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Cyclodextrin-membrane interaction in drug delivery and membrane structure maintenance

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Abstract

Cyclodextrins (CDs) are cyclic oligosaccharides able to improve drug water solubility and stability by forming CD/drug inclusion complexes. To further increase drug entrapment and delay its release, the CD/drug inclusion complex can be embedded in the aqueous phase of a liposome, a lipid vesicle composed of phospholipid bilayer surrounding an aqueous compartment. The resulting carrier is known as drug-in-cyclodextrin-in-liposome (DCL) system. CDs and DCLs are recognized as effective drug delivery systems; therefore, understanding the interaction of CDs with liposomal and biological membranes is of great importance. CDs are able to extract phospholipids, cholesterol, and proteins from membranes; the effect depends on the membrane structure and composition as well as on the CD type and concentration. Under definite conditions, CDs can affect the membrane fluidity, permeability, and stability of liposomes and cells, leading to the leakage of some of their internal constituents. On the other side, CDs demonstrated their beneficial effects on the membrane structure, including preservation of the membrane integrity during freeze-drying. In this paper, we review the literature concerning the interaction of CDs with biomimetic and biological membranes. Moreover, the impact of CDs on the membrane properties, mainly fluidity, stability, and permeability, is highlighted.

Keywords: cholesterol; cyclodextrin; liposome; membrane; phospholipid.

56 **Introduction**

57 Liposomes are phospholipid (PL) vesicles containing one or more lipid bilayers and an aqueous
58 internal cavity. They can encapsulate hydrophilic and hydrophobic drugs in their aqueous core
59 and lipid bilayer, respectively, constituting an effective drug delivery system (Gharib et al.,
60 2015).

61 Another drug delivery system is based on cyclodextrins (CDs), oligosaccharides formed of
62 glucopyranose units. CDs have a truncated funnel shape with a hydrophobic internal cavity and
63 a hydrophilic outer surface (Gharib et al., 2015). Thus, CDs can entrap hydrophobic drugs in
64 their cavities forming CD/drug inclusion complexes that improve drug solubility and
65 bioavailability, enhance physical and thermal stability of drugs, and limit drug toxic effects (Baek
66 et al., 2013; Loftsson and Masson, 2001; Zhang et al., 2013).

67 Drug-in-CD-in-liposome (DCL), a combined system made of CD and liposome, was proposed
68 by McCormack and Gregoriadis (1994) to increase loading rates of hydrophobic molecules and
69 to provide their prolonged release compared to conventional liposomes and CD/drug inclusion
70 complexes. In DCL, hydrophobic drugs are loaded into the aqueous phase of liposome in the
71 form of CD/drug inclusion complex.

72 CDs boost drug delivery by interacting with membrane components (Babu and Pandit, 2004;
73 Mura et al., 2014; Nakanishi et al., 1992; Tilloy et al., 2006; Ventura et al., 2001). This
74 interaction may induce a perturbation in the lipid bilayer affecting the membrane properties such
75 as fluidity (Gharib et al., 2018a; Grammenos et al., 2010) and permeability (Piel et al., 2007;
76 Wang et al., 2011). A deep understanding of CD interaction with biomimetic, i.e. liposomal, and
77 biological membranes is crucial in pharmacology for controlling CD-mediated drug delivery and
78 release.

79 Freeze-drying of liposomes is essential to extend their shelf life (Gharib et al., 2018b). Also,
80 sperm cryopreservation has been extensively applied in artificial insemination programs (Mocé
81 et al., 2010). However, the freezing process can cause membrane damage (Drobnis et al.,
82 1993); therefore, suitable cryoprotectants should be added. CDs are able to form hydrogen
83 bonds with polar groups of membrane lipids, thereby stabilizing the ordered conformation of
84 liposomes and spermatozoa during freeze-drying. Furthermore, CD/cholesterol inclusion
85 complexes serve as cholesterol (Chol) donors to load membranes with Chol for membrane

stabilization. Consequently, free CDs (Gharib et al., 2018b; Madison et al., 2013; Zeng and Terada, 2000) and Chol-loaded CDs (Salmon et al., 2016) are capable to maintain the integrity of a membrane during freeze-drying, thereby ensuring its protection.

To the best of our knowledge, this is the first review that focuses on CD interaction with biomimetic and biological membranes considering the factors that may affect this interaction such as the type and concentration of CD as well as the membrane structure and composition. The main techniques applied to study the CD-membrane interaction are introduced, and the literature data are resumed into conclusive tables. Furthermore, the literature data on CD-mediated extraction of membrane components (PLs, Chol, and proteins) are discussed. We also summarize the effects of CDs on the membrane properties such as permeability, fluidity, and stability. In addition, an overview of the beneficial effects of CDs as membrane cryoprotectants is presented. The last section of this review discusses recent data on DCLs development.

1. Biological membranes

1.1. Membrane structure based on fluid mosaic model

The fluid mosaic model of cell membranes is a fundamental concept in membrane biology. According to this model, the basic structure of a biological membrane is a lipid bilayer associated with proteins, often glycosylated (Singer and Nicolson, 1972). The variety of lipids and proteins experience both rotational and translational freedom within the bilayer plane and are asymmetrically distributed between the membrane leaflets (Holthuis and Levine, 2005).

1.2. Membrane composition

1.2.1. Phospholipids and sphingolipids

PLs are amphiphilic molecules comprising a glycerol backbone esterified at first and second positions with two fatty acids; the third alcohol of glycerol is esterified by a phosphoric acid which, in turn, is esterified by a polar group such as choline, ethanolamine, glycerol, inositol, or serine. The membrane surface charge depends on the polar head groups of PLs and sphingolipids constituting the membrane. The PLs phosphatidylcholine (PC) and phosphatidylethanolamine (PE) containing, respectively, positively charged groups choline and ethanolamine, are neutral. On the contrary, when the polar group is zwitterionic (serine) or non-ionizable (glycerol, hydrogen, or inositol), the resulting PL is negatively charged. Furthermore,

116 the fatty acids which esterify the primary and secondary alcohols of glycerol vary in their length
117 and degree of saturation (Li et al., 2015). Like PLs, sphingolipids are composed of a polar head
118 group and a nonpolar moiety, which is a fatty acid linked to a long-chain amino alcohol,
119 sphingosine. They include sphingomyelins (SM) and glycosphingolipids: cerebrosides,
120 sulfatides, globosides, and gangliosides. Gangliosides and sulfatides are negatively charged
121 due to the presence of sialic acid and sulfate groups, respectively, contributing to the global
122 membrane charge. Sphingolipids have generally much more saturated hydrocarbon chains than
123 PLs allowing them to be packed tightly together (Brown and London, 2000).

124 The structure of a lipid influences its geometry and membrane curvature. Thus, PC and SM
125 both possess a cylindrical shape based on the head-to-tail ratio (the same head and tail cross
126 sectional areas) resulting in a lamella (bilayer) when mixed in an aqueous medium. However,
127 lysophosphatidylcholine (LPC) and phosphatidylinositol (PI) are of inverted conical shape
128 (higher head-to-tail ratio) and form micelles in an aqueous environment. Conversely, PE,
129 phosphatidic acid (PA), and phosphatidylserine (PS), containing relatively small head groups,
130 are cone-shaped lipids which adopt in water an inverted micellar structure. The fact that
131 biological membranes have a lamellar structure explains the choice of PC for the preparation of
132 biomimetic lipid bilayers.

133 As mentioned earlier, membrane components are asymmetrically distributed between the inner
134 and outer leaflets of membrane. In general, the inner leaflet is rich in PLs containing amine or
135 serine moieties and the signaling lipids such as PI and PA, whereas PC and SM are densely
136 located in the outer leaflet (Zalba and ten Hagen, 2017).

137 **1.2.2. Cholesterol**

138 Cellular membranes contain up to 40 % sterols (Chol in mammals) in relation to the total
139 membrane lipids. Chol has a four-ring nucleus with a double bond between C-5 and C-6, an
140 iso-octyl side chain at C-17, two methyl groups at C-18 and C-19, and a hydroxyl group at C-3.
141 Its hydroxyl group is oriented toward the aqueous phase while the hydrophobic moiety is
142 located alongside PL acyl chains. Chol plays an essential role in controlling membrane fluidity,
143 permeability, receptors function, and ion transport (Burger et al., 2000; Cooper, 1978; Kaddah
144 et al., 2018; Simons and Toomre, 2000).

145 **1.2.3. Proteins**

Membrane proteins are classified into integral (or intrinsic) and peripheral (or extrinsic). Integral proteins intercalate into the membrane hydrophobic matrix where they are tightly bound by hydrophobic interactions. Integral proteins are also suggested to be amphipathic with their hydrophobic domains embedded in the hydrophobic interior and their hydrophilic domains protruding from the hydrophobic region of the lipid bilayer into the surrounding aqueous environments. Peripheral proteins are loosely bound to hydrophilic parts of membranes by electrostatic and other non-hydrophobic interactions (Nicolson, 2014).

1.2.4. Lipid rafts

Lipids are not only asymmetrically distributed between the membrane leaflets but also heterogeneously dispersed within a single layer. Cellular membranes contain highly ordered stable structures called “lipid rafts” surrounded by a liquid disordered matrix. Lipid rafts are small-sized domains rich in sphingolipids and Chol, closely packed, functional, and dynamic. They are resistant to solubilization by mild detergents (Brown and London, 1998; Simons and Ikonen, 1997). Also, rafts contain a specific group of membrane proteins linked to saturated acyl chains either using glycosylphosphatidylinositol (GPI) anchor or through acylation with myristate or palmitate (Brown and London, 1998). Lipid rafts were proved to be implicated in many cellular processes such as sorting of lipids and proteins (McIntosh et al., 2003), signal transduction and trafficking (Hanzal-Bayer and Hancock, 2007; Stauffer and Meyer, 1997), and transmission of viral and bacterial infections (Wang et al., 2009).

2. Biomimetic membranes

Due to the complex organization of biological membranes, simple biomimetic membranes are used as models. In the case of CD–membrane interaction studies, lipid monolayers and liposomes are utilized (Grauby-Heywang and Turllet, 2008; Milles et al., 2013; Ohvo-Rekilä et al., 2000).

2.1. Lipid monolayers

Lipid monolayers, also referred as Langmuir monolayers, are formed by spreading amphiphilic molecules at the surface of a liquid; they consist of a single lipid type or a mixture of lipids. This system displays many advantages in comparison with other biomimetic membranes allowing control of parameters such as temperature, nature and packing of lipids, and compositions of the liquid medium (pH, ionic strength) (Maget-Dana, 1999).

2.2. Liposomes

Liposomes are spherical self-closed structures where a lipid bilayer encloses an aqueous inner cavity. Liposomes are mainly prepared from PLs, with or without Chol. They are generally classified according to their size and number of bilayers. Small unilamellar vesicles (SUV) range between 20 and 100 nm while large unilamellar vesicles (LUV) are greater than 100 nm, and giant unilamellar vesicles (GUV) exceed 1000 nm; all these types having a single lamella. Multilamellar vesicles (MLV) are large vesicles ($> 0.5 \mu\text{m}$) possessing more than 5 concentric lamellae (Gharib et al., 2015).

Liposomes are biocompatible, biodegradable, non-immunogenic, and non-toxic structures. All these characteristics make them suitable for drug delivery. Hydrophobic and hydrophilic substances can be entrapped, respectively, within the lipid bilayer and the aqueous internal cavity, and amphiphilic molecules are located at the water-bilayer interface (Figure 1). These properties make liposomes effective as carriers of bioactive molecules in cosmetic, pharmaceutical, food, and farming industries (Sherry et al., 2013).

