Vesicles as antibiotic carrier: state of art

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This article contains 3 figures, 1 table and 7098 words (including the abstract)

Abstract

Antimicrobial resistance (AMR) has become a global health problem. Bacteria are able to adapt to different environments, with the presence or absence of a host, forming colonies and biofilms. In fact, biofilm formation confers chemical protection to the microbial cells, thus making most of the conventional antibiotics ineffective. Prevention and destruction of biofilms is a challenging task that should be addressed by a multidisciplinary approach from different research fields. One of the medical strategies used against biofilms is the therapy with drug delivery systems. Lipidic nanovesicles are a good choice for encapsulating drugs, increasing their pharmacodynamics and reducing side effects. These soft nanovesicles have many advantages for their compositions similar to the cell membrane, physical and chemistry properties, good affinity with drugs and easy route of administration.

This review summarizes the current knowledge on different types of vesicles which may be used as antibiotic carriers. The main preparation and purification methods for the synthesis of these vesicles are also presented. The advantages of drug encapsulation are critically reviewed. In addition, recent works on endolysin formulations as novel, “greener” and efficient antibiofilm solution are included. This paper can provide useful background for the design of novel efficient formulations and synergistic nanomaterials and could be also useful at the pharmaceutical industry to develop wastewater treatments and reduce the antibiotics in the environmental waters.

Keywords: antibiotic carrier, vesicles synthesis, purification method, encapsulation efficiency, antimicrobial resistance, encapsulation advantages, drug delivery.

1. Introduction

Bacteria have developed new forms of resistance and they have the ability to change the way to colonize surfaces, aggregate and form biofilms. A biofilm is a complex community of bacteria cells that can form on living (as the dental plaque in animals and humans) or non-living surfaces and can be prevalent in natural, industrial and hospital environments. The formation of a biofilm begins with the reversible attachment of free bacterial cells to the surface via van der Waals forces. This step is followed by an “irreversible attachment”. Multiplication of bacteria and even the integration of external microorganisms lead to a multicellular mature form. The cells are enclosed in a matrix composed by exopolysaccharides, amyloid fibers and extracellular DNA. These aggregates are characterized by the presence of nutrients and oxygen that lead to heterogeneity and bacterial cell differentiation. Biofilms are able to grow on any surface, to survive to environmental stresses and to allow bacteria to infect the host in a more aggressive and resistant manner (Penesyan et al., 2015; Tacconelli et al., 2018).

In recent years, the inappropriate use of antibiotics and the new defence mechanisms of bacteria have rendered ineffective the action of traditional antibiotics. The associated antimicrobial resistance (AMR) is at present a major global economical and health problem (https://ec.europa.eu/health/amr/antimicrobial-resistance_en) and an EU priority concern (https://ecdc.europa.eu/en/publications-data/summary-latest-data-antibiotic-resistance-

42 european-union). Infections associated with biofilms and AMR are considered to be a leading
43 cause of morbidity within hospitals and nursing homes, causing additional healthcare costs
44 exceeding one billion €/year in the EU (https://www.ecdc.europa.eu/en/annual-epidemiological-
45 reports).

46 The continuous search for new antibiotics has a high cost and requires a long time for the
47 pharmaceutical industries. Therefore, there is a high interest on developing novel routes to kill
48 bacteria and avoid AMR mechanisms. With this aim, recent studies have used endolysins as an
49 antibacterial agent (Cha et al., 2019; Yan et al., 2019) . Endolysins are peptidoglycan hydrolytic
50 enzymes. They have a strong bactericidal activity against gram + bacteria, including *Streptococcus*
51 *Pneumoniae*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis, Clostridium*
52 *perfrigens,* and other antibiotic-resistant bacteria. They specifically attacks bacterial cells, not
53 human cells, and rarely enter into the gram- cell wall. Endolysin should be treated with chelating
54 agents such as EDTA, but they are carcinogenic to the human body and they are inactive at acid pH
55 (Bai et al., 2019; Lim et al., 2014).

