place in the case of fluoroquinolones (ciprofloxacin). After liposome adhesion to the outer membrane, a large number of drug molecules can diffuse through the inner membrane into the cell and reach the target by means of hydrophobicity and microspeciation (Hernandez-Borrell and Montero, 2003; Montero et al., 1996; Piddock, 1998). Presumably, better antimicrobial efficacy of liposomal ciprofloxacin in comparison with the activity of the free drug was therefore noted. As for aminoglycosides (gentamicin, amikacin, tobramycin), the mode of action is more complicated. The initial step during the uptake of aminoglycosides such as gentamicin is passive ionic binding of the molecule to the cell surface (Kadurugamuwa et al., 1993a,b; Martin and Beveridge, 1986). The outer membrane's affinity for gentamicin depends on the lipopolysaccharide structure. Ionic binding of aminoglycosides to the outer membrane of cell surfaces is very important in the bactericidal activity of these drugs (Kadurugamuwa et al., 1993a,b; Kotra et al., 2000). In the case of the fusion of gentamicin encapsulated in liposomes, the initial step of uptake is omitted. This is probably why these authors observed low antibacterial efficacy against most of the tested Gram-negative isolates compared with free drug.

Drulis-Kawa et al. (2009) determined the role of the bacterial outer-membrane structure, especially outer-membrane proteins and LPS, and envelope properties (hydrophobicity and electrostatic potential) in the fusion of *P. aeruginosa* cells and lipid vesicles. The interactions between PC:Chol:DOTAP (3:4:3) liposomes and bacterial cells were tested under fluorescence microscopy using rhodamine-labelled vesicles. The cells' negative charge and hydrophobic properties promote interaction with the cationic lipid vesicles (Campanha et al., 1999; Carmona-Ribeiro, 2000), but no specific correlation was noted for the tested strains. A similar situation concerned LPS structure, in which parent strains and their mutants possessing identical ladder-like band patterns in SDS-PAGE analysis exhibited totally different results with fluorescent microscopy. Outer-membrane protein analysis showed that an 18-kDA protein occurred in the isolates demonstrating fusion with rhodamine-labelled vesicles and, conversely, strains lacking the 18-kDA protein exhibited no positive reaction (red emission). This suggests that even one protein may be responsible for favouring stronger interactions between *P. aeruginosa* cells and cationic liposomes. Unexpectedly, the antibiotic-resistant clinical strains showed the most intensive red emission (visible fusion) by fluorescent microscopy. These strains exhibited a 2–4-fold increase in MIC for liposomal meropenem compared with the free drug. This could mean that if the drug resistance mechanisms were very efficient, even direct drug transport into the periplasm did not affect bacterial killing.

4. Disadvantages of liposomal antibiotics

One of the disadvantages of liposomal antibiotics is the short shelf-life of lipid vesicles, which limits drug stability. Short shelf-lives can be conditioned by both physical and chemical processes.

Chemical instability can be observed mainly due to the hydrolysis of ester bonds or the oxidation of unsaturated acyl chains of the lipids used to construct the liposomal vesicles (Sharma and Sharma, 1997; Storm and Crommelin, 1998). These processes occur in both synthetic and natural phospholipids. Moreover, besides hydrolysis and oxidation, peroxidation of unsaturated acyl chain bonds is also possible (e.g. egg and soy bean PC) (Storm and Crommelin, 1998). Phospholipids from natural sources also exhibit structural diversity in their acyl chains, which causes differences in the stability and unique composition of liposomes. In this regard, the stability of liposomal drugs *in vitro* depends mainly on lipid composition, with storage temperature playing an important role. It is possible to prevent oxidation by adding antioxidant components or by freeze-drying. A low storage temperature also prevents hydrolysis (Storm and Crommelin, 1998).