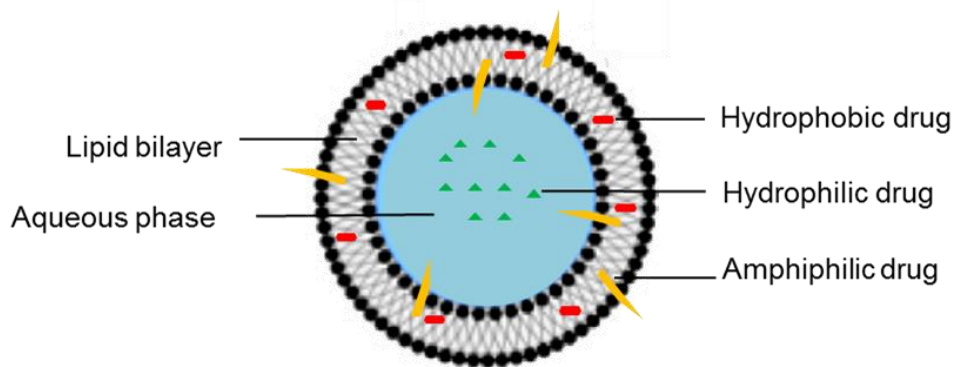


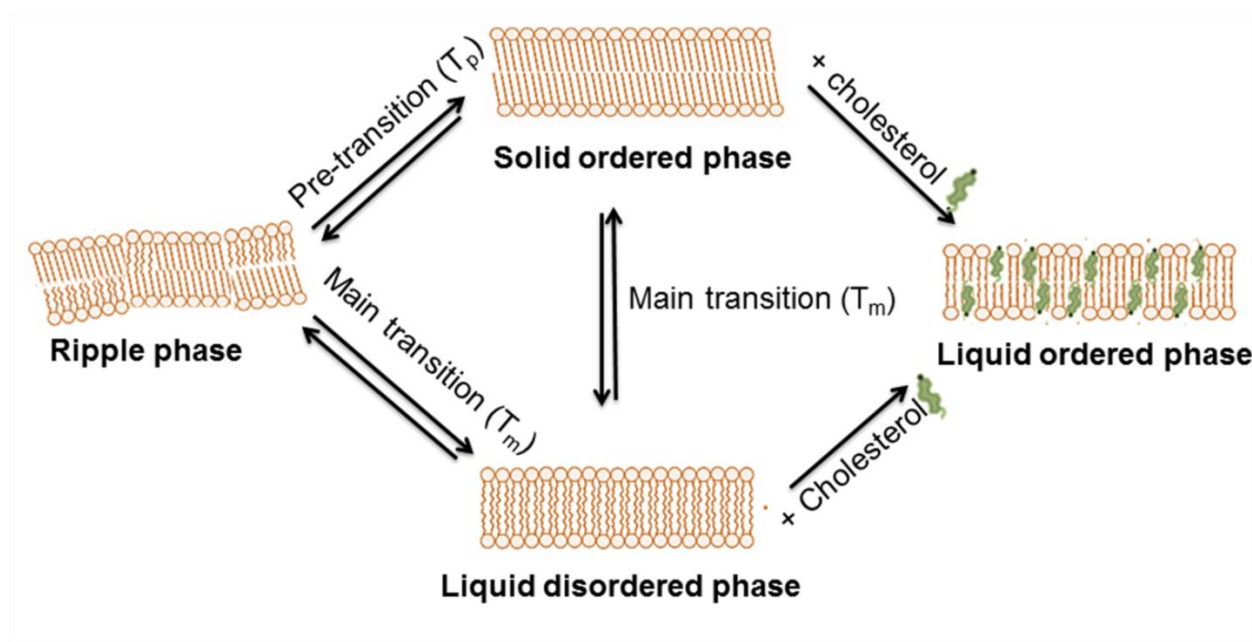
Figure 1: Schematic representation of a liposome constituted of a lipid bilayer enclosing an aqueous phase. Hydrophobic drug (red) is entrapped in the lipid bilayer. Hydrophilic drug (green) is embedded in the aqueous phase. Amphiphilic drug (orange) is located at the water-bilayer interphase.

3. Membrane fluidity

The fluidity of a membrane is one of its important properties; it strongly depends on the temperature and the membrane composition, in particular the presence of Chol and its content.

199 Depending on the temperature, a lipid bilayer can adopt distinct physical states (Figure 2) which
 200 are characterized by different lateral organization, molecular order, and mobility of lipids
 201 constituting the bilayer (Eeman and Deleu, 2010). At low temperatures, the lamellar gel phase
 202 (also called 'solid ordered' (So) phase) is formed where the hydrocarbon chains are elongated
 203 to the maximum in all-trans configuration. Upon temperature elevation, a lipid bilayer
 204 demonstrates structural changes called 'thermotropic transitions': the pre-transition in which the
 205 lipid bilayer passes from the lamellar gel phase to the rippled gel phase, and the main transition
 206 which represents the transition from the rippled gel phase to the 'liquid disordered' (Ld) phase.
 207 Ld phase is characterized by the presence of numerous gauche conformers along the acyl
 208 chains; therefore, it shows a great increase in membrane fluidity and molecular disorder
 209 compared to the rippled gel phase (Abboud et al., 2018).

210 In the presence of Chol, a lipid bilayer can acquire a new phase, called 'liquid ordered' (Lo)
 211 phase. In this case, the acyl chains have intermediate properties between those of So and Ld
 212 phases. Vist and Davis (1990) presented the dynamic of DPPC membrane at various
 213 temperatures and Chol levels. The authors showed that for intermediate membrane content of
 214 Chol (7-30 %), the So phase coexists with the Lo phase below the transition temperature.
 215 Above the transition temperature, the Lo phase coexists with the Ld phase. Beyond 30 mol %
 216 membrane Chol content and whatever the temperature, the Lo phase is reached.



217

218 Figure 2: The different physical states of a lipid bilayer in an aqueous environment. At low
 219 temperature, the solid ordered phase exists. At pre-transition temperature (T_p), a lipid bilayer

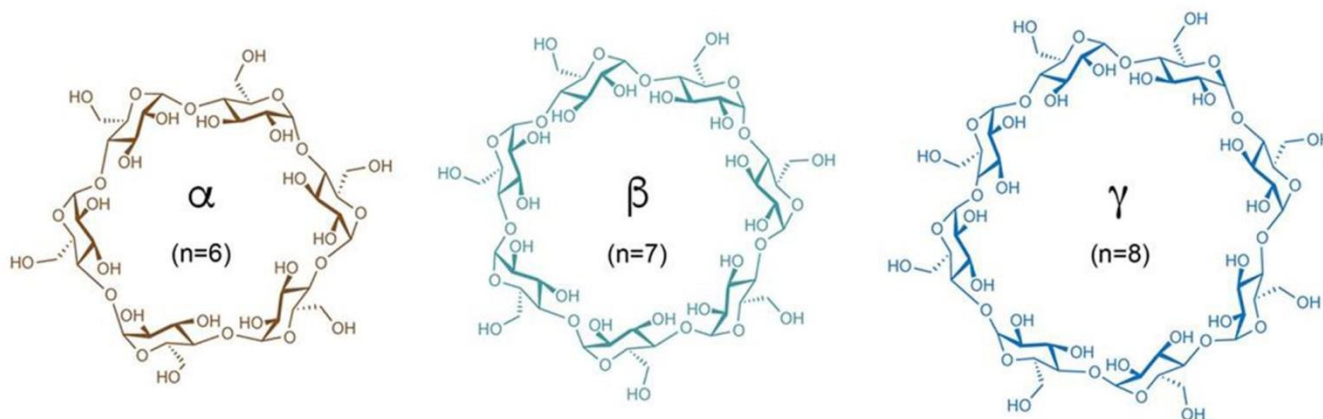
220 passes from the solid ordered phase to the rippled phase (left). At main transition temperature
221 (T_m), a lipid bilayer passes to the liquid disordered phase (down). Adding Chol (green) to a lipid
222 bilayer induces the formation of the liquid ordered phase (right).

223 Membrane fluidity is also influenced by the membrane PL composition. First, the nature of polar
224 head group affects the membrane lateral organization; membrane lipids with small polar heads
225 allow a more compact lipid assembly due to a reduced steric hindrance (Eeman and Deleu,
226 2010). Additionally, saturation status of acyl chains strongly affects the membrane lateral
227 organization; namely, saturated lipids have straight tails, thus promoting their tight packing.
228 However, the cis double bonds of unsaturated lipids prohibit their tight packing through steric
229 hindrance resulting in a more fluid membrane. In addition, membrane fluidity depends on the
230 length of the acyl chains; longer alkyl chains are easily held together via Van der Waals and
231 hydrophobic interactions in comparison to those with shorter ones (Zalba and ten Hagen,
232 2017).

233 4. Cyclodextrins

234 4.1. Structure

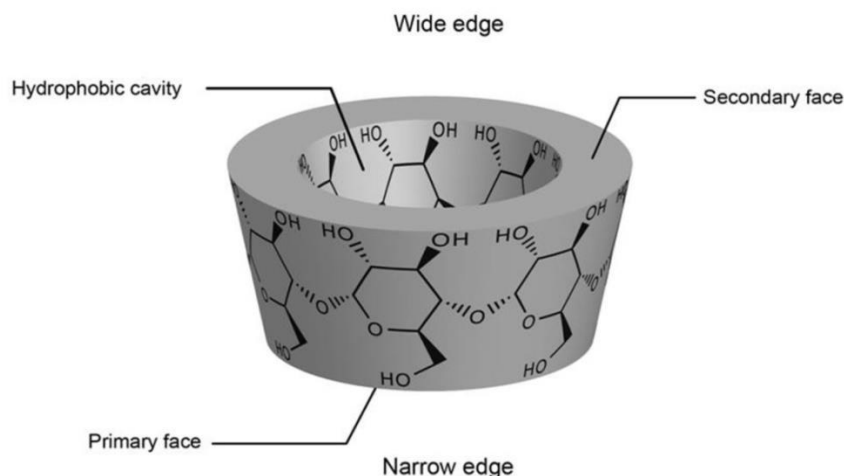
235 Cyclodextrins (CDs) are non-toxic cyclic oligosaccharides formed of α -1,4-linked D-
236 glucopyranose units. They are obtained from starch by means of enzymatic degradation. Due to
237 the 4C_1 chair conformation of glucopyranose, CDs have a bottomless bowl shape (truncated
238 cone) of various sizes according to the number of glucose units. The most common native CDs
239 are formed of 6 (α -CD), 7 (β -CD), or 8 (γ -CD) glucose subunits, with a respective cavity size of
240 approximately 0.5, 0.6, and 0.8 nm (Figure 3) (Gharib et al., 2015).



241

242 Figure 3: The chemical structure of the most common native cyclodextrins.

243 Based on X-ray studies, CDs dispose their hydroxyl functional groups to the cone exterior
244 extending the primary hydroxyl group (C6) of glucopyranose from the narrow edge of the ring
245 and the secondary hydroxyl groups (C2 and C3) from the wider edge as shown in Figure 4 (Del
246 Valle, 2004). This arrangement provides CD a hydrophilic outer surface, whereas the interior
247 cavity is hydrophobic.



248

249 Figure 4: The truncated cone-shaped structure of a cyclodextrin molecule with its hydroxyl
250 groups disposed outside.

251 4.2. Derivatives

252 Natural CDs, especially β -CD, have limited solubility in water because of their relatively strong
253 intermolecular hydrogen bonding in the crystal state. The aqueous solubility of CDs determined
254 at 25 °C is 0.1211, 0.0163, and 0.1680 mol/L for α -CD, β -CD, and γ -CD, respectively (Connors,
255 1997). Chemical modifications such as amination, etherification, methylation, and esterification
256 of the primary and secondary hydroxyl groups are applied to synthesize various hydrophobic,
257 hydrophilic, and ionic CD derivatives with an improved aqueous solubility compared to the
258 native CDs (Gharib et al., 2015).

259 In addition to the native CDs, various CD derivatives have been used to study their interaction
260 with membranes. They include the hydroxypropyl derivatives (HP- α -CD, HP- β -CD, and HP- γ -
261 CD), maltosylated derivatives (G₂- α -CD and G₂- β -CD), sulfobutylether- β -CD (SBE- β -CD),
262 carboxyethylated- γ -CD (CE- γ -CD), and the methylated derivatives (Me- α -CD, DM- α -CD, Me- β -
263 CD, dimeb, trimeb, Me- γ -CD). The latters also include the randomly methylated β -CD
264 derivatives (rameb) and the partially methylated crystallized- β -CD (crysmeb). Recently, interest

265 in multi-substituted- β -CDs such as hydroxypropyl-sulfobutyl-ether- β -cyclodextrin (HP_n-SBE_m- β -
266 CD), which is substituted by hydroxypropyl and sulfobutyl groups: n-(2,3,6-O-2-hydroxypropyl)-
267 m-(2,3,6-O-sulfobutyl)- β -CD has emerged. Two HP_n-SBE_m- β -CDs (HP₂-SBE₃- β -CD and HP₃-
268 SBE₂- β -CD) were evaluated for their effects on biological membranes (Wang et al., 2011).

269 **4.3. Cyclodextrins used as drug delivery enhancers**

270 CDs are able to entrap hydrophobic drugs in their cavities forming CD/drug inclusion
271 complexes. As a result, CDs favor drug dissolution in the aqueous phase, making them suitable
272 to diffuse in an aqueous medium, to come in contact with membrane surface, and to permeate
273 through membrane. Moreover, encapsulation in CD protects the drug from chemical and
274 enzymatic degradation (Babu and Pandit, 2004; Rong et al., 2014; Rosa Teixeira et al., 2013).

275 **5. Drug-in-cyclodextrin-in-liposome**

276 Van der Waal forces, hydrogen bonds, and hydrophobic interactions are involved in the
277 CD/drug inclusion complex formation (Gidwani and Vyas, 2015). Therefore, the inclusion
278 complex will rapidly dissociate following intravenous administration where blood components
279 may displace the encapsulated drug. As for liposomes, highly lipophilic drugs incorporated in
280 the liposomal PL bilayer would be also rapidly released after intravenous and transdermal
281 administration (Kirby and Gregoriadis, 1983; Maestrelli et al., 2005; Takino et al., 1994). To
282 ensure a stable encapsulation of hydrophobic drugs, an approach has been proposed
283 (McCormack and Gregoriadis, 1994) where the drugs are encapsulated in the aqueous phase
284 of liposomes in the form of CD/drug inclusion complexes. This approach combines the relative
285 advantages of both carriers in a single “drug-in-CD-in-liposome” (DCL) system. Indeed, the
286 entrapment of a water-soluble CD/drug inclusion complex into liposomes would allow
287 accommodation of insoluble drugs in the aqueous phase of vesicles (Figure 5) (Gharib et al.,
288 2017).

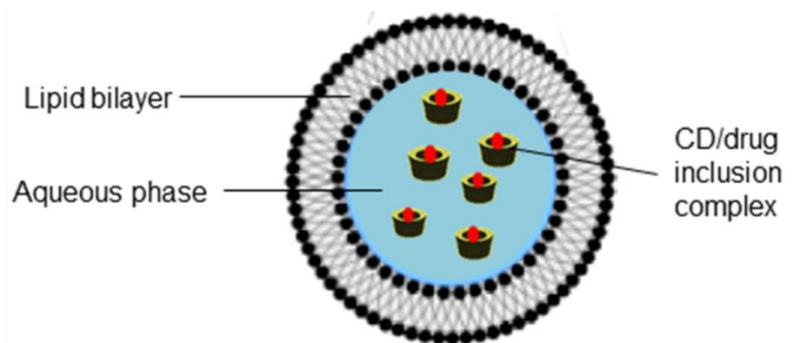


Figure 5: Schematic representation of drug-in-cyclodextrin-in-liposome system composed of lipid bilayer and an aqueous inner cavity. The drug in the form of CD/drug inclusion complex is loaded in the aqueous phase.