56 Novel drug delivery systems have been developed as alternative mechanisms to systemic
57 administration and for a controlled release of a pharmaceutical compound. These mechanisms
58 include vesicle systems, such as liposomes or niosomes. More recently, extracellular vesicles (EV)
59 have also been considered as potential drug delivery vehicles due to their role in intercellular
60 communication and the transfer of biological information (Vader et al., 2016). The novel drug
61 delivery nanotechnology aims to modify drug release profile, absorption, distribution and
62 elimination, in order to improve product efficacy and safety (Tibbitt et al., 2016).

63 An efficient targeted delivery system requires maintaining its integrity under challenging
64 conditions. Plasma proteins, such as lipoproteins, may interact with liposomes and cause a
65 leakage of their content. Liposomes may also be targeted by the mononuclear phagocyte system
66 (MPS) in different locations (Pattni et al., 2015). In addition, the system has to properly reach its
67 target site of action with minimal side effects. Vesicular systems represent an appropriate option,
68 since they can transport biological molecules and have potential benefits. They are stable in blood
69 and they can confer immune tolerance. In addition, vesicular systems can be site specific and exert
70 their effect on the target by means of surface modification/functionalization with specific
71 molecules (e.g. ligands or antibodies). Types of sustained release formulations include liposomes,
72 drug loaded biodegradable microspheres and drug polymer conjugates (Srikanth and Sivaiah,
73 2012).

74 Nanovesicles are nanocolloids consisting of an aqueous core surrounded by a lipid layer that are
75 being used for drug encapsulation with several types of applications, such as, pharmaceutical,
76 food or cosmetic industries (Sakai-Kato and Takechi-Haraya, 2018). Vesicles are suitable carriers
77 for hydrophilic and lipophilic drugs, since molecules can be encapsulated either in the inner
78 aqueous core or in the lipid layer. Therefore, vesicles are appropriate drug carriers for a wide type
79 of applications.

80 For these reasons, encapsulation of traditional antibiotics in nanovesicles based on the
81 compatibility of the chemical-physical properties between antibiotics and vesicles could be a
82 feasible challenge (Ghafelehbashi et al., 2019). In addition, the routes for nanovesicles formulation

would be also useful at the pharmaceutical industry to develop wastewater treatments, and reduce the antibiotics in the environmental waters.

The present article summarizes the recent works regarding antibiotics and endolysins encapsulation using vesicles as a drug carrier. The types of vesicles have been discussed, followed by the techniques used for vesicles preparation and purification.

2. Type of vesicles

Synthetic and natural vesicles can be used as drug carriers. In recent years, synthetic vesicles have been developed in the pharmaceutical and Nano medicine fields, due to the possibility of producing them on a large scale and using elements such as polymers that can control drug release. More often, the efficacy in vitro is not proved in experiments in vivo (Van Der Meel et al., 2014).

The possibility of using natural vesicles, such as extracellular vesicles, is being explored. Their interesting features are the ability to circulate in the body longer without being detected by the immune system; exploit the action of pre-existing proteins, different according to the type of cell or tissue from which they come, at the level of the membrane or inside the vesicle itself; cross barriers such as blood-brain barrier (BBB), which cannot be crossed by synthetic vesicles (Van Dommelen et al., 2012).

Vesicles can be classified based on their size and the amount of lamellas present in their membrane layer. Small unilamellar vesicles are in the range of 20-100 nm, large unilamellar vesicles have larger size than 100 nm and multi-lamellar vesicles have minimum size of 500 nm.

However, vesicles can also be classified according to their membrane chemical composition; a detailed description of each type of vesicles, synthetics and natural, is presented in the following sections. Although more types of vesicles can be found in the literature, only the most commonly used in antibiotic encapsulation are described in the present work. Figure 1 shows different types of vesicles used for antibiotic encapsulation.