The physical instability of liposomal drugs leads to drug leakage from the lipid vesicles. The highest membrane permeability and fastest leakage of content can be observed at the lipid phase transition. It is higher in the liquid than in the gel phase (Mouritsen and Jorgensen, 1998). The problem with liposomal drug stability occurs mainly in cases of *in vivo* administration. Under physiological conditions, stability is usually low and depends on the interaction of the liposomal membranes with components of body fluids (Gregoriadis, 1995). Lipids that are transferred from the liposomal membrane to plasma lipoproteins in blood cause changes in liposome properties and release the drug. This process is most evident in liposomal formulations containing short-chain lipids or those with fluid membranes. Fluid liposomes release their content a few minutes after intravenous administration. The stability of liposomal vesicles can be enhanced by the addition of cholesterol, which stabilise their membrane and fluidity (Ulrich, 2002). Drug leakage increases *in vivo* in liposomes carrying a net charge (Ulrich, 2002). This is a very unfavourable situation, especially as the best results of antibacterial activity of liposomal drugs *in vitro* are observed for positively charged or fluid liposomes (Drulis-Kawa et al., 2006a; Gubernator et al., 2007). The presence of anionic lipids in liposomal vesicles also favours the binding of serum proteins to the vesicle surface (Briones et al., 2008). Strong adsorption was observed between positively charged bovine serum proteins and negatively charged lipids due to electrostatic attraction. In contrast, bovine serum proteins did not adsorb to the surface of cationic liposomes (Yokouchi et al., 2001). Another aspect of the physical instability of liposomes is the aggregation and fusion of liposomal vesicles. Both lead to changes in liposome size, which influences the *in vivo* therapeutic efficacy of the drug (Sharma and Sharma, 1997).

The encapsulation process has a very important influence on the therapeutic utility of liposomes. Liposomal drug formulations are only useful if there is a therapeutic amount of drug and a reasonable amount of lipids. Lipids in high doses can be toxic and can compromise the pharmacokinetics of liposomal drugs (Sharma and Sharma, 1997). Depending on the characteristics of the drug molecules, an active drug-loading method or the thin lipid film method can be used. Other methods yielding high-efficiency encapsulation, such as reverse-phase evaporation, are usually not suitable because of the presence of residual toxic organic solvents in the liposomal preparations. Another issue could be the use of the freeze-dried liposome rehydration method, which often offers higher encapsulation efficiency compared with the thin lipid film method. Gubernator et al. (2007) obtained meropenem and gentamicin (hydrophilic drugs) encapsulation efficiency in the range of 2.7–5.7% for a cationic fluid formulation. Encapsulation efficiency using the thin lipid film method is usually relatively low and highly associated with the substantial bilayer fluidity. The low encapsulation efficiency can also be explained by electrostatic repulsion between positively charged liposomes and drug molecules (Campanha et al., 1999; Lutwyche et al., 1998). For quinolones (hydrophobic molecules), an active drug encapsulation method is usually chosen. The ammonium sulphate method yields as much as 85–95% encapsulation efficiency and a drug-to-lipid ratio close to 1:6 (Gubernator et al., 2007). Encapsulation efficiency also depends on the type of lipids. Anionic liposomes containing the fusogenic lipid DOPE show lower drug encapsulation (2–3%). Lutwyche et al. (1998) showed that 25–33% of total gentamicin was associated with the outer surface of anionic liposomes composed of DOPE lipid, so a gentamicin encapsulation capacity of 2.8% was obtained in the anionic formulation DOPE/DOPS/PEG. The encapsulation efficiency of the drug was thus relatively low, but similar results were obtained by other investigators (Lutwyche et al., 1998; Omri and Ravaoarinoro, 1996b). Low encapsulation also makes liposomal drug application much more expensive than conventional antibiotic treatment (Kshirsagar et al., 2005).

Liposome preparation methods on the laboratory scale are very complex and expensive, making it not always possible to scale up the processes. A few of the laboratory preparation methods are being used in industry, i.e. the detergent removal method, the ethanol injection method, and the lyophilisation of bilayer-forming lipids in the presence of drug (Storm and Crommelin, 1998).