6. Cyclodextrin-lipid membrane interaction

The interaction of native and modified CDs with fatty acids, Chol and PLs was recently reviewed by Szente and Fenyvesi (2017). In the sections below, we will focus, in particular, on the interaction between CDs and both biomimetic and biological membranes, with respect to the composition of biomimetic membrane, the type of biological membrane, the lipid to CD molar ratio, as well as the CD type and concentration.

6.1. The effect of cyclodextrins on membrane fluidity

The effect of CDs on the fluidity and stability of liposome membranes and biological systems (stratum corneum and colon carcinoma cells) was evaluated using differential scanning calorimetry (DSC), fluorescence anisotropy, and electron spin resonance (ESR) techniques.

DSC is used in lipid membrane research to study the thermal behavior of lipid bilayers in the presence of active agents, i.e. CDs. The thermodynamic parameters such as pre-transition temperature (T_p), main transition temperature (T_m), main transition enthalpy (ΔH_m), and temperature width at half peak height ($\Delta T_{1/2}$) can be determined using DSC (Demetzos, 2008).

T_p is represented by a flat endothermic peak and its disappearance reflects drug interaction with the polar head groups of PLs. T_m is a sharp endotherm represented by the apex of the peak (Demetzos, 2008; Gharib et al., 2018a). ΔH_m is the heat required for the entire transition; it is calculated from the area under the main transition peak. The decrease in ΔH_m suggests an increase in the membrane fluidity and disorder (increase in the number of acyl chains in the gauche conformation) while its increase reflects an interaction of the drug with the upper glycerol head group region of the lipid bilayer. Furthermore, $\Delta T_{1/2}$ reflects the cooperativity of

315 the transition, being inversely proportional to it. It is very sensitive to the presence of additives
316 (Gharib et al., 2018a).

317 Most studies in the literature evaluated the effect of CDs on DPPC liposome membrane. The
318 latter, in the absence of CD, displayed a pre-transition at approximately 35 °C and a main
319 transition at around 41 °C (Gharib et al., 2018a). The interaction of CDs with liposomal
320 membranes induced alterations in the membrane calorimetric parameters. Table 1 summarizes
321 the literature data on the DSC results obtained with CD-loaded liposomes, showing the
322 liposomal membrane composition, CD type and concentration as well as lipid:CD molar ratio.
323 As can be seen from Table 1, CDs influence the membrane fluidity.

324 Table 1: The effect of cyclodextrins on the thermotropic parameters of the liposome membranes
325 determined by DSC
326

327 [CD]: Cyclodextrin concentration, Chol: cholesterol, DMPC: dimyristoyl phosphatidylcholine;
 328 DPPC: dipalmitoyl phosphatidylcholine, NI: not indicated.

Membrane composition	Lipid:CD molar ratio	CD type	[CD] (mM)	Variation in thermotropic parameters	Ref
-DPPC -DPPC:Chol (90:100)	1:0 1:3 1:7 1:16 1:32	- β -CD - HP- β -CD - Dimeb - Trimeb	NI	Effects of CDs on DPPC vesicles: - Dimeb, β -CD, and trimeb: T_m was increased with increasing lipid:CD molar ratio - Dimeb: ΔH_m was decreased while $\Delta T_{1/2}$ was increased - Trimeb: an increase in $\Delta T_{1/2}$ without affecting ΔH_m Effects of CDs on DPPC:Chol vesicles: - Trimeb and β -CD: ΔH_m was increased - Dimeb: an increase in ΔH_m up to DPPC:Chol molar ratio of 1:7; after that it was decreased - HP- β -CD: no effect on both vesicles.	Puglisi et al., 1996
DPPC	8:17 8:27 8:37 8:50	- α -CD - β -CD - HP- β -CD - Dimeb - Trimeb - γ -CD	0-50	- α -CD and dimeb: ΔH_m was decreased with increasing CD concentration while T_m was not affected - Trimeb: a slight decrease in T_m without affecting ΔH_m - β -CD, γ -CD, and HP- β -CD: T_m and ΔH_m were barely affected.	Nishijo and Mizuno, 1998
DMPC	ND	β -CD	0-167	- no effect at low CD concentration - at 167 mM, the pre-transition peak was abolished and T_m was increased	Castelli et al., 2006
DPPC	80:20	HP- β -CD	NI	- both pre-transition and main transition peaks were preserved - T_m and T_p were reduced - ΔH_m was reduced.	Liossi et al., 2017
DPPC	100:181 100:454 100:909 100:1363	HP- β -CD	29 - 221	- the pre-transition peak was abolished - T_m was increased as a function of [CD] - an increase in ΔH_m at low molar ratios (100:181 and 100:454) but it was decreased at high ratios (100:909 and 100:1363) - $\Delta T_{1/2}$ was increased.	Gharib et al., 2018a

329
 330 Indeed, β -CD (Castelli et al., 2006) and HP- β -CD (Gharib et al., 2018a) abolished the pre-
 331 transition peak values of DMPC and DPPC liposomes, respectively, suggesting an interaction
 332 of CDs with the polar head groups of PLs. Regarding the effect of CDs on T_m value, it was

333 reported that dimeb, β -CD, trimeb (Puglisi et al., 1996), and HP- β -CD (Gharib et al., 2018a)
334 increased the T_m of DPPC membranes as a function of lipid:CD molar ratio (Figure 6). In
335 addition, β -CD, at a concentration of 167 mM, increased the T_m of DMPC liposome membrane
336 (Castelli et al., 2006). Thus, CDs appear to stabilize the liposome lipid bilayer by hydrogen
337 bonding to polar lipids. In contrast, Nishijo and Mizuno (1998) showed that the T_m value of
338 DPPC membrane was reduced in the presence of trimeb, while β -CD, γ -CD, and HP- β -CD
339 barely influenced the T_m of this membrane model. The authors suggested that CDs exhibit a
340 membrane fluidizing effect and may extract PLs from the membrane. On the other side, Liossi
341 et al (2017) showed a lowering effect of HP- β -CD towards DPPC membrane. The ΔH_m of DPPC
342 membrane was significantly decreased in the presence of α -CD (Nishijo and Mizuno, 1998),
343 dimeb (Nishijo and Mizuno, 1998; Puglisi et al., 1996), or HP- β -CD (Liossi et al., 2017)
344 suggesting an increase in the membrane fluidity and disorder. Conversely, Gharib et al. (2018a)
345 demonstrated an increase in ΔH_m of DPPC liposome at low HP- β -CD molar fraction (1.81 and
346 4.54); an HP- β -CD interaction with the upper chain/glycerol/head group region of the lipid
347 bilayer was proposed. However, at higher molar fraction (9.09 and 13.63), HP- β -CD exerted a
348 lowering effect on the ΔH_m ; HP- β -CD was suggested to interact with the hydrophobic core of
349 the lipid bilayer leading to perturbation of DPPC packing order. Furthermore, adding Chol
350 appears to modulate the effect of CDs on DPPC membranes. Thus, β -CD and trimeb increased
351 the ΔH_m of DPPC:Chol (90:10) membrane at all the studied lipid to CD molar ratios. Whereas
352 the effect of dimeb differed depending on its molar fraction; the ΔH_m increased to its maximal
353 value (4.94 ± 0.18 kcal/mol) at lipid:CD molar ratio of 1:7; however, a further increase in its
354 molar fraction (1:32) led to a decrease in ΔH_m (2.88 ± 0.31 kcal/mol). According to the authors,
355 dimeb is able to extract both DPPC and Chol at higher CD molar fraction (Puglisi et al., 1996).

356 Few studies have determined the effect of CD on the cooperativity of transition. The presence
357 of dimeb, trimeb (Puglisi et al., 1996), or HP- β -CD (Gharib et al., 2018a) induced an increase in
358 the $\Delta T_{1/2}$ of DPPC vesicles. This could be explained by the interaction between CD and the
359 hydrophobic region of DPPC bilayer which causes membrane disruption (Gharib et al., 2018a).

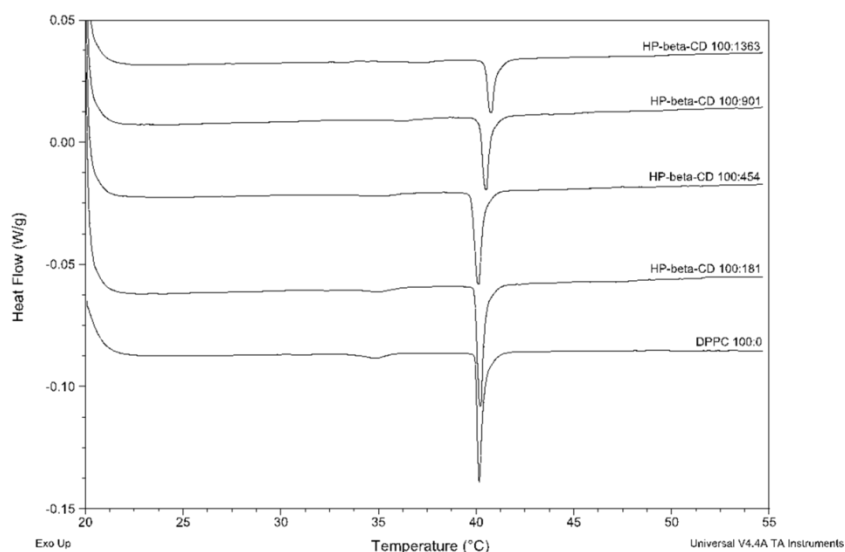


Figure 6: DSC scans of blank and HP- β -CD-loaded DPPC liposomes prepared at DPPC:HP- β -CD molar ratios of 100:181; 100:454; 100:909 and 100:1363 (Gharib et al., 2018a)

Angelini et al. (2017) determined the ratio of pyrene fluorescence intensities in excimer and monomer state for palmityl-oleoyl-phosphatidylcholine (POPC) and β -CD-loaded-POPC liposomes. The results showed that β -CD increased the membrane fluidity in comparison with the control.

The anisotropy value is known to be inversely proportional to the membrane fluidity. To our knowledge, the study of Gharib et al. (2018a) is the only one using fluorescence anisotropy to evaluate the CD effect on the membrane fluidity. The authors used 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe since membranes do not exhibit a natural intrinsic fluorescence. Due to its low aqueous solubility, DPH inserts in the bilayer core; the depolarization property of DPH depends on the packing of the acyl chains. Thus, the fluorescence anisotropy of DPH in liposomes gives information about the organization of the membrane environment around the fluorescent probe (Gharib et al., 2018a). The authors determined the DPH anisotropy values of blank and HP- β -CD-loaded DPPC liposomes prepared at different DPPC:HP- β -CD molar ratios (100:181, 100:454, 100:909, and 100:1363) at 28, 41, and 50 °C. The results showed that HP- β -CD reduced the DPH anisotropy of DPPC membrane at all the studied temperatures in a concentration-dependent manner (Figure 7), suggesting an increase in the membrane fluidity of DPPC liposomes in the presence of HP- β -CD (Gharib et al., 2018a).

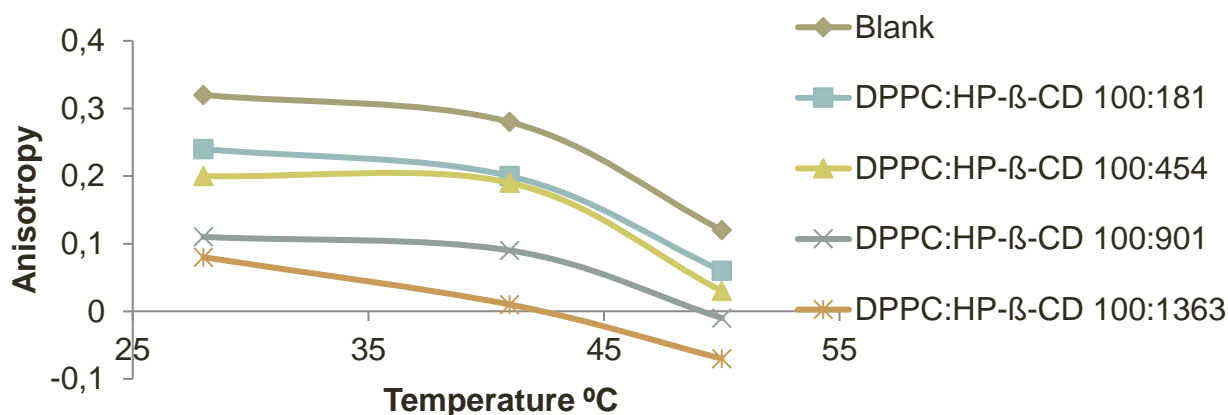


Figure 7: DPH anisotropy values for blank and HP-β-CD-loaded DPPC liposomes prepared at different DPPC:HP-β-CD molar ratios at 28, 41, and 50 °C (Gharib et al., 2018a).

Moreover, Gharib et al. (2018a) determined the anisotropy values of DPH inserted in liposomes composed of saturated PLs and Chol or unsaturated PLs and Chol; in addition, different PL:Chol:HP-β-CD molar ratios were used in this study. HP-β-CD was found to increase the membrane fluidity of liposome membranes composed of unsaturated PLs, while no effect was exerted on those composed of saturated lipids. Thus, the packing state of PLs can modulate the CD effect on membrane model systems.