Figure 1

2.1.1 Liposomes

Liposomes are characterized by a lipid bilayer formed by naturally or synthetic phospholipids such as phophatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol. Liposomes are considered nanoliposomes when their size is smaller than 200 nm. The size, lamellarity and encapsulation efficacy are influenced by their formulation and the preparation method used (Pattni et al., 2015; Srikanth and Sivaiah, 2012).

Liposomes can have a charge positive or negative depending on the additives used, for instance the addition of N-[1(2,3- dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) will confer a liposome positively charged what is interesting for some applications in which the vesicle should be attached to a negative molecule (Du et al., 2019).

Lipids exist in either a fluid state ($T > T_M$) or a gel state ($T < T_M$), the fluid state of the lipids is more permeable to water and can be exploited to encapsulate drugs during liposome production. At body temperatures ($T \approx 37$ °C), a fluid state will make the liposomes leaky, and the encapsulated

122 drugs are likely to escape before reaching the site of action. Cholesterol is frequently added to the
123 liposome formulation in order to stabilize the lipid layer structure.

124 Liposomes can interact with cells with different mechanisms: exchange or transfer of lipids,
125 adsorption, merging with their membrane, endocytosis (Pattni et al., 2015). The main advantages
126 of the liposomes are: biocompatibility with the human organism; increase the solubility of the
127 encapsulated drugs; prevent a chemical and biological degradation under storage conditions of
128 agents and during patient administration; they have a specific site of action with the reduction of
129 the toxicity by the encapsulated drug; they can be chemically modified adding to the basic
130 composition polymers that can give greater stability or elasticity (Al-mahallawi et al., 2017).

131 ***2.1.2 Niosomes***

132 Niosomes are formed mainly by non-ionic surfactants. As in the case of liposomes, their
133 encapsulation ability will be regulated by the conditions and preparation methods, a part from
134 their main composition. Membrane stabilizer such as cholesterol are widely used since it has been
135 demonstrated that it enhances encapsulation efficiency and long term stability (González-
136 Menéndez et al., 2018; Moghassemi and Hadjizadeh, 2014). The main advantages of this type of
137 vesicles compared to liposomes are their osmotical activity, chemical stability, long storage time,
138 low toxicity and biodegradability.

139 Surfactants that have charge on the hydrophilic head can be also used for vesicles preparation and
140 thus yield positively or negatively charged nanoparticles (Menina et al., 2019).

141 ***2.1.3 Polymersomes***

142 Polymersomes (Ps) are vesicles formed by synthetic amphiphilic block copolymers, which contains
143 an aqueous core. The bilayer membrane is like a corona composed of hydrated hydrophilic both at
144 the inside and outside of the hydrophobic middle part of the membrane separating and protecting
145 the fluidic core from the outside medium (Lee and Feijen, 2012a).

146 The polymers used are poly (ethylene glycol) (PEG) blocks, amphiphilic block copolymers with a
147 high molecular weight. They allow to obtain a thin (3-4 µm) and robust membranes and minimize
148 the interfacial free energy and the steric repulsion. The release of the drug from polymersomes is
149 guided by the concentration gradient between the drug in the PS and the external environment.
150 However, the most interesting feature is that polymers change their chemical-physical properties
151 due to external stimuli, such as temperature, pH, radiation, allowing a controlled release of the
152 drug at the target site.

153 The advantages of polymersosomes compared to liposomes are their biological stability due to
154 their similarity to human extracellular vesicles; prolongation of blood circulation times; easy
155 controlled release of the drug due to their sensitivity to extremal stimuli and their
156 biodegradability.

157 ***2.1.4 Copolymer micelles***

158 This type of nanovesicles is included in the present work since their physical properties and
159 applications are similar to those of the traditional vesicles.

