Title: Supramolecular Assistance between Cyclodextrins and Didecyldimethylammonium Chloride against Enveloped Viruses: Toward Eco-biocidal Formulations

Author: Loïc Leclercq Anny Dewilde Jean-Marie Aubry Véronique Nardello-Rataj

PII: S0378-5173(16)30807-9
DOI: http://dx.doi.org/doi:10.1016/j.ijpharm.2016.08.057
Reference: IJP 16033

To appear in: *International Journal of Pharmaceutics*

Received date: 25-7-2016
Revised date: 23-8-2016
Accepted date: 26-8-2016

Please cite this article as: Leclercq, Loïc, Dewilde, Anny, Aubry, Jean-Marie, Nardello-Rataj, Véronique, Supramolecular Assistance between Cyclodextrins and Didecyldimethylammonium Chloride against Enveloped Viruses: Toward Eco-biocidal Formulations.International Journal of Pharmaceutics http://dx.doi.org/10.1016/j.ijpharm.2016.08.057

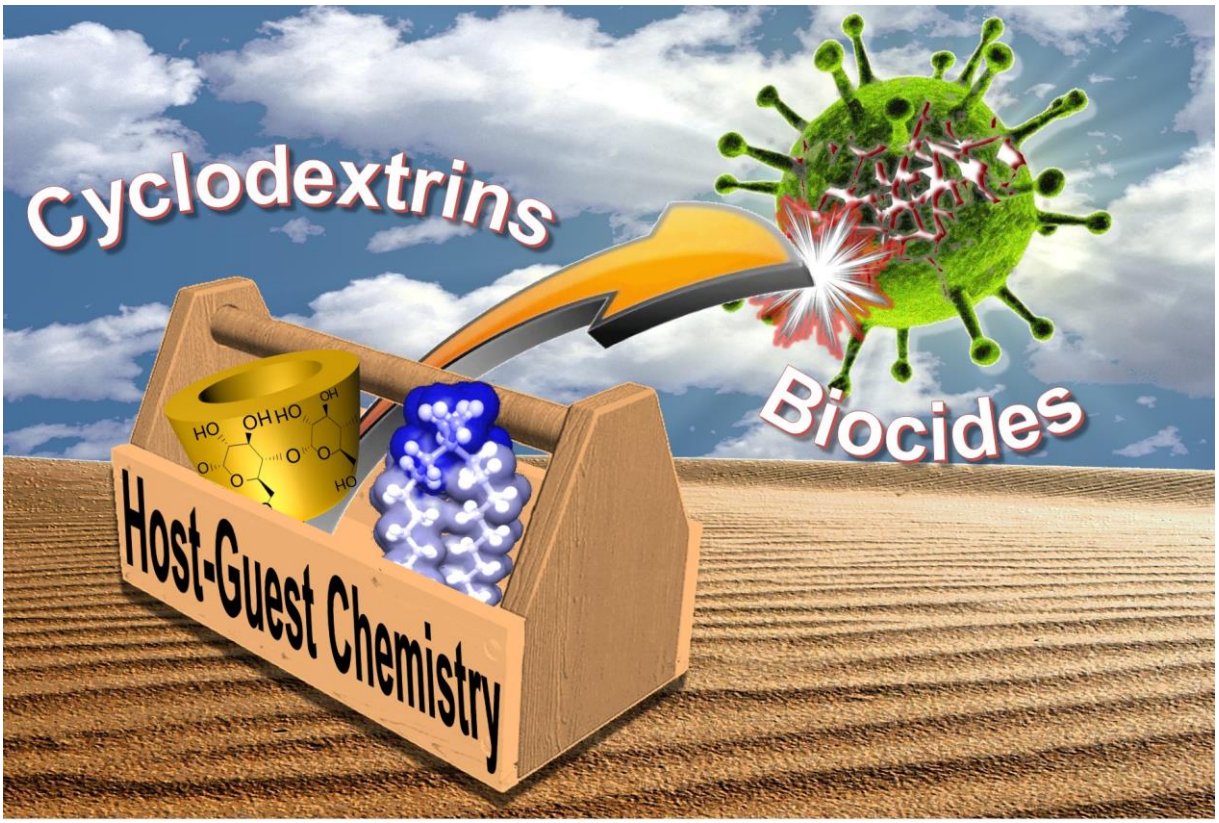
Supramolecular Assistance between Cyclodextrins and Didecyldimethylammonium Chloride against Enveloped Viruses: Toward Eco-biocidal Formulations

Loïc Leclercq,[a] Anny Dewilde,[b] Jean-Marie Aubry,[a] and Véronique Nardello-Rataj*[a]*

[a] Univ. Lille, CNRS, ENSCL, UMR 8181 – UCCS - Equipe CÏSCO, F-59000 Lille, France. Phone: (+33) 320 434 448, Fax: (+33) 320 434 260, E-mail: veronique.rataj@univ-lille1.fr and loic.leclercq@univ-lille1.fr

[b] Univ. Lille, Faculté de Médecine, UPRES EA 3610, Institut de Microbiologie, Laboratoire de Virologie, CHRU Lille, F-59037 Lille Cedex, France.