ESR spectroscopy is another technique used to provide information about the structure and dynamic of biological membranes. The fatty acid spin-label agents, 5-doxyl stearate (5-DSA) and 16-doxyl stearate (16-DSA), are generally used as paramagnetic probes. 5-DSA is located at the lipid-aqueous interface of the membrane while 16-DSA is inserted into its hydrophobic core. The nitroxide group of the spin probes moves around the point of attachment. Hence, the ESR spectra allow the identification of changes in the probe rotational mobility in biological membranes and can be further correlated with membrane fluidity (Abboud et al., 2018; Grammenos et al., 2010).

ESR was applied to examine the effect of rameb (0-10 mM) on the microviscosity of human colon carcinoma cell membrane. In the absence of rameb, the microviscosity was found to be 298 cP. This value decreased with increasing CD concentration and stabilized at 265 cP for a rameb concentration of 2.5 mM. Then, the values remained constant until 10 mM (Grammenos et al., 2010).

Finally, it is worthy to note that the membrane Chol content was not considered in the above mentioned studies. Nevertheless, it is well known that Chol has a key role in maintaining the membrane fluidity; its content can modulate CD-induced membrane fluidity changes.

6.2. Extraction of lipid membrane components from biomimetic and biological membranes

Several studies evaluated the extent of lipid extraction mediated by CDs. Following the incubation of membrane with CD, the suspension is subjected to centrifugation, and the supernatant is collected to determine the amount of extracted PLs and Chol in the suspension. In general, CDs enable rapid extraction of membrane lipids. Alpha-CDs were found to extract mainly PLs; β -CDs, in particular methylated β -CDs, extract preferably Chol; γ -CDs are less lipid selective compared to the other CDs (Figure 8).

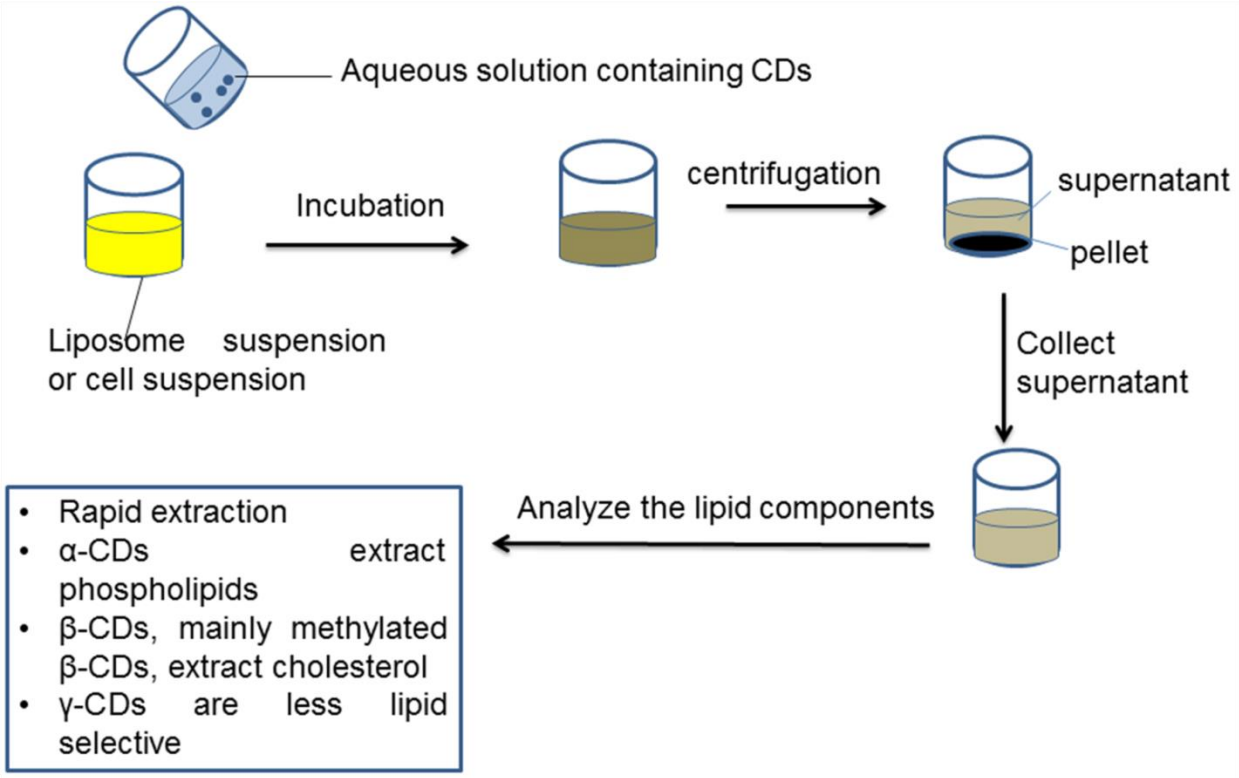


Figure 8: A scheme presenting a general technique for evaluating the extent of cyclodextrin-mediated lipid extraction

The fluorescent analogs of PLs and Chol have been widely used to investigate the structural and dynamic properties of membranes. The interaction of CDs with fluorescent-labeled Chol (Milles et al., 2013) and PLs (Denz et al., 2016) bearing 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)

or dipyrromethene boron difluoride (BODIPY) moieties induced a modification in Förster resonance energy transfer (FRET) signals. The latter occurred between a rhodamine moiety linked to phosphatidylethanolamine (Rh-PE) and the NBD or BODIPY moieties linked to liposomal PLs. Excitation of the BODIPY or NBD moieties induced a large FRET signal; large rhodamine fluorescence was obtained when the fluorophores came close together within the membrane. Consequently, the CD-mediated extraction of the fluorescent analogs reduced their concentration in the membrane, resulting in a decrease of FRET signals (Denz et al., 2016).

Table 2 resumes the literature data regarding CD-mediated extraction of lipid components from biomimetic and biological membranes; the studies on biomimetic membranes are presented first (liposomes then monolayers) followed by those on biological membranes. Furthermore, Table 2 shows the lipid composition of liposomes and monolayers, the cell type from which membranes are extracted as well as the type and concentration of CD used in each study.

Table 2: Extraction of lipid membrane components by cyclodextrins

Membrane	CDs type	[CD] (mM)	Outcomes	Ref
Liposomes				
-POPC:DHE (70:30) -SM:DHE (70:30) -POPC:cholestatrienol (70:30) -SM:cholestatrienol (70:30)	HP- β -CD	0-4	<ul style="list-style-type: none"> - CD-mediated sterol extraction in a concentration dependent manner - Slower sterol extraction rate from SM vesicles compared to POPC vesicles - Better extraction of cholestatrienol compared to DHE. 	Ohvo-Rekilä et al., 2000
-DOPC -DOPC:Chol (70:30) -DOPC:Chol (55:45) -DOPC:SM:Chol (53:17:30) -DOPC:SM:Chol (40:40:20) -DOPC:SM:Chol (30:30:40)	Me- β -CD	0.002-8	<ul style="list-style-type: none"> - No DOPC extraction at all CD concentrations used - Fast Chol extraction rate - Reduced Chol extraction rate in the presence of SM. 	Besenica et al., 2008
-DOPC:Rh-PE:NBD-Chol (99:0.5:0.5) -DOPC:Rh-PE:BODIPY-Chol (99:0.5:0.5)	<ul style="list-style-type: none"> - α-CD - β-CD - HP-β-CD - Me-β-CD - γ-CD 	0-10	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - Rapid exponential kinetics of Chol efflux with release rate constants ranging from 0.2 to 0.6 s⁻¹ - Greater Chol extraction by Me-β-CD compared to other CDs. Slight extraction of NBD-Chol by HP-β-CD, 	Milles et al., 2013

			β -CD, and γ -CD; no effect was exerted on BODIPY-Chol.	
-POPC:Rh-PE:C6/C12 NBD-PC (99:0.5:0.5) -POPC:Rh-PE:C6/C12 NBD-PS (99:0.5:0.5) -POPC:Rh-PE:C6/C12 NBD-PE (99:0.5:0.5) -POPC:Rh-PE:C6/C12 NBD-SM (99:0.5:0.5)	- Me- α -CD - Me- β -CD - Me- γ -CD	0-10	- CD-mediated PL extraction in a concentration dependent manner - Bi-exponential kinetics of PL efflux - High extraction of PL by Me- α -CD and Me- β -CD whereas Me- γ -CD was less effective - Better extraction of short chain PLs (C6) compared to the long ones (C12) - Better extraction of NBD-PC and NBD-SM compared to NBD-PS and NBD-PE.	Denz et al., 2016
Monolayers				
-Chol -DPPC -DDPC -SM -DPPC:Chol (0.5:1 to 9:1) -DDPC:Chol (0.5:1 to 9:1) -SM:Chol (0.5:1 to 9:1)	β -CD	0-1.6	- CD-mediated Chol extraction in a concentration dependent manner - Insignificant PL extraction compared to Chol extraction - β -CD-induced disappearance of Chol rich domains in DDPC:chol membranes - Reduced Chol extraction in the presence of PL and SM; SM effect > PL effect.	Ohvo and Slotte, 1996
-DHE -Cholestatrienol -Chol -POPC:DHE (70:30) -POPC:Cholestatrienol (70:30) -POPC:Chol (70:30)	HP- β -CD	1.6: pure layers 16: mixed layers	- Lipid extraction from pure sterol monolayers in the order: cholestatrienol > dehydroergosterol > Chol - Slower extraction rate from mixed monolayers compared to the pure ones.	Ohvo-Rekilä et al., 2000
-DPPC -DMPC -POPC -DMPG -SM	β -CD	0.7	- Lipid extraction in the order: SM > POPC > DPPC - No extraction of DMPG was obtained.	Grauby - Heywang and Turlet, 2008
Biological membranes				
Human erythrocytes	- α -CD - β -CD - γ -CD	0-40	CD-mediated membrane Chol extraction in the order: β -CD > γ -CD > α -CD.	Irie et al., 1982
Human erythrocytes	- α -CD - β -CD - γ -CD	0-40	- CD-mediated PLs extraction in the order: α -CD > β -CD >> γ -CD - CD-mediated extraction of Chol in the order: β -CD >> γ -CD; no effect of α -CD.	Ohtani et al., 1989

Rabbit erythrocytes	<ul style="list-style-type: none"> - α-CD - HP-α-CD - DM-α-CD 	3	<ul style="list-style-type: none"> - PLs were extracted by DM-α-CD and α-CD but no effect of HP-α-CD - No Chol extraction by all CDs used. 	Motoyama et al., 2006
Rabbit erythrocytes	<ul style="list-style-type: none"> - β-CD - Me-β-CD - Dimeb 	β -CD: 3 Me- β -CD: 1 Dimeb: 0.8	<ul style="list-style-type: none"> - Only dimeb induced PL and SM release - CD-mediated Chol extraction in the order: dimeb = Me-β-CD > β-CD. 	Motoyama et al., 2009b
-Mouse L-cell fibroblast -Human fibroblast -Rat hepatoma cells	<ul style="list-style-type: none"> - B-CD - HP-β-CD - Me-β-CD 	0-10	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - CD-mediated Chol extraction in the order: Me-β-CD > β-CD > HP-β-CD - HP-β-CD-induced a rapid Chol extraction (up to about 2 hours) - Me-β-CD and HP-β-CD induced cellular release of desmosterol as a function of CD concentration; Me-β-CD effect > HP-β-CD effect - No significant PLs release was obtained - Extent of Chol extraction was the same for all the cell types. 	Kilsdonk et al., 1995
-Mouse L-cell fibroblast -Human skin fibroblast -Rat hepatoma cells	HP- β -CD	0-200	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner till reaching saturation at high CD concentration (50 mM for rat hepatoma cells and 75 mM for L-cell fibroblasts) - Bi-exponential kinetics of Chol efflux. The range of half-times for the fast pool: 19–23 s, and that for the slow pool: 15–35 min - The Chol extraction rate from cells in the order: rat hepatoma cells > mouse L-cell fibroblasts > human skin fibroblasts. 	Yancey et al., 1996
Human skin fibroblasts	HP- β -CD	5-15	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - Chol extraction was stimulated by SM degradation (using sphingomyelinase), while PC degradation (using PC-PLC) had no effect on Chol extraction. 	Ohvo et al., 1997
Human sperm	Me- β -CD	0-10	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner. 	Cross, 1999
Goat sperm	β -CD	0-16	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner 	Iborra et al.,