160 This kind of micelle is formed by self-assembled copolymers. They have an effect on increasing the
161 solubility and decreasing the toxicity of hydrophobic drugs. Self-assembly is determined by the
162 mass and composition of the copolymer backbone, the concentration of polymer chains, and the
163 properties of encapsulated or pendant drugs and targeting agents. The polymer chains interact
164 with water and reduce the interfacial free energy of the polymer—water system allowing micelle
165 formation. The most common polymeric micelles used in drug delivery are amphiphilic di-block
166 (hydrophilic—hydrophobic) or tri-block (hydrophilic—hydrophobic—hydrophilic) polymers.

167 The critical micelle concentration (CMC) is the minimum concentration of polymer necessary to
168 produce micelle; in fact, it is the number of polymer chains that saturates the solution in bulk.

169 The main characteristics of this systems are their small size (~10—200 nm) to effectively
170 penetrate into tissue; to be unrecognizable by the mononuclear phagocyte system (MPS) for a
171 sufficient time to allow accumulation in target tissue. Moreover, they can easily be degraded from
172 the organism and they can be easily tuneable. These features could improve their encapsulation
173 pharmacokinetic profile. Copolymer micelles can be easily synthesized in a reasonably inexpensive
174 and reproducible method (Grotz et al., 2019; Owen et al., 2012).

175 ***2.2 Extracellular vesicles***

176 Extracellular vesicles are cell-derived membranous structures which a key role in intercellular
177 communication. EV contain proteins, nucleic acids and lipids that may be functional in recipient
178 cells and may vary depending on the cell type of origin.

179 Exosomes are a subtype of EV with an endosomal origin. They originate from budding of early
180 endosomes (intraluminal endosomal vesicles, ILV) and they mature in multivesicular bodies (MVB).
181 ILV are eventually released into the extracellular space upon fusion of the MVB with the plasma
182 membrane, generating the exosomes (Zhang et al., 2019). Exosomes are secreted by all cell types
183 and may be found in body fluids such as blood, saliva, cerebrospinal fluid and urine. Typically,
184 exosomes are enriched in proteins with various functions, such as tetraspanins, a family of
185 transmembrane proteins which regulate a variety of cellular processes related to plasma
186 membrane dynamics (morphology, adhesion, cell migration), protein trafficking, and cell signaling;
187 heat shock proteins, as part of the stress response that are involved in antigen binding and
188 presentation; MVB formation proteins, which are involved in exosome release. They are also
189 enriched in phosphatidylserine, phosphatidic acid, cholesterol, sphingomyelin, arachidonic acid
190 and other fatty acids, prostaglandins, and leukotrienes, which account for their stability and
191 structural rigidity. Moreover, exosomes also have some functional lipolytic enzymes, which can
192 produce units of bioactive lipids autonomously (Ha et al., 2016).

193 EV have been shown to regulate or participate in a variety of biological processes, both under
194 physiological and pathological conditions, as reviewed elsewhere (Yáñez-Mó et al., 2015).
195 Exosomes communicate with the target cell by different mechanisms: direct stimulation of target
196 cells via surface-bound ligands; transfer of activated receptors to recipient cells; and epigenetic
197 reprogramming of recipient cells via delivery of functional proteins, lipids, RNAs. Due to their
198 biological nature and their small size, exosomes can elude phagocytosis from macrophages. This is
199 an advantage over liposomes and other types of vesicles. Studies have confirmed the possibility of
200 encapsulating drugs such as doxorubicin in anticancer therapy (Tian et al., 2014) and the ability to

cross the blood-brain barrier (BBB), but this has only been confirmed in vitro (Yang et al., 2015). Exosomes could be potentially used as hydrophilic and lipophilic drug carriers. Although a proper mechanism of drug encapsulation must still be studied, they are considered as potential innovative vehicles for drug delivery (Batrakova and Kim, 2015; Tan et al., 2013). Recent studies show that exosomes are involved in the immune response caused by bacterial infections, and their role trapping toxins involved at virulence factors could open the path to novel treatments to combat pathogen infections (Keller et al., 2020).

3. Preparation methods

In this section, the most common techniques used for vesicle preparation with encapsulated drug are described. A schematic description of the most used methods described is presented in Figure 2 and Figure 3.