A very important aspect of drug preparation is sterility. Sterilization procedures for liposomal antibiotics cannot involve the use of heat, irradiation, or chemical agents. Lipids are very sensitive to high temperatures and easily undergo oxidation and hydrolysis (Zuidam et al., 1993). Heat sterilization can be consider only for thermostable and lipophilic drugs (Storm and Crommelin, 1998; Zuidam et al., 1993). A useful method of liposome sterilization is mechanical filtration, but only for liposome vesicles smaller than bacterial cells, and it still does not guarantee the removal of viral particles (Sharma and Sharma, 1997).

5. Clinical trials

Preliminary reports of human treatment using liposomes as antibiotic carriers were provided by Peyman et al. (1988). The authors successfully cured chronic intraocular inflammatory disorders using a single intravitreal dose of different drugs. A few years later, simultaneously with widely performed experiments *in vitro* and *in vivo* on animal models, clinical phase I/II trials with liposomal antibiotics also started. The first results of tuberculosis treatment were described by Nightingale et al. (1993) and Wiley et al. (1994). Nightingale et al. (1993) tested liposomal gentamicin in treating *M. avium–Mycobacterium intracellulare* complex (MAC) bacteraemia in AIDS patients. Gentamicin encapsulated in conventional LUV vesicles of egg phosphatidylcholine (TLC G-65; The Liposome Company, Princeton, NJ, USA) were administrated by intravenous infusion twice weekly for 25 days in three different doses: 1.7, 3.4, and 5.1 mg/kg. The numbers of bacterial colonies were reduced in blood specimens by 25% or more at all the given doses, but no total bacterial eradication was noted. The activity of liposomal gentamicin depended on the duration of treatment, not on the dose. The MIC of the isolated bacteria remained at the same level for the whole study period. This means that liposomal gentamicin did not generate bacterial resistance. In only 1 patient among 21 participants did liposomal gentamicin exhibit nephrotoxicity when given in the highest dose. During the therapy, bone marrow specimens from the patients were also examined (Wiley et al., 1994); no reduction in colony count was noted in the evaluable samples. The phase I/II study showed a good antibacterial activity of liposomal gentamicin in MAC bacteraemia, but the duration of therapy, dose frequency, and combination with other drugs should still be determined for effective antimycobacterial treatment. Brandissou et al. (1997) and Sesin et al. (1996) described the efficacy of liposomal

gentamicin and amikacin, respectively, in the treatment of MAC infections in AIDS patients. They concluded that liposomal aminoglycosides could be useful, but only in a three- or four-drug regimen including clarithromycin, rifampin, ethambutol, and clofazimine.

Liposomal amikacin MiKasome® (NeXstar Pharmaceuticals, USA), intended for mycobacteria eradication, also passed the second phase of clinical trials. Whitehead et al. (1998) treated patient with multidrug-resistant tuberculosis with liposomal amikacin for 11 weeks and subsequently with conventional amikacin. In this study the efficacies of the free and liposomal forms of the antibiotic were compared. MiKasome® was well tolerated and an even 30 times greater accumulation of liposomal amikacin in serum and sputum was found compared with the free drug. The elimination half-life was 180 h for MiKasome® and 6 h for conventional amikacin. No renal or significant hearing disorders were noted, in contrast to the free drug. However, in spite of the improved pharmacokinetics of the liposomal antibiotic, no eradication of *Mycobacterium tuberculosis* from the sputum was observed. During the 11 weeks of treatment, no reduction in the drug sensitivity of the bacteria was detected, in contrast to conventional amikacin, for which the MIC increased from <2 to 8 mg/l. It can be concluded that in the case of concentration-dependent eradication with antibiotics such as aminoglycosides, liposomal delivery was beneficial, exhibited significantly lower toxicity, and did not generate drug resistance, but the early bactericidal activity and dosing of MiKasome® should still be clarified and further tested.