			<ul style="list-style-type: none"> - Rapid exponential kinetics of Chol efflux with half time of about 10 min - No PL extraction was obtained. 	2000
T lymphocytes Jurkat cell lines	Me- β -CD	0.5-15	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - Rapid Chol efflux at a single rate; a plateau was reached after 15 min. 	Maham mad and Parmry d, 2008
Rod outer segment	Me- β -CD	0-40	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - Significant PL extraction at CD concentration above 15 mM. 	Niu et al., 2002
Rod outer segment	Me- β -CD	15	<ul style="list-style-type: none"> - CD-mediated membrane Chol extraction - No effect of CD on the PL membrane content. 	Elliott et al., 2003
Blood brain barrier model	<ul style="list-style-type: none"> - α-CD - β-CD - γ-CD 	γ -CD: 0-50 other: 0-5	<ul style="list-style-type: none"> - Selective extraction of PC by α-CD compared to other CDs - Selective Chol extraction by β-CD in a concentration dependent manner - α-CD and β-CD mediated extraction of SM - γ-CD was less lipid selective. 	Monna ert, 2004
Human umbilical vein endothelial cells	<ul style="list-style-type: none"> - B-CD - HP-β-CD - Me-β-CD - Dimeb - Trimeb - Rameb - Crysmeb 	0-10	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - CD extraction ability was as follows: trimeb < HP-β-CD < β-CD = crysmeb < dimeb = Me-β-CD < rameb. 	Castag ne et al., 2009
Human embryonic kidney-derived HEK293A cells	<ul style="list-style-type: none"> - HP₂-SBE₃-β-CD - HP₃-SBE₂-β-CD - SBE-β-CD - Me-β-CD - Dimeb 	0-20	<ul style="list-style-type: none"> - CD-mediated Chol extraction in a concentration dependent manner - For the same CD concentration, the effect of CDs on Chol extraction was in the order: HP₂-SBE₃-β-CD < HP₃-SBE₂-β-CD < SBE-β-CD < Me-β-CD < dimeb. 	Wang et al., 2011
Caco-2 cells	<ul style="list-style-type: none"> - α-CD - β-CD - γ-CD - G₂-α-CD - G₂-β-CD 	β -CDs: 0-15 other CDs: 0-150	<ul style="list-style-type: none"> - No effect of β-CD and G₂-β-CD on PLs extraction at low CD concentrations (< 15 mM) - The majority of membrane PLs were extracted by α-CD - Moderate PLs extraction was obtained at CD 37.5 mM for G₂-α-CD and G₂-β-CD 	Ono et al., 2001

			- No effect of γ -CD.	
<ul style="list-style-type: none"> - Human embryonic kidney derived HEK293T cells - Human cervical cancer-derived HeLa cells - T lymphocyte Jurkat cell lines 	<ul style="list-style-type: none"> - HP-β-CD - HP-γ-CD - SBE-β-CD - Dimeb - Rameb 	50	<ul style="list-style-type: none"> - The Chol extraction by CDs was in the order: Dimeb > Rameb >> HP-β-CD > SBE-β-CD - No effect of HP-γ-CD. 	Szente et al., 2018

434 BODIPY: dipyrromethene boron difluoride; Chol: cholesterol; DDPC: didecanoyl-
435 phosphatidylcholine; DHE: dehydroergosterol; Dimeb: dimethylated- β -cyclodextrin; DM- α -CD:
436 dimethyl- α -cyclodextrin; DMPC: dimyristoyl-phosphatidylcholine; DMPG: dimyristoyl-
437 phosphatidylglycerol; DOPC: dioleoyl-phosphatidylcholine; DPPC: dipalmitoyl-
438 phosphatidylcholine; NBD: 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PC: phosphatidylcholine; PE:
439 phosphatidylethanolamine; PL: phospholipid; PLC: phospholipase C; POPC: palmitoyl-oleoyl-
440 phosphatidylcholine; PS: phosphatidylserine; Rh: Rhodamine; SM: sphingomyelin; Trimeb:
441 trimethylated- β -cyclodextrin

442

443 6.2.1. Kinetics of cyclodextrin-induced lipid extraction from membranes

444 Several studies examined the ability of CDs to induce lipid extraction from liposomal and
445 biological membranes; the extraction kinetics was determined by monitoring the extraction as
446 a function of time; the release rate constants or half times were evaluated.

447 Generally, Chol release from liposomes and biological membranes displayed a rapid
448 exponential kinetics (Iborra et al., 2000; Milles et al., 2013). However, Yancey et al. (1996)
449 reported that HP- β -CD-mediated Chol release from mouse L-cell fibroblasts, human
450 fibroblasts, and rat hepatoma cells followed a bi-exponential kinetics, revealing a fast pool
451 with half-times of 19–23 s and a slow pool with half-times ranging from 15 to 35 min.
452 Moreover, Kilsdonk et al. (1995) demonstrated that HP- β -CD-induced a rapid Chol release
453 from mouse L-cell fibroblasts over an initial period (up to about 2 h) until the equilibrium
454 between Chol in the outer medium and in the membrane compartment was reached. Also, the
455 kinetics of the PL extraction from liposomes by Me- α -CD and Me- β -CD was fitted to a bi-
456 exponential equation (Denz et al., 2016).

457

458 6.2.2. The factors affecting lipid extraction from membranes

The data presented in Table 2 show that CDs have a potential to extract lipid components from biomimetic and biological membranes; the extent of extraction depends on CD type and concentration, PL structure, overall lipid membrane composition, and cell type.

6.2.2.1. Cyclodextrin type and concentration

The interaction of methylated CDs such as Me- α -CD, Me- β -CD, and Me- γ -CD, with liposome membrane containing NBD-labeled PLs was examined by detecting FRET between the NBD and Rh-PE as described earlier (Denz et al., 2016). Me- α -CD and Me- β -CD were similarly efficient in inducing a high efflux of fluorescent-labeled PLs embedded in the membrane, whereas Me- γ -CD produced no effect. Also, the interaction of different CDs (α -CD, β -CD, HP- β -CD, Me- β -CD, and γ -CD) with NBD and BODIPY-labeled Chol was characterized (Milles et al., 2013). The results demonstrated that Me- β -CD induced the greatest Chol extraction while α -CD had no effect.

Moreover, many studies analyzed the effect of CDs on the erythrocyte membrane. Beta-CD induced greater Chol extraction from human erythrocytes relative to other native CDs (Irie et al., 1982; Ohtani et al., 1989). On the other hand, α -CD was more potent in inducing PL extraction (Ohtani et al., 1989). The influence of diverse CD derivatives on rabbit erythrocyte membrane was studied; α -CD and DM- α -CD were shown to extract PLs while HP- α -CD had no effect (Motoyama et al., 2006). In addition, methylated β -CD derivatives caused a greater Chol efflux from the erythrocyte membrane, as compared to β -CD (Motoyama et al., 2009b).

The presence of β -CD and its derivatives, HP- β -CD and Me- β -CD, did not modify the PL content of mouse L-cell fibroblast membrane but reduced the Chol content in the order of Me- β -CD > β -CD > HP- β -CD (Kilsdonk et al., 1995). Moreover, the impact of CD type on the lipid release was demonstrated using a blood brain barrier model. Indeed, α -CD preferentially promoted PL extraction, β -CD selectively extracted Chol, while both CD types induced SM release (Monnaert, 2004). Using various β -CD derivatives, it was shown that CDs enhanced Chol efflux from human umbilical vein endothelial cells, with rameb inducing the greatest effect (Castagne et al., 2009). In addition, β -CD derivatives (HP₂-SBE₃- β -CD, HP₃-SBE₂- β -CD, SBE- β -CD, Me- β -CD, and dimeb) caused Chol extraction from human embryonic kidney cells with dimeb exerting the strongest effect (Wang et al., 2011). The impact of the three native CDs as well as G₂- α -CD and G₂- β -CD on Caco-2 cell membrane was also investigated (Ono et al., 2001); the authors demonstrated that β -CD and G₂- β -CD did not affect the PL content while α -

CD extracted most of the PLs from cell membrane, and γ -CD produced no effect. According to Szente et al. (2018), the cavity size and the substitution groups of CDs influenced their ability to extract Chol from biological membranes and to evoke cell damage. Methylated CDs (dimeb and rameb) were more potent in solubilizing Chol compared to HP- β -CD and SBE- β -CD, whereas HP- γ -CD was not found to extract Chol.

CD concentration can also influence the CD-mediated lipid extraction. All studies on this subject, both on biomimetic and biological membranes, highlight the importance of CD concentration. Indeed, increasing the CD concentration increases the extent of lipid release from membranes (Denz et al., 2016; Milles et al., 2013; Ohvo and Slotte, 1996; Ohvo-Rekilä et al., 2000; Yancey et al., 1996).

6.2.2.2. Phospholipid type

The effect of PL acyl chain length and saturation as well as PL head group type on the strength of CD-mediated PL extraction have been discussed in the literature (Denz et al., 2016; Grauby-Heywang and Turlet, 2008). Short chain lipids are better extracted than long ones from liposome membrane when various methylated CDs were applied. In addition, PC was more effectively removed from liposome membrane in comparison with PE and PS (Denz et al., 2016). Moreover, β -CD was capable to release PC from monolayers without any effect on phosphatidylglycerol (PG) monolayers (Grauby-Heywang and Turlet, 2008).

Concerning the saturation status of acyl chains, the presence of an unsaturated acyl chain was reported to favor the β -CD-induced PLs desorption from monolayers (Grauby-Heywang and Turlet, 2008); a double bond creates a kink in the carbon chain rendering the structure less tightly packed. In addition, the lipid backbone influences its extraction; thus, SM (sphingosine backbone) was easier extracted from biomimetic membranes than PLs (glycerol backbone) (Grauby-Heywang and Turlet, 2008). This difference can be explained by the fact that SM can act as both H-bond donor and acceptor while PC is only a H-bond acceptor; H-bonds between lipids and CDs stabilize lipid-CD complex, thereby favoring lipid extraction (Boggs, 1987).

6.2.2.3. Membrane lipid composition

CD-mediated Chol desorption from pure Chol monolayer was studied and compared to those composed of Chol mixed with PLs or SM. The results showed that CDs induced minimal efflux of Chol from mixed monolayers in comparison with pure Chol monolayers (Ohvo and Slotte,

1996; Ohvo-Rekilä et al., 2000), and SM exerted a greater effect in lowering Chol desorption rate compared to PLs (Ohvo and Slotte, 1996). The rate of HP- β -CD-mediated sterol extraction was higher from POPC:sterol vesicles than that from SM:sterol vesicles (Ohvo-Rekilä et al., 2000). Besenicar et al. (2008) reported that the addition of SM to dioleoyl-phosphatidylcholine:Chol (DOPC:Chol) vesicles slowed the Me- β -CD-mediated Chol extraction. These findings are corroborated by the study of Ohvo et al. (1997). The authors stated that reducing SM content in human skin fibroblasts by about 76 %, using sphingomyelinase, stimulated the HP- β -CD-induced Chol removal from the plasma membrane (23 % compared to 13 % from untreated cells), while reducing PC content by about 12 % using PC-phospholipase C, had no effect on membrane Chol level. Hence, Chol interaction with other membrane components may retard its CD-induced extraction.

6.2.2.4. Cell type

Two studies compared the effect of CDs on the Chol extraction rate from three different cell types: mouse L-cell fibroblast, human skin fibroblast, and rat hepatoma cells (Kilsdonk et al., 1995; Yancey et al., 1996). Kilsdonk et al. (1995) used β -CD, HP- β -CD, and Me- β -CD (in the range of 0–10 mM), and Yancey et al. (1996) used HP- β -CD (0–200 mM). According to the first study, the rate of Chol release did not differ among the cell types. In contrast, Yancey et al. (1996) found that Chol release rate varied between the cells as follows: rat hepatoma cells > mouse L-cell fibroblasts > human skin fibroblasts. This difference might be due to the disparate CD concentrations tested; at high concentrations such as 200 mM, HP- β -CD might have various Chol efflux capacities towards different cell types.

6.2.3. The mechanisms of cyclodextrin-mediated lipid extraction

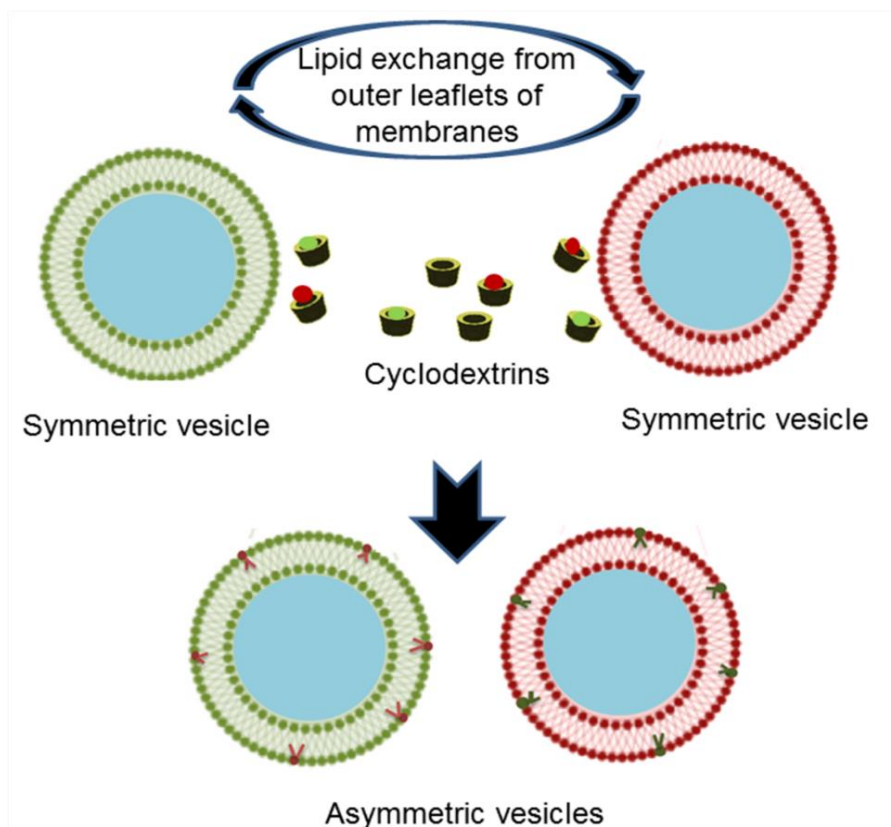
The mechanisms of Chol and PL extraction by CDs were discussed in several studies. Thus, Fauvelle et al. (1997) evaluated the effect of α -CD-induced PI extraction using ^1H -NMR, ^2H -NMR, and ^{31}P -NMR spectroscopy techniques (1997). Fauvelle and coworkers showed that α -CD was attracted to membrane surface by electrostatic interaction between the positively charged primary hydroxyl side of α -CD and the negatively charged PI at the bilayer surface. This promoted the PI molecule extraction; in addition, another α -CD molecule was observed to include the PI unsaturated sn-2 acyl chain.