3.1 Thin-film hydration

Thin-film hydration is a method widely used for the formation of liposomes and niosomes. The components of the lipid bilayer are dissolved in the organic phase in a flask (usually chloroform or ethanol). After the organic phase is removed through vacuum evaporation, with the usual use of the rotary evaporator, a thin layer is formed inside the flask. To obtain a homogeneous, transparent and dry film, certain temperatures, pressures, rotation and time are chosen. While the drug is dissolved in the aqueous phase (commonly deionized water) and is then added to the flask to hydrate the thin layer previously formed. Finally, it is incubated at a transition temperature above the surfactants in a water bath (Gonzalez Gomez et al., 2019; Mozafari, 2005).

3.2 Reverse-phase evaporation

The reverse- phase evaporation method consists in diluting the lipids in chloroform in a flask. The aqueous phase is added to the formed organic solution. The drug is usually diluted to the phase were has more solubility. The mixture is sonicated for 35-40 minutes at around 10°C in order to obtain a homogeneous dispersion.

Finally, the organic solvent is removed under reduced pressure at room temperature using a rotary evaporator. The formation of bubbles can be avoided by increasing the pressure (Ge et al., 2019; Kaddah et al., 2018; Nele et al., 2019).

3.3 Ethanol injection

Ethanol injection method is also a common method in which the membrane components are dissolved in ethanol in a water bath at 60 ° C. The drug is dissolved in the phase that has larger solubility (frequently aqueous phase), then the organic phase with the lipids is injected into the aqueous phase with a syringe under stirring and the formation of the vesicles is observed. The organic solvent is removed by evaporation with the rotary evaporation and the size and shape of the vesicles are adjusted (García-Manrique et al., 2016; Mozafari, 2005).

Figure 2

3.4 Freezing-Annealing-Thawing (FAT)

238 The FAT method includes the thin film hydration. The vesicles were prepared by the thin film
239 method, as described above, but before the extrusion phase, three freeze-thaw cycles were
240 added. A single freeze-thaw cycle consisted of freezing vesicles for 5 minutes at around -200 °C
241 using liquid nitrogen and thawing them in a bath sounder at room temperature for 5 min. The
242 vesicles are stored at 4 ° C for 30 minutes after the three cycles of freezing and thawing. After
243 storage are annealing at room temperature for 30 minutes and further extrusion for size reduction
244 (Gonzalez Gomez et al., 2019; Hope et al., 1986; Qiao et al., 2017).

245 ***3.5 Heating Method***

246 The heating method is a faster method of preparations of liposomes without the use of dangerous
247 chemical products. There is a first hydration of the liposomal components in aqueous medium,
248 followed by heating with glycerol (3% v / v), up to a temperature of 60-120 °C. Glycerol is soluble
249 in water and physiologically acceptable with the capability of increase the vesicles stability.
250 Glycerol does not need to be removed from liposomes. It was confirmed that no degradation of
251 the lipids occurred using high temperatures and the heat abolishes the need to carry out any
252 sterilisation procedure (Mozafari, 2005).

253 The glycerol is non-toxic agent and bio-acceptable. It serves as dispersant and prevents
254 coagulation or sedimentation of the vesicles thereby enhancing the stability of the liposome
255 preparations; it also improves the stability of the liposome preparations against freezing and
256 thawing.

257 The drug can be added to the reaction medium with liposomal components and glycerol; when
258 temperature has dropped to a point not lower than the transition temperature of the lipids or
259 after the preparation of liposomes at room temperature.

260 The heating temperatures have to be higher than the Tc of the lipids, because below Tc lipids are
261 in the gel state and cannot usually form closed continuous bilayered structures.

262 When cholesterol is used as membrane stabilizer, the temperature to obtain liposomes is ~120°C.
263 Since the majority of the phospholipid molecules employed as liposomal constituents have
264 transition temperatures below 60°C, in the absence of cholesterol HM-liposomes can be prepared
265 for example at 60-70°C (Laouini et al., 2012; Nkanga and Krause, 2019).