MiKasome® vesicles were also tested in the treatment of pulmonary tuberculosis in patients in a seriously epidemic area in South Africa (Donald et al., 2001). Amikacin was administrated intravenously once daily for 3 days and then conventional therapy was applied. Unfortunately, amikacin did not show early bactericidal activity (EBA), in contrast to tuberculosis treatment on animal models (Donald et al., 2001). It was confirmed that liposomal amikacin, similar to the free drug, showed poor antibacterial activity in pulmonary and chronic tuberculosis, but in humans an EBA effect was not observed. An explanation of this was proposed by Whitehead et al. (1998) who suggested that in the animal model the mycobacteria accumulated mostly in macrophages, so the liposomal antibiotic reach the bacteria after fusion between intracellular vacuoles containing bacilli and liposomal vesicles. In human tuberculosis with cavitary disease there are also large numbers of extracellular free mycobacteria and the liposomal drug did not obtain a high concentration in the extracellular matrix. Another explanation, proposed by Donald et al. (2001), concerned the acidic micro-environment in lesions due to acute inflammation in which aminoglycosides were inactive. Amikacin loaded in long-circulating vesicles (MiKasome®) was also studied in diseases other than tuberculosis. Krieger et al. (1999) tested the activity of the liposomal drug in treating urinary tract infections. The once-daily high-dose application exhibited a low toxicity rate and a gave satisfactory cure efficacy, although only 6% of the drug 1 day after application and 12% a week after injection were cleared through the kidneys. Unfortunately, NeXstar Pharmaceuticals was acquired by Gilead Company in 1999 and further clinical trials with MiKasome® were postponed.

Intensive study was also focused on the liposomal amikacin formulation Arikace™ (Transave, Inc., Monmouth Junction, NJ, USA), designed for inhaled application as an alternative treatment of pulmonary *P. aeruginosa* infections in cystic fibrosis (CF) patients. Arikace™ was designed with small (0.3 μm) uncharged liposomes that enable penetration of the biofilm formed by *P. aeruginosa* and delivery of the drug in the vicinity of the bacteria. Li et al. (2008) concentrated on aspects of the administration of nebulised liposomal amikacin. They described the most effective application system to obtain the proper droplet size, which had significant influence on antibiotic deposition in the lung area. Dupont et al. (2008) treated CF patients with 280-mg and 560-mg daily doses administrated by eFlow® Electronic Nebulizer inhalation. Patients receiving the 560-mg dose achieved a 2.2 log reduction in *P. aeruginosa* density. The sustained release of amikacin exhibited a beneficial effect for another 4 weeks after treatment in the high-dose group. In 2009, Okusanya et al. described clinical trials of Arikace™. The drug inhalations (500 mg) were administrated once daily for 14 days to 24 patients. The pharmacokinetics and pharmacodynamics of the drug were evaluated. Decreased numbers of *P. aeruginosa* colonies in sputum and positive changes in pulmonary function were significant after 14 days of treatment. Arikace™ allowed obtaining a high concentration of amikacin above the minimum inhibitory concentration for *P. aeruginosa*, which inhibited the potential development of drug resistance. Additionally, enzymes presented in the sputum and excreted by the pathogen caused liposomal vesicle disintegration, and amikacin was released in a high concentration, targeting the drug to the bacterial microenvironment. The second phase of the clinical trials was successful because Arikace™ delivered once daily for 28 consecutive days produced a significant improvement in lung function, was well tolerated, and had a side-effect profile comparable to placebo. Dubus and Ravilly (2008) considered inhalation of liposomal amikacin as well as other antibiotics in the treatment of chronic colonization and early infection with *P. aeruginosa.* They obtained positive effects, but the heterogeneous and variable deposition in the lungs strictly depended on the inhalation procedure and technique. They concluded that the inhaled route could be particularly attractive in treating cystic fibrosis, despite the expensive procedure with nebulised liposomal drugs.