549 Lopez et al. (2011) applied molecular dynamic (MD) simulations to study the mechanism of
550 Chol extraction by β -CD. It is known that β -CDs in aqueous solution are associated to form
551 dimers which are bound to the membrane surface assuming either a tilted or untilted
552 configuration. Only the untilted configuration was appropriate to extract Chol allowing the
553 formation of a membrane-bound CD/Chol complex. Although Chol is inserted quite deeply
554 within the channel, the dimethyl end of its hydrophobic tail remains in contact with the
555 monolayer surface until the CD/Chol complex tilts by 90°. Finally, the complex is desorbed
556 where each dimer extracts one Chol molecule from membrane. It is worthy to mention that the
557 CD monomer is able to interact with membrane but the strength of this interaction is insufficient
558 to extract Chol. Also, Yancey et al. (1996) proposed that the CD-mediated Chol extraction
559 occurs by its desorption from the surface directly into CD hydrophobic core (without passage
560 through the aqueous phase). In addition, Steck et al. (2002) suggested that Chol efflux induced
561 by CDs takes place through an activation-collision mechanism where the reversible partial
562 projection of Chol molecules out of the erythrocyte lipid bilayer precedes their collisional capture
563 by CD.

564 Furthermore, Sanchez et al. (2011) used Laurdan generalized polarization to investigate the
565 specificity of Me- β -CD (0.25, 1, and 2 mM) in removing Chol from the coexisting macro-
566 domains (liquid-ordered and liquid-disordered domains) of GUV membranes composed of
567 DOPC:DMPC:Chol (1:1:1). The authors proved that Me- β -CD selectively remove Chol from the
568 liquid disordered phase of the liposome bilayer.

569 **6.2.4. Cyclodextrin-mediated lipid exchange between membranes**

570 Based on the capacity of natural CDs and their derivatives to extract membrane lipid
571 components, they had been widely used to catalyze the lipid exchange between different
572 membrane vesicles or between a lipid vesicle membrane and a biological cell membrane. CD-
573 mediated lipid exchange has been mainly performed to build up lipid vesicles with lipid
574 asymmetry (Figure 9); indeed, in natural biological membranes, the lipid composition of the
575 inner and the outer leaflets is different (Huang and London, 2013). CDs promote the lipid
576 molecule efflux from a donor membrane surface partially or fully forming a soluble complex with
577 this lipid in aqueous medium and carrying it to an acceptor membrane (Tanhuanpää and
578 Somerharju, 1999).



579

580 Figure 9: A scheme for cyclodextrin-mediated formation of asymmetric vesicles: cyclodextrins
 581 can promote lipid efflux (red and green) from symmetric donor membranes, form soluble
 582 complexes in aqueous phase, and carry the lipids to acceptor membranes.

583 Various studies were carried out to investigate the transfer of PLs and Chol between donor and
 584 acceptor membranes. Donor vesicles, containing unlabeled lipids as well as labeled ones
 585 bearing a fluorescent moiety (NBD, BODIPY, pyrene, or other), a heavy isotope, or a
 586 radioactive molecule, were incubated with CDs; the unlabeled acceptor membranes are
 587 generally cells or lipid vesicles. After incubation, the acceptor membranes were separated from
 588 the donor membranes, subjected to lipid extraction, and analyzed. Besides, Huang and London
 589 (2013) studied CD-mediated lipid exchange assessing FRET between NBD-labeled lipids and
 590 rhodamine-labeled lipids embedded in donor vesicles. FRET was also used to monitor the DHE
 591 transfer from CD/DHE complex to lipid vesicles containing dansyl-PE (McCauliff et al., 2011).

592 Table 3 summarizes the literature regarding the CD-induced lipid exchange between
 593 membranes. The membrane lipid compositions of donor and acceptor vesicles, the type and
 594 concentration of CD used in each study are presented as well.

Donor membrane	Acceptor membrane	CD type	[CD] (mM)	Outcomes	Ref
Liposomes of POPC, TNP-PE, and pyrene-labelled PL with various head groups and acyl chain length	-POPC: POPA (500:20) liposomes -Human fibroblasts -Baby hamster kidney cells	CE- γ -CD	0-30	<ul style="list-style-type: none"> - Rate of lipid transfer was proportional to the CD concentration - Increasing acyl chain length (6-14 C) decreased the PL transport rate - Transport of PI was enhanced compared to the other PLs - Rate of SM transport was 3 folds greater than that of PC. 	Tanhuanpää and Somerharju, 1999
Negatively charged liposomes SOPC:SOPG (85:15) containing ^3H -Chol and ^3H -DPPC	Uncharged SOPC vesicles	<ul style="list-style-type: none"> - α-CD - β-CD - γ-CD - Me-β-CD 	0-1	<ul style="list-style-type: none"> - CDs, except α-CD, induced Chol transfer. Chol transfer rate was 53, 64, 63 folds higher when treated with 1 mM Me-β-CD, γ-CD or β-CD, respectively, compared to untreated samples - Rate of Chol transfer was proportional to the CD concentration - Me-β-CD and α-CD induced DPPC transfer rather than Chol transfer - β-CD and γ-CD induced Chol transfer rather than DPPC transfer. 	Leventis and Silvius, 2001
POPC:Chol: heavy isotopes labelled PL of various head group and acyl chain length liposomes	Baby hamster kidney cell	Me- β -CD	0-10	<ul style="list-style-type: none"> - CD induced PL transfer - Rate of PL transfer was proportional to CD concentration - Increasing acyl chain length decreased the PL transfer rate but the nature of the head group of PL had no effect. 	Kainu et al., 2010
EPC:DHE (75:25) liposomes	EPC:dansyl-PE (97:3) liposomes	HP- β -CD		<ul style="list-style-type: none"> - CD-induced the DHE transfer - The rate of DHE transfer was proportional to the CD concentration - The DHE transfer occurred by a collisional transfer mechanism. 	McCauliff et al., 2011

Liposomes with NBD labelled lipids	Liposomes with unlabelled lipids	<ul style="list-style-type: none"> - Me-β-CD - HP-β-CD - HP-α-CD 	0-150	<ul style="list-style-type: none"> - Lipid exchange was observed at Me-β-CD concentration < 10 mM - CD induced vesicle solubilisation at a concentration > 50 mM - Me-β-CD induced lipid exchange more than the other CDs. 	Huang and London, 2013
Liposomes containing NBD or ^3H labelled PLs with various head group and length of acyl chain	A549 lung carcinoma cells	Me- α -CD	0-40	<ul style="list-style-type: none"> - Various % of exchange was found for different lipids: ~75 % for SM, 10-15% for PC and PE; there was no exchange of PS and PI - Increasing the length of acyl chain decreased the SM transport rate. 	Li et al., 2016

596 Chol: cholesterol; DHE: dehydroergosterol; DPPC: dipalmitoyl-phosphatidylcholine; EPC: egg
597 phosphatidylcholine; NBD: 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PC: phosphatidylcholine; PE:
598 phosphatidylethanolamine; PI: phosphatidylinositol; PL: phospholipid; POPA: palmityl-oleoyl-
599 phosphatidic acid; POPC: palmitoyl-oleoyl-phosphatidylcholine; PS: phosphatidylserine; Rh:
600 rhodamine; SM: sphingomyelin; SOPC: 1-stearoyl-2-oleoyl-phosphatidylcholine; SOPG: 1-
601 stearoyl-2-oleoyl-phosphatidylglycerol; TNP-PE: N-trinitrophenyl-PE
602

603 As it is obvious from Table 3, CDs are able to mediate efficient transfer of PLs and Chol
604 between lipid vesicles or between a lipid vesicle and a cell; the transfer is controlled by several
605 parameters. First, the level of CD-mediated lipid exchange between vesicles is directly
606 proportional to the CD concentration (Huang and London, 2013; Kainu et al., 2010; Leventis
607 and Silviu, 2001; McCauliff et al., 2011; Tanhuanpää and Somerharju, 1999). However, at high
608 CD concentrations, it may induce membrane damage and vesicle solubilization (Huang and
609 London, 2013). Second, the level of lipid exchange between membranes is affected by the CD
610 type. Me- β -CD is more efficient towards lipid exchange between liposomes compared to HP- β -
611 CD and HP- α -CD (Huang and London, 2013). Moreover, Me- β -CD and α -CD are found to be
612 more potent in mediating DPPC transfer between vesicles compared to that of Chol; on the
613 contrary, β -CD and γ -CD are more efficient transfer inducers of Chol in comparison with DPPC
614 (Leventis and Silviu, 2001). Another factor affecting the lipid exchange is the type of PL. For
615 instance, Me- α -CD induced the transport of SM (75 %), PC and PE (10-15 %) but not PI and
616 PS. Also, SM molecules with shorter acyl chains (fewer than 36 C) were exchanged to a greater
617 extent than the longer ones (Li et al., 2016). These findings are consistent with those of Kainu
618 et al. (2010) reporting that the transport of PLs triggered by Me- β -CD depends on length of

their acyl chains; at the same time, there was no consistent relation with the PLs head group. Similarly, the lipid transport rate mediated by CE- γ -CD (5 mM) decreased as the acyl chain length of PC increased from 6 to 14 C atoms (Tanhuanpää and Somerharju, 1999). Furthermore, CE- γ -CD enhanced the PI transport rate by about twice compared to PG, PE, PS, and PC; besides, the SM transport mediated by this CD was about 3 folds more rapid than that of PC. The authors concluded that the overall molecular hydrophobicity of PLs is an essential factor controlling their CE- γ -CD-mediated transport.

McCauliff et al. (2011) studied the mechanism of transfer of DHE, a fluorescent Chol analogue, from HP- β -CD/DHE complex to PL membranes. They demonstrated that the DHE transfer occurred by a collisional transfer mechanism involving a direct interaction of HP- β -CD with the membrane. HP- β -CD acts like Niemann–Pick C2 protein; the latter is involved in Chol transport and metabolism.

6.2.5. The effects of cyclodextrins on signal transduction pathways

To the best of our knowledge, CDs do not interact with specific membrane receptors. They act through a non-classical mechanism which is based on the extraction of membrane components (Chol, PL, lipid metabolites, and proteins), and/or the disruption of raft domains (Kabouridis et al., 2000; Matassoli et al., 2018). This may result in the alteration of signaling pathways; for example, Chol extraction by CDs leads to several changes including: *i*) the activation of phosphatidylinositol-3-kinase/protein kinase B/ Bcl-2-associated death promoter (PI3K/Akt/Bad) pathways (Motoyama et al., 2009); *ii*) the stimulation of ERK (extracellular signal-regulated kinase) phosphorylation and the inhibition of PI3K/Akt phosphorylation, thereby reducing the expression of the transcription factor c-Jun in colon carcinoma cells (Scheinman et al., 2013); *iii*) the suppression of resveratrol-induced activation of kinase-dependent signaling pathways (c-Jun NH₂-terminal kinase/ERK/Akt) and caspase-dependent apoptosis in colon cancer cells (Colin et al., 2011); *iv*) the activation of Wnt/ β -catenin pathway which plays multiple roles at different stages of development of *Xenopus laevis* (Reis et al., 2016); *v*) the activation of protein kinase A and tyrosine kinase resulting in protein phosphorylation and sperm capacitation (Osheroff, 1999; Visconti et al., 1999); *vi*) the enhancement of antigen receptor-mediated intracellular calcium release in Ramos B cells (Awasthi-Kalia et al., 2001), and its inhibition in Jurkat T cells (Kabouridis et al., 2000); *vii*) the enhancement of signaling pathways associated with β 1-adrenergic receptors resulting in compartmentalized cyclic adenosine monophosphate (cAMP) response in cardiac monocytes (Agarwal et al., 2011); *viii*) the suppression of cAMP-

dependent protein kinase (PKA) modulation of voltage-gated potassium channel (Kv) current in rat mesenteric artery smooth muscle cells (Brignell et al., 2015); ix) the suppression of signaling pathways associated with chemokine receptor (CCR5), leading to the inhibition of calcium release and abrogation of inhibition of forskolin-stimulated cAMP accumulation in Chinese hamster ovary cells and human embryonic kidney cells (Cardaba et al., 2008), x) the suppression of insulin-stimulated Akt phosphorylation in mice hepatocytes (Key et al., 2017); xi) other signaling pathways may also be involved. Additionally, CD-induced extraction of other membrane components (PLs or proteins) may affect several signal transduction pathways. For instance, DM- α -CD suppressed nitric oxide and tumor necrosis factor- α (TNF- α) production as well as nuclear factor- κ B (NF- κ B) activation in stimulated macrophages due to the efflux of PLs and cluster of differentiation 14 from lipid rafts (Motoyama et al., 2005).

The signaling pathways changes would depend on the cell line (Awasthi-Kalia et al., 2001; Kabouridis et al., 2000; Motoyama et al., 2005), the CD type and concentration (Colin et al., 2011; Motoyama et al., 2005), the CD forms (free or CD/guest complex) (Key et al., 2017), etc. This illustrates the vastness of this topic and requires separate consideration in a special review.

668

669 6.3. Protein extraction

670 Table 4 resumes the results of several studies, as examples, on the ability of CDs to extract
671 proteins from different cell membranes. The type and concentration of CD are also presented.