266 ***3.6 Solid-dispersion method***

267 In solid-dispersion method, lipid components are diluted in chloroform or ethanol with surfactants
268 to stabilize the structures of vesicles. The solution changes from liquid to solid state by
269 evaporating the solvent at 54 °C.

270 Finally, the mixtures were stored in a dryer at minimum 24 h to form liposomes (de Freitas et al.,
271 2019; Huang and Dai, 2014).

272 ***3.7 Ultrasonication method***

273 Ultrasonication is a physicochemical dispersion method. The multillamelar are prepared by a
274 conventional method, as the ones previously described then there is the sonication with a bath
275 type or a probe type sonicator under an inert atmosphere, usually nitrogen or argon (Manca et al.,
276 2019; Melis et al., 2016). The principle of sonication involves the use of pulsed, high frequency

sound waves (sonic energy) to agitate a suspension of the MLVs. MLVs produces SUVs with diameter in the range of 15–50nm. The purpose of sonication, therefore, is to produce a homogenous dispersion of small vesicles with a potential for greater tissue penetration (Lee and Feijen, 2012a; Walvekar et al., 2019).

A probe tip sonicator has some disadvantages: one of them is the lipid degradation due to the overheating. On the other hand, when the probe is worn, it tends to release titanium particles into lipid suspension, which must be removed by centrifugation.

Bath sonications can also be used. A test tube containing the suspension is placed in the bath sonicator and sonicating for 5–10 minutes above the transitional temperature of the lipid. The advantages are temperature control to minimize thermo degradation of the lipid; no contaminations like the probe; the position of the tube and water level in the bath is can be also regulated for maximum efficiency. The disadvantage is the denaturation or inactivation of some thermolabile substances, such as, DNA or proteins to be entrapped (Zhang and Zhu, 1999).

3.8 Solvent- diffusion technique

Solvent- diffusion is a technique used to encapsulate drug in polymeric micelles (Owen et al., 2012). The micelles can be formed with amphiphilic polymer like Solupus, polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol copolymers (Grotz et al., 2019). The micelles were prepared by dissolving the appropriate amount of polymer in distilled water under magnetic stirring (50 rpm) at 25 °C.

The drug is dissolved in the organic phase, like acetone, under sonication. Then the organic solution is added with a syringe pump to the aqueous dispersion with polymeric micelles. The mixture is left under magnetic stirring at room temperature overnight to ensure acetone evaporation (Karami et al., 2016; Zhang et al., 2014).

Figure 3

4. Purification methods

Regardless of the preparation method used, small contaminating molecules, non-encapsulated drug or other free molecules belonging to components of the vesicles membrane can be found in the suspension together with the vesicles containing drug. Therefore, purification methods are used, in which particles with encapsulated drug are separated from no-encapsulated drug and from molecules described above. This step is important for determining the encapsulation efficiency, a calculation that allows to quantify the amount of drug inside vesicles. Separation is typically achieved by filtration or ultra-centrifugation, which can be challenging for the large-scale purification. Other possible routes for removal of non-encapsulated material include dialysis, gel-permeation chromatography, ion-exchange chromatography and size exclusion chromatography.

In this section, the most common techniques used are described.

4.1 Filtration

This method involves the filtering of vesicles through filtration units under pressure. Screen filters made from polycarbonate or depth filters made from cellulose acetate can be used to retain particles that are larger than 200 nm, allowing vesicles smaller than 200 nm to pass through. This

316 type of technique is useful for thermolabile products, while the high pression can make some
317 problems for vesicles degradation (Dimov et al., 2017; Grotz et al., 2019).

318 ***4.2 Ultrafiltration and centrifugation***

319 Ultrafiltration is the common method used to separate free and encapsulated drug. The vesicles
320 dispersion is added to a centrifugal filter, which contains an ultrafiltration membrane. The sample
321 is located at the tops part of the tube and centrifuged.