The Aradigm Company (Hayward, California) designed liposomal ciprofloxacin formulations for the treatment of infections in patients with cystic fibrosis (ARD-3100) and non-CF bronchiectasis (ARD-3150). In 2008 the second phase of clinical trials were performed in Australia, New Zealand, and the United Kingdom with CF patients and with non-CF bronchiectasis patients infected by *P. aeruginosa*. Liposomal ciprofloxacin was administrated via a nebulizer once daily for 2 weeks in the CF and for 28 days in the non-CF bronchiectasis patients. Inhalation was well tolerated, no serious side effects were noted, and the serum concentration of the drug was much lower than by conventional ciprofloxacin injection. The antibacterial efficacy of inhaled ARD-3100 was significant because a 1.5 log reduction in bacterial CFU was reported after 14 days even if the antibiotic was applied for only 1 week. Satisfactory therapeutic effects were achieved as measured by the forced expiratory volume in one second (FEV1), which increased by 7% from baseline after 14 days of treatment ($p = 0.04$) (Bruinenberg et al., 2008).

The clinical results of treating cystic fibrosis patients presented above encourage further investigations and trials on liposomal antibiotics for inhalation.

6. Concluding remarks

The discovery of liposomes as universal carriers allowed the development of various delivery systems for enzymes, DNA, drugs, and other chemical compounds. The possibility of modifying the vesicles formulation plays a major role in their wide application. The manipulation of different physicochemical properties of liposomes, such as size, lipid type, the lipid composition of the liposomal formulation, the charge on the liposomal surface, and fluidity of the liposomal membrane, enables the design of particular carriers with the desired pharmacokinetic and pharmacodynamic properties. Most studies regarding liposomal antibiotics deal with aminoglycosides, quinolones, polypeptides, and betalactames. Some of the studies focused on improving pharmacokinetics and reducing toxicity, while others involved enhancing antibacterial activity. Liposome vesicles can be designed to treat intracellu-

lar infection in which conventional liposomes are taken up by the mononuclear phagocyte system and transported directly to infected cells. On the other hand, using PEG to cover the vesicles completely changes the biodistribution of liposomal drugs because long-circulating liposomes release their contents for a long period of time, allowing the maintenance of a constant concentration of antibiotic in the serum. At the same time, STEALTH liposomes can accumulate at the site of infection because local inflammatory conditions increase capillary permeability. The liposome surface can be modified by adding charged lipids, immunoglobulins, proteins, or saccharides to direct them to specific targets such as particular tissue cells, pathogens, or bacterial biofilm. The application of fluid vesicle formulations allows enhancing the antibacterial activity of encapsulated antibiotics against extracellular pathogens such as *P. aeruginosa*, *B. cepacia*, and *E. coli*. Direct interactions or fusion between fluid vesicles and the bacterial outer membrane lead to increased penetration of the drug into the bacterial cell. There is much *in vitro* and *in vivo* evidence verifying the significant improvement of applying liposomes as antibiotic carrier systems, but until now most *in vivo* experiments were performed on animal models. A few clinical studies were performed only with encapsulated aminoglycosides (gentamicin and amikacin) in the treatment of mycobacterial bacteraemia and tuberculosis or in the cure of pseudomonal infections in cystic fibrosis patients. The results of clinical trials with liposomal antibiotics were not spectacular in some cases, but promising effects were obtained in eradicating *P. aeruginosa* cells from sputum samples of cystic fibrosis patients treated with liposomal amikacin for inhalation. When discussing the clinical trial results it should be pointed that only two or three liposomal formulations from among the large number tested in the laboratory were used. It is obvious that not only clinical trials, but also liposome preparation is extremely time and cost consuming, which could severely limit the wide application of liposomal antibiotics in the health service.

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