672 **Table 4: Some examples of cyclodextrin-induced protein extraction**

Cell type	CD type	[CD] (mM)	Outcomes	Ref
Human erythrocytes	- α -CD - β -CD - γ -CD	0-40	- Proteins were randomly extracted, without preference to any particular ones - The CD-induced protein extraction was in the order: β -CD > γ -CD > α -CD.	Ohtani et al., 1989
T lymphocytes	Me- β -CD	0-50	- Me- β -CD-induced extraction of the proteins: Thy-1 (cluster of differentiation-90), cluster of differentiation-45, cluster of differentiation-26, MHC-1, PTK-1, Lck, and Fyn.	Ilanguvaran and Hoessli, 1998
Caco-2 cells	- α -CD	β -CD:	- Proteins were randomly extracted,	Ono et al.,

	<ul style="list-style-type: none"> - β-CD - γ-CD - G_2-α-CD - G_2-β-CD 	0-15 Others: 0-150	without preference to any particular ones <ul style="list-style-type: none"> - The CD-induced protein extraction was in CD concentration dependent manner - At 150 mM, the CD-induced protein extraction was in the order: γ-CD < G_2-β-CD < G_2-β-CD << α-CD - At 15 mM, β-CD exhibited no ability for protein extraction. 	2001
B lymphocytes	Me- β -CD	10	<ul style="list-style-type: none"> - Me-β-CD induced extraction of BCR and cluster of differentiation-20. 	Awasthi-Kalia et al., 2001
Rod outer segment	Me- β -CD	15	<ul style="list-style-type: none"> - Me-β-CD induced extraction of transducin α subunit ($T\alpha$) from detergent resistant domains. 	Elliott et al., 2003
Rabbit erythrocytes	<ul style="list-style-type: none"> - α-CD - HP-α-CD - DM-α-CD 	3	<ul style="list-style-type: none"> - Proteins were randomly extracted, without preference to any particular ones - DM-α-CD induced protein extraction - No extraction was detected with other CDs. 	Motoyama et al., 2006
Rabbit erythrocytes	<ul style="list-style-type: none"> - β-CD - Me-β-CD - Dimeb 	β -CD: 3 Me- β : 1 Dimeb: 0.8	<ul style="list-style-type: none"> - Proteins were randomly extracted, without preference to any particular ones - Me-β-CD and dimeb induced protein extraction - No extraction was obtained with β-CD. 	Motoyama et al., 2009
Rod outer segment	Me- β -CD	0-20	<ul style="list-style-type: none"> - Me-β-CD induced extraction and purification of prenylated proteins GRK1 and PDE6. 	Saito et al., 2012

673 BCR: B cell receptor; GRK1: rhodopsin kinase; Lck: lymphocyte-specific protein tyrosine
 674 kinase; MHC 1: major histocompatibility complex 1; PDE 6: cGMP phosphodiesterase 6; PTK:
 675 protein tyrosine kinase; Thy-1: Thymocyte antigen 1
 676

677 As shown in Table 4, CDs are able to solubilize membrane proteins; the CD concentration and
 678 type are modulating factors of the process (Ono et al., 2001; Ohtani et al., 1989; Motoyama et
 679 al., 2006; Motoyama et al., 2009). Thus, β -CD was more potent in inducing protein extraction
 680 from human erythrocytes compared to the other native CDs (Ohtani et al., 1989). On the other
 681 side, α -CD was more effective in inducing protein extraction from Caco-2 cell membrane, and
 682 no effect was exerted by β -CD (Ono et al., 2001). Furthermore, DM- α -CD (Motoyama et al.,
 683 2006) and the methylated derivatives of β -CD (Me- β -CD and dimeb), unlike the native α - and β -

684 CDs (Motoyama et al., 2009b), were found to induce the extraction of proteins spanning rabbit
685 erythrocytes membranes.

686 It is worthy to note that Me- β -CD was the only CD used in the literature to selectively extract
687 membrane proteins from T lymphocytes (Ilangumaran and Hoessli, 1998) and B lymphocytes
688 (Awasthi-Kalia et al., 2001). Also, it was observed to selectively extract prenylated proteins
689 GRK1 and PDE6 from rod outer segment membrane. These proteins were demonstrated to
690 have different EC50 values (effective concentration to extract 50 % of total protein amount); it
691 depends on the strength of protein-membrane association. For GRK1, EC50 was 0.17 mM
692 while for PDE6 it was 5.1 mM (Saito et al., 2012). Moreover, Elliott et al. (2003) reported that
693 adding Me- β -CD resulted in the extraction of the peripheral protein transducin α subunit ($T\alpha$)
694 from the detergent resistant domains of rod outer segment membrane without any effect on
695 caveolin-1.

696 **6.4. The effect of cyclodextrins on the membrane permeability**

697 Cell membrane permeability is essential for many biological processes. Therefore, liposome
698 membrane permeability was examined in the presence of various CDs by analyzing the release
699 of fluorescent dyes incorporated into liposomes such as carboxyfluorescein (Nishijo et al.,
700 2000) and calcein (Besenicar et al., 2008; Hatzi et al., 2007; Piel et al., 2007). Regarding
701 cellular membranes, permeability was evaluated by analyzing the release of intracellular
702 components such as hemoglobin (Ohtani et al., 1989) and the enzyme lactate dehydrogenase
703 (Wang et al., 2011). Table 5 summarizes the results of the literature search regarding the effect
704 CDs on the membrane permeability with respect to the membrane lipid composition or cell type,
705 CD type and concentration, and intravesicular fluorescent dye or intracellular component used
706 to assess the membrane permeability.

707 **Table 5: The effect of cyclodextrins on liposomal and biological membrane permeability**

Membrane	CD type	[CD] (mM)	Released substance	Outcomes	Ref
Liposome					
- DPPC - DSPC - DMPC	- α -CD - β -CD - HP- β -CD - Dimeb - Trimeb - γ -CD	0-10	CF	- Release of CF was proportional to the CD concentration - Release of CF was in the order: DPPC liposome: Dimeb > α -CD > Trimeb DSPC liposome: Dimeb > Trimeb > α -CD	Nishijo et al., 2000

				<p>DMPC liposome: α-CD > Dimeb > Trimeb</p> <p>Other CDs had no effect</p> <ul style="list-style-type: none"> - α-CD induced the CF release as function of time - Dimeb and Trimeb induced a rapid release of CF at the initial stages followed by a slow release which finally levelled off. 	
<ul style="list-style-type: none"> - SPC:SA (90:10) - SPC:Chol:SA (60:30:10) 	<ul style="list-style-type: none"> - β-CD - Dimeb - Trimeb - Rameb - Crysmeb - HP-β-CD - SBE-β-CD - γ-CD - HP-γ-CD 	<p>β-CD: 0-7.5</p> <p>Crysmeb: 0-50</p> <p>others: 0-100</p>	Calcein	<ul style="list-style-type: none"> - Release was proportional to the CD concentration - Release was in the order: SPC: SA liposome: Dimeb > Rameb > Trimeb - SPC:Chol:SA liposome: Dimeb > Rameb > Trimeb > γ-CD - Other CDs had no effect - Dimeb induced total release immediately after addition. Other CDs induced the release as a function of time. 	Piel et al., 2007
<ul style="list-style-type: none"> - PC - PC:Chol (1:1) - HPC - HPC:Chol (1:1) - DSPC - DSPC:Chol (1:1) 	<ul style="list-style-type: none"> - HP-β-CD - Me-β-CD - HP-γ-CD 	<p>HP-β-CD: 27</p> <p>Me-β-CD: 31</p> <p>HP-γ-CD: 25</p>	Calcein	<ul style="list-style-type: none"> - Release of calcein was in the order: Me-β-CD > HP-β-CD = HP-γ-CD - Release of calcein was in the order: PC > HPC > DSPC - Release of calcein: MLV > SUV - The presence of Chol reduced the permeability of PC liposomes but increased or did not affect that of other liposome types - CDs induced a significant release of calcein immediately after its addition; no further effect was produced. 	Hatzi et al., 2007
<ul style="list-style-type: none"> - DOPC - DOPC:Chol (60:40) 	Me- β -CD	0-4	Calcein	<ul style="list-style-type: none"> - Me-β-CD did not induce the calcein release from the mentioned liposomes. 	Besenic et al., 2008
Biological cells					
Human erythrocytes	<ul style="list-style-type: none"> - α-CD - β-CD - γ-CD 	0-40	Potassium and hemoglobin	<ul style="list-style-type: none"> - Release was proportional to the CD concentration - Release was in order of β-CD > α-CD > γ-CD - Higher CD concentration was needed for hemoglobin release compared to potassium release. 	(Ohtani et al., 1989)

Mouse L-cell fibroblast	<ul style="list-style-type: none"> - B-CD - HP-β-CD - Me-β-CD 	0-10	¹⁴ C-adenine	<ul style="list-style-type: none"> - Release was proportional to the CD concentration - Effect of Me-β-CD > HP-β-CD - β-CD had no effect. 	(Kilsdonk et al., 1995)
Human embryonic kidney 293A cells	<ul style="list-style-type: none"> - HP₃-SBE₂-β-CD - HP₂-SBE₃-β-CD - SBE-β-CD - Me-β-CD - Dimeb 	0-20	LD	<ul style="list-style-type: none"> - Release was proportional to the CD concentration - Release was in the order: Me-β-CD = Dimeb > HP₃-SBE₂-β-CD = HP₂-SBE₃-β-CD = SBE-β-CD. 	(Wang et al., 2011)

708 CF: carboxyfluorescein; Chol: cholesterol; DMPC: dimyristoyl-phosphatidylcholine; DOPC: 709 dioleoyl-phosphatidylcholine; DPPC: Dipalmitoyl-phosphatidylcholine; DSPC: distearoyl- 710 phosphatidylcholine; HPC: hydrogenated phosphatidylcholine; LD: lactate dehydrogenase; PC: 711 phosphatidylcholine; SA: stearylamine; SPC: soybean phosphatidylcholine.

712
713 The interaction of CDs with lipid membrane components increased the membrane permeability; 714 the extent of increase in permeability is directly proportional to the CD concentration (Kilsdonk 715 et al., 1995; Nishijo et al., 2000; Ohtani et al., 1989; Piel et al., 2007; Wang et al., 2011). On the 716 other side, Besenicar et al. (2008) showed that Me- β -CD (0-4 mM) did not influence the 717 membrane permeability of DOPC and DOPC:Chol liposomes. This is probably due to relatively 718 low CD concentrations (0-4 mM) unlike other studies using higher CD concentrations up to 10 719 mM (Kilsdonk et al., 1995; Nishijo et al., 2000), 20 mM (Wang et al., 2011), and 100 mM (Piel et 720 al., 2007).

721 Additionally, several studies stated that the CD-induced membrane permeability increase 722 depends on the type of CD, namely its hydrophobicity and the cavity size. For instance, α -CD, 723 dimeb, and trimeb were shown to induce carboxyfluorescein release from DPPC, distearoyl- 724 phosphatidylcholine (DSPC), and dimyristoyl-phosphatidylcholine (DMPC) liposomes while no 725 effect was produced by β -CD, HP- β -CD, and γ -CD (Nishijo et al., 2000). Besides, the 726 permeability of soybean phosphatidylcholine (SPC):stearylamine (SA) liposome, assessed by 727 studying the kinetics of calcein release, was found to increase after exposure to methylated β - 728 CDs such as dimeb, rameb, and trimeb (Piel et al., 2007). However, other CDs used in this 729 work, β -CD, cysmeb, HP- β -CD, SBE- β -CD, γ -CD, and HP- γ -CD, did not significantly induce 730 calcein leakage from liposomes. As suggested by the authors, calcein molecules may escape 731 from the liposomes as a result of the interaction of methylated CDs with membrane lipids. 732 Moreover, the same authors showed that the methylated CDs increased the permeability of 733 liposomes containing or not Chol; this result allowed them to conclude that Chol does not

734 protect membranes from the effect of CDs (Piel et al., 2007). Furthermore, Hatzi et al. (2007)
735 demonstrated that the CD-mediated membrane permeability increase was greater for Me- β -CD
736 than for HP- β -CD or HP- γ -CD; thus, the effect of CD on the membrane permeability depends
737 on the lipophilicity of CD rather than its cavity size.

738 Concerning the effect of CDs on the permeability of biological membranes, the native CDs were
739 able to increase the permeability of human erythrocytes, with β -CD exerting the greatest effect
740 (Ohtani et al., 1989). In addition, methylated CDs such as Me- β -CD (Kilsdonk et al., 1995;
741 Wang et al., 2011) and dimeb (Wang et al., 2011) were demonstrated to be more potent in
742 enhancing the membrane permeability of mouse L cell fibroblasts (Kilsdonk et al., 1995) and
743 human embryonic kidney 293A cells (Wang et al., 2011) compared to other β -CD derivatives.

744 Other factors controlling the CD-induced membrane permeability are the type of PL constituting
745 the liposomal membrane and the size of liposomal vesicles (Hatzi et al., 2007). Indeed,
746 liposomes composed of saturated PLs (HPC) were found to be less affected by CDs in relation
747 to the unsaturated ones. Moreover, the presence of Chol in the membrane lowered the
748 permeability of unsaturated PC liposomes, while it increased or did not affect that of HPC or
749 DSPC liposomes (Hatzi et al., 2007).

750 Moreover, for the same membrane lipid composition, the CD-induced calcein release from MLV
751 was greater relative to SUV. The greater SUV stability is evidently due to the curvature of lipid
752 molecules which does not allow lipids to establish an optimal contact angle to interact with CDs
753 (Hatzi et al., 2007).