322 While the free drug will pass through the membrane, the vesicles will retained on the top part of
323 the tube. To recover the vesicles, the filtration tube can be flipped upside down and centrifuged
324 again (Ghafelehbashi et al., 2019; Gonzalez Gomez et al., 2019; Osman et al., 2019; Walvekar et
325 al., 2019a).

326 ***4.3 Dialysis***

327 The vesicles are placed in a container in which there is a solution and a dialysis membrane. Dialysis
328 membranes have a cut-off value smaller than the vesicles size, but larger than the drug, so that
329 the drug can pass through the membrane.

330 The free drug crosses the membrane moving towards the compartment in which the drug is
331 absent or in lower concentration. The vesicles remain in the first compartment (Ghosh et al., 2019;
332 Gonzalez Gomez et al., 2019; Grotz et al., 2019; Moyá et al., 2019).

333 Table 1

334 **5. Encapsulation efficiency (EE)**

335 The encapsulation efficiency (EE) is the percentage of the incorporated drug that has been
336 entrapped into the nanovesicle. To calculate this parameter, the non-encapsulated drug must be
337 removed from the vesicle by any of the purification methods described in the previous section.
338 Once the vesicles are purified Organic solvents such as acetonitrile, ethanol, methanol, Triton X-
339 100, are used to break the vesicles. The system with the broken vesicles should be purified again
340 in order to extract the amount of drug that was encapsulated.

341 Depending on the type of drug, different techniques are used to estimate the concentration such
342 as UV and / or fluorescence spectroscopy, enzymes or protein based assays, gel electrophoresis
343 and high pressure liquid chromatography are the most efficient.

344 Encapsulation efficiency is given by the percentage difference between the total antibiotic
345 (encapsulated and non-encapsulated) and the free antibiotic (non-encapsulated) (Eq 1).

346 $$EE\ \% = \frac{total\ drug - free\ drug}{total\ drug}\ x\ 100 \qquad [Eq\ 1]$$

347

348 The more common techniques used for purification and drug determination for each antibiotic
349 studied are summarized in Table 1.

350 **6. Advantages and disadvantages of drug encapsulation**

Encapsulation is an excellent solution for getting a drug to a specific site in a time interval that follows a pharmacokinetics sequence different from that of the free drug. This improves effectiveness by reducing side effects and protects drug from possible degradation. Nanoencapsulation is useful for solving problems of physical instability: solubility, oxidation, pH variation, temperature, moisture, ionic strength changes, absorption, diffusion; biochemical reactions such as enzymatic degradation, denaturation and controlled release. Nanocarriers have a higher thermodynamic, kinetic stability and have a higher surface-to-volume ratio (De Azeredo, 2013), they exhibit a better role in improving the bioavailability and solubility (Rezaei et al., 2019).

In recent years the use of vesicles such as liposomes, niosomes and polymersosomes as release systems for conventional antibiotics has achieved remarkable success in overcoming antibiotic resistance both in vitro and in vivo.

An essential advantage of nanocarriers is the possibility of administrating drugs with a concentration necessary to obtain the desired therapeutic effect. This concept is applicable to antibiotics, as one of the forms of bacterial resistance is to degrade the antibiotic enzymatically. It has been reported that by using a formulation with the antibiotic in the nanocarrier, the enzymatic degradation was avoided and the required amount of antibiotic arrived at the specific site (Kelly et al., 2020; Ma et al., 2013).

Liposomes and niosomes are the most used vesicles for target drug delivery, due to their composition of the membrane similar to the eukaryotic and prokaryotic cell. The double layer of phospholipids melts easily with bacterial cell membranes to release in high antimicrobial doses directly inside bacteria. A size <100-200 nm prevents reticuloendothelial rejection and allows penetration into infectious biofilms (Eleraky et al., 2020). In spite of that, liposomes that penetrate the entire thickness of biofilms killing all bacteria present is not yet possible.