754 Furthermore, Monnaert (2004) measured the endothelial permeability coefficient (Pe) of [14 C]-
755 sucrose across blood brain barrier at 0- 5 mM for α -CD and β -CD, and at 0-50 mM for γ -CD.
756 The sucrose permeability was determined to be dependent of CD concentration; the effect of
757 CDs was in the order of α -CD > Me- α -CD = HP- α -CD = β -CD = Me- β -CD = HP- β -CD > γ -CD, Me-
758 γ -CD > HP- γ -CD.

759 Table 5 presents the data on the kinetics of intravesicular components release from liposomes
760 induced by different CDs. We can notice that most CDs induced an instant release of
761 intravesicular components after CD adding to liposomes followed by a slow (Nishijo et al., 2000)
762 or negligible (Hatzi et al., 2007) release stage. This finding can be explained by the fact that at
763 the initial stage, the CD-induced extraction of membrane lipids results in membrane
764 permeabilization. Following this stage, CD/lipid complexes may act as lipid donors towards
765 membrane allowing its re-organization (Hatzi et al., 2007).

766

6.5. Vesicles solubilization upon cyclodextrin – membrane interaction

The particle size analysis by dynamic light scattering (Hatzi et al., 2007) and turbidity measurements (Boulmedarat et al., 2005; Hatzi et al., 2007) revealed that CDs, in particular methylated β -CDs, induced vesicle solubilization due to the CDs ability to draw PLs and Chol out of the liposome membrane (Anderson et al., 2004).

Hatzi et al. (2007) showed that Me- β -CD was more potent in solubilizing PC and PC:Chol liposomes than HP- β -CD, at a CD concentration starting from 60 mM. This result was attributed to the ability of Me- β -CD to mediate a faster extraction of Chol with respect to HP- β -CD. Also, POPC vesicles were solubilized in a concentration-dependent manner when mixed with rameb solution (15-150 mM); the increase in rameb concentration from 80 to 100 mM led to a 2-fold increase in the amount of extracted POPC (Anderson et al., 2004). In addition, rameb (0-152 mM) caused a progressive decrease in the turbidity of SPC:Chol:SA (60:30:10) liposomes until a transparent solution was obtained (above a CD concentration of 50 mM). Furthermore, Huang and London (2013) used FRET to study CD-induced vesicle solubilization. Their results showed that, among different CDs (Me- β -CD, HP- β -CD, and HP- α -CD), a significant vesicle solubilization (abolishment of FRET signals) was achieved by Me- β -CD; the effect was depending on the acyl chain length and saturation degree as well as on the PL head group.

Based on the data discussed in this section and in the preceding sections, we can conclude that CDs are inactive at concentrations below 0.5 mM. In contrast, at moderate concentrations, they can induce lipid extraction and catalyze lipid exchange between membranes. CDs can also promote vesicles solubilization at relatively high concentrations (> 50 mM). It is worthy to mention the “shuttle-sink” model for lipid efflux described by Atger et al. (1997). According to this model, the rate of Chol exchange (influx and efflux) between cell membrane and medium compartment, as it is the case with serum lipoproteins, increases in the presence of low CD concentrations; the equilibrium is not changed and there is no clear change in the membrane and medium Chol pools. However, in the presence of lipid vesicles and CDs, the vesicles act as a sink shifting the equilibrium to favor net efflux of Chol from the cell membranes.

6.6. Cyclodextrins act as cryoprotectants during freeze-drying

Freeze-drying, also known as lyophilization, is essential to ensure the long-term storage of liposomes and biological cells. Nevertheless, it may cause membrane disruption owing to ice crystal formation, membrane phase transition, and osmotic dehydration (Chen et al., 2010; Ingvarsson et al., 2011; Wolkers, 2013). Freeze-drying appeared to be effective for storage of

several cell types including erythrocytes (Arav, 2013), platelets (Crowe and Fitzpatrick, 2013), and sperms (Keskin-tepe and Eroglu, 2015). It was mostly applied on spermatozoa; cryopreservation may promote damage in all sperm compartments, resulting in the loss of sperm motility, viability, acrosomal integrity, and the fertilizing capacity of the frozen-thawed sperms (Mocé et al., 2010).

Hence, maintaining the integrity of the lipid bilayer structure during freezing of liposomes and biological cells (particularly sperm cell) is highly recommended. Several papers studied the effect of CD as a cryoprotectant to preserve liposomal and biological membranes during the aforementioned process.

6.6.1. Cryopreservation of liposomes using cyclodextrins as cryoprotectants

Van den Hoven et al. (2012) demonstrated a stabilizing effect of HP- β -CD, during freeze-drying, towards PEGylated DPPC:Chol liposomes (lipid:CD w:w ratio of 1:6) loaded with prednisolone; its effect was superior to that of sucrose or trehalose, other known cryoprotectants. Sebaaly et al. (2016) compared the effect of different cryoprotectants (HP- β -CD, sucrose, trehalose, maltodextrin Glucidex6D and 19D, stearylamine, and cetyl alcohol) on phospholipon 90H-liposomes loading eugenol, an essential oil component. HP- β -CD showed the best protective effect; the mean vesicle size, pDI, Zeta potential, and eugenol encapsulation efficiency values were maintained when freeze-drying was conducted in the presence of HP- β -CD.

The effects of composition and saturation of soybean PLs on the liposome stability during freeze-drying was evaluated by Gharib et al. (2018b) using HP- β -CD as a cryoprotectant. HP- β -CD (10-100 mM) added to suspensions of conventional liposomes or present in the interior aqueous phase of CD-in-liposome system, protected hydrogenated liposomes during freeze-drying, while this effect was not observed for liposomes composed of unsaturated PLs.

HP- β -CD has a unique structure with numerous H-bond donors and acceptors. It may replace water molecules at the liposome surface during freeze-drying, thereby protecting the liposomal membrane from damage and keeping its structure intact (van den Hoven et al., 2012).

6.6.2. Cryopreservation of sperm cells using cyclodextrins as cryoprotectants

The effect of CDs on sperm cell cryopreservation was documented in a set of studies. In general, HP- β -CD and Me- β -CD, at optimal concentrations, have an ability to increase sperm cell viability after thawing, as compared to the control. However, CD-induced resistance of

spermatozoa to the damage caused by freezing and thawing was shown to vary between species; the variation is due to membrane PL:Chol ratio that differs between species (Madison et al., 2013).

The study of Madison et al. (2013) on jack and stallion sperm cells demonstrated that adding HP- β -CD (60 mM) resulted in an improved cell viability and motility of both sperm cell types. In the presence of HP- β -CD, the post thaw motility was 23 ± 0.7 % for jack and 17 ± 0.4 % for stallion relative to control (20 ± 0.7 % for jack; 15 ± 0.4 % for stallion). Moreover, for both species, HP- β -CD induced the sperm post thaw acrosome reaction. Zeng and coworkers studied the effects of HP- β -CD (Zeng and Terada, 2000) and Me- β -CD (Zeng and Terada, 2001) on boar sperm cell viability, motility, and acrosomal status during freeze-drying. According to the authors, the pretreatment with HP- β -CD (up to 40 mM) and Me- β -CD (up to 20 mM) increased the number of sperm cells with intact acrosomes and enhanced the sperm motility compared to untreated cells. However, further elevation of CD concentration up to 80 mM and 40 mM for HP- β -CD and Me- β -CD, respectively, lowered the sperm viability and motility.

6.6.3. Cryopreservation of sperm cells pretreated with cyclodextrin/cholesterol complex

Chol-loaded CDs were suggested to facilitate Chol transfer into the sperm plasma membrane. The effect of Me- β -CD/Chol inclusion complex applied prior to the freeze-drying procedure, on sperm membrane integrity, sperm motility, acrosome reaction, and sperm fertility was extensively studied in the literature. Purdy and Graham (2004) showed that pretreating bull sperm with Me- β -CD/Chol can increase the number of survived sperm cells and preserve their fertilizing potential. In addition, adding treated or untreated sperm to oocytes gave similar percentages of oocytes able to cleave and develop into embryos. Similarly, Salmon et al. (2016) reported that Me- β -CD/Chol, added prior to the cryopreservation procedure, improved the goat sperm cryosurvival rate showing greater percentages of motile and live sperms with intact acrosomes relative to untreated samples. However, contradictory results were published by Pinho et al. (2016) who demonstrated that Me- β -CD/Chol application did not improve, it even decreases, the quality of Piau swine sperm.

The effect of Me- β -CD/Chol inclusion complex was concentration dependent. Pretreating boar sperm with Me- β -CD/Chol (1 mg/ 120×10^6 sperm) increased the percentages of viable (40 ± 3

861 %) and total motile sperm ($25 \pm 3\%$) determined after 1 h, in comparison with untreated
862 samples (viability $32 \pm 3\%$ and total motility $19 \pm 3\%$). However, higher concentrations of Me-
863 β -CD/Chol (above 3 mg/120 $\times 10^6$ sperm) resulted in lower percentages of viable and motile
864 sperm; thus, at the concentration of 6 mg/120 $\times 10^6$ sperm, the cell viability and total motility
865 were $24 \pm 3\%$ and $11 \pm 3\%$, respectively (Blanch et al., 2012). Furthermore, freezing stallion
866 sperm in the presence of Me- β -CD/Chol (0-7.5mg/ 120 $\times 10^6$ sperm) enhanced sperm
867 membrane integrity, as compared to the control; the optimal Chol-Me- β -CD concentration being
868 1.5 mg/120 $\times 10^6$ sperm (Moore et al., 2005).

869 One of the damaging effects during freeze-drying is osmotic stress. The use of a CD/Chol
870 inclusion complex as a cryoprotectant was investigated by evaluating its effect on the osmotic
871 tolerance of sperm cells incubated in both hypo-osmotic and hyper-osmotic conditions. For
872 example, the pretreatment of ram sperm with Chol-loaded Me- β -CD protected membrane
873 integrity after short-term (15 min) exposure to osmotic challenges and significantly increased
874 the percentages of living and intact sperm cells (Ahmad et al., 2013). Also, Me- β -CD/Chol
875 addition increased rabbit sperm cell viability without affecting their functional integrity during a
876 set of osmotic challenges (Aksoy et al., 2010).

877 **7. Recent data in DCLs development**

878 The DCLs systems have been characterized in many publications for their morphology, size,
879 encapsulation efficiency (EE), and rate of drug release. In this section, the focus is done on the
880 effect of CD/drug inclusion complex on the physiochemical properties and stability (based on
881 membrane integrity and drug retention) of liposomes by comparing the characteristics of DCLs
882 to those of blank and drug-loaded liposomes. In 2015, a review had emerged discussing this
883 topic (Gharib et al., 2015). Hence, only the literature data reported between 2015 and 2018 will
884 be presented in this section.

885 Many studies showed that the incorporation of a drug into liposomes in the form of CD/drug
886 inclusion complex resulted in an improvement of encapsulation efficiency (EE) and/or loading
887 rate values, and in a delay of drug release compared to conventional liposomes. Thus, the
888 loading rate was found to be $31.5 \pm 4.2\%$ for eugenol-loaded phospholipon90H:Chol liposomes
889 and it doubled ($63.54 \pm 2.28\%$) when using the HP- β -CD/eugenol-loaded
890 phospholipon90H:Chol liposomes (Sebaaly et al., 2015). Similarly, for anethole, the loading rate
891 was 2 times higher for phospholipon90H:Chol DCLs ($0.83 \pm 0.15\%$) compared to

phospholipon90H:Chol conventional liposomes (0.48 ± 0.07 %). Moreover, DCLs demonstrated slower drug release behavior, retaining 38 % of anethole initially present in the vesicles, with respect to 22 % of anethole retained by conventional liposomes (Gharib et al., 2017). Furthermore, the EE values of flurbiprofen-loaded DCLs systems prepared with SBE- β -CD or HP- β -CD, were lower than that of flurbiprofen-loaded liposomes; on the other hand, DCLs delayed the flurbiprofen release (Zhang et al., 2015). Additionally, nerolidol was efficiently embedded into both lipid E80 conventional liposomes and DCLs with high EE and loading rate values (> 90 %), and DCLs was more effective to prolong the nerolidol release in comparison to conventional liposomes (Azzi et al., 2018). DCLs exhibited higher estetrol EE and slower estetrol release rate in comparison with conventional liposomes; nevertheless, both formulations were characterized by a high extent of drug release (80 %) after 6 h (Palazzo et al., 2019). Conversely, the EE of HP- β -CD/risperidone-loaded SPC:Chol liposomes was less than that of risperidone-loaded SPC:Chol liposomes, and risperidone was released faster from DCLs compared to conventional liposomes (Wang et al., 2016).

Conclusion

In this review, the interaction of CDs with biomimetic and biological membranes was analyzed. The CD effect on membranes is mediated by several factors including membrane structure and composition, in particular acyl chain length and saturation of PLs and structure of their head groups, as well as CD type and concentration. CDs can extract membrane lipids; their extraction capacity depends on CD concentration which appears to be efficient above 0.5 mM. CDs induce vesicle solubilization at concentrations near or above 50 mM. The interaction of CDs with membrane lipids promotes an increase of membrane permeability and fluidity. None of the studies evaluating CD effect on membranes considered the Chol content of lipid bilayer and few studies determined lipid:CD molar ratio. Therefore, these parameters should be analyzed in future studies. Finally, CDs and CD/Chol inclusion complexes were proven to have cryoprotective properties being able to preserve the integrity of liposomal and biological membranes during freeze-drying.

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