Nevertheless liposomes have fusogenicity, which is the ability to merge with bacterial membranes, and it is related to the fluidity of the lipid bilayer. The components used to make liposomes more fusogenic are: cholesterol hemisuccinate combined with dioleyl phosphatidylethanolamine (DOPE) and dipalmitoyl phosphatidylcholine (DPPC)(Mi et al., 2018; Pignatello et al., 2011).

Liposomes have some disadvantages such as chemical-physical instability during storage, with consequent degradation of the components forming the membrane and loss of load. It is also difficult to have a large-scale reproducibility, lack of uniformity when loading, requirement of surfactants in high concentrations, and the possibility of aggregation and precipitation.

In that sense polymeric vesicles offers a clear advantage since stability is guaranteed as the physiological and chemical characteristics of the polymers can be controlled with greater precision (Lee and Feijen, 2012).

Polymersomes conjugated with hyaluronic acid-oleylamine (HA-OLA are developed by Walvekar and coworkers (Walvekar et al., 2019). They showed a slow and prolonged release of vancomycin (VCM) for 72 hours. In vitro antibacterial activity against MRSA revealed that VCM-encapsulated had a higher action compared to free VCM thanks to a synergistic activity of

391 polymers and antibiotic. In fact, loaded VCM polymers had a greater impact on MRSA
392 membrane rupture than free VCM.

393 However, polymersomes can be toxic, restriction of production in high volume, low
394 bioavailability, and requirement of organic solvents unlike liposomes (Walvekar et al., 2019).

395 In recent years, chitosan has been used to functionalize vesicles surface. Negatively charged
396 liposomes have been developed attaching chitosan-modified gold nanoparticles for
397 vancomycin encapsulation (Pornpattananangkul et al., 2011). A synergistic effect of
398 antimicrobial gold nanoparticles and antibiotic effect was observed on growth inhibition of S.
399 aureus in a skin bacterial infection.

400 Another advantageous liposomes surface modification could be achieved by antibody
401 conjugation (so called "immunoliposomes") or lectins, for different purpose such as
402 Streptococcus oralis.(oral bacteria) treatment (Robinson et al., 2000).

403 Control release can be achieved using PEG, a biodegradable polymer that allows a longer
404 permanence of liposomes in blood (Kelly et al., 2020). Moreno-Sastre et al. presented a study
405 on the aerosol of ciprofloxacin incorporated in PEGylated liposomes, addressed to the lungs.
406 The analysis of drug distribution in epithelial coating liquid (ELF) after aerosol of PEGylated
407 liposomes and uncoated liposomes confirmed the slower elimination of ciprofloxacin in the
408 tissue uncoated liposomes and a lower activity. Nevertheless, PEGylated liposomes did not
409 show cytotoxic effects (Moreno-Sastre et al., 2015).

410 **7. Conclusions**

411 This review article provides a general overview on the most suitable formulations for antibiotic
412 transport. Encapsulation in soft nanovesicles is important to develop more efficient formulations
413 against biofilms, increase pharmacokinetics, and reduce side effects. These strategies would help
414 also to control the antibiotics released to environmental waters, and could contribute to a global
415 action on AMR. The most important methods for the synthesis of vesicles more compatible to the
416 human organism were described, with a list of vesicle types, their different synthesis and
417 purification methods as well as encapsulation efficiency. The choice of the vesicle components is
418 related to the hydrophilic or hydrophobic nature of the active principle to be encapsulated, on the
419 steric hindrance of the antibiotic and on their mechanism of action against the microorganism
420 concerned. The versatility of the nanovesicle design enables personalised therapies for drug
421 delivery. In recent years, bacteriophage components with bactericidal activity such as endolysins
422 have been developed, looking for efficient antimicrobial treatments.

423 **8. Acknowledgements**

424 This work is part of a project that has received funding from the European Union's Horizon 2020
425 research and innovation program under the Marie Sklodowska-Curie Grant Agreement No. 813439
426 (Break Biofilms).

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