Graphical abstract.



Abstract. Nosocomial infections have emerged as important causes of morbidity and mortality in immunocompromised individuals. In this respect, biocides are widely used in hospitals leading to resistant microorganisms. We show here that cyclodextrins can remarkably boost the virucidal activity of di-*n*-decyldimethylammonium chloride. These oligosaccharides synergistically work with the biocide affording a noticeable reduction of the active virucide concentration between 40 and 85%. Partial replacement of a significant amount of the biocide by eco- and bio-compatible cyclodextrins whilst maintaining the same activity is of great interest as it allows the reduction of the toxicological drawbacks of classical biocide mixtures.

Keywords. Di-*n*-decyldimethylammonium chloride, Cyclodextrins, Host-Guest Chemistry, Lipids Extraction, Virus inactivation, Synergistic Formulation, Eco-biocide.

1. Introduction

Amphiphilic Quaternary Ammonium Compounds (QACs) are organic cations that contain one, two or three long alkyl chain(s) (typically from C_8 to C_{18}) and methyl or benzyl groups covalently attached to a central positively charged nitrogen atom. Due to their property of adsorption especially on negatively charged surfaces, they are mainly used as disinfectants, fabric softeners and antistatic agents but they can also be found as additives for various technical applications. They are also widely used in catalysis, better known under the name of Phase Transfer Catalysts (PTCs) like the commercially important benzyltrimethylammonium or methyltrioctylammonium chlorides. In 2004, their world-wide annual consumption was reported as 500,000 tons (Hauthal, 2004). Benzalkonium, [BA], benzethonium, [BZ], and di-*n*-decyldimethylammonium, [DiC_{10}], chlorides are widely used in hospitals to prevent and control pathogen infections (Nardello-Rataj & Leclercq, 2014). Indeed, QACs are membrane-active agents interacting with lipids and proteins prior to the plasma membrane disruption (Denyer, 1995; Nardello-Rataj & Leclercq, 2014). Unfortunately with the long-term used of biocides, a certain tolerance among some types of pathogens occurs (Gerba, 2015). Consequently, some pathogens were found to have reduced susceptibility to QACs and the disinfectant can no longer be used against this particular strain (Moore et al., 2008). In some cases, the resistance against biocides can also lead to resistance to antibiotics (Gilbert & McBain, 2003) Furthermore, the use of biocides is regulated in both the United States of America and the European Union. In order to improve efficacy and decrease resistance effect, mixtures of biocides are often encountered in formulations but they may also lead to an increase of ecotoxicity and/or sensitivity due to cumulative damages. Moreover, nosocomial infections are not limited to bacteria or fungi. Indeed, viral infections can be acquired in healthcare settings by person to person or by fomites transmission. Viruses represent 32% of pediatric nosocomial infections (Valenti et al., 1980). Respiratory syncytial virus (RSV) is the

major cause of lower respiratory tract infections and hospital visits during infancy (Hall, 1977). Another common viral disease is caused by herpes simplex (HSV) leading to several distinct medical disorders (orofacial and genital herpes, whitlow, keratitis or encephalitis, Aitken & Jeffries, 2001). People with immature or suppressed immune systems (newborns, transplant recipients, AIDS) are prone to severe complications from HSV infections. More recently, between 2013 and 2016, Ebola virus epidemic has resulted in at least 28 646 suspected cases and 11 323 confirmed deaths in West Africa (Ebola situation report, 2016). To avoid Ebola transmission, infection-control programs have been developed with the use of disinfectants including QACs (Chepurnov, 2003) but as pointed out by Moore and Payne (2004): "*viruses are more resistant than bacteria or fungi to the QACs*".

In this context, natural cyclodextrins (CDs) have been shown to be effective for the inactivation of pathogens including viruses. CDs constitute a family of macrocyclic compounds built up from six, seven or eight α-D-glucopyranoside units linked 1→4 (α-, β- and γ-CD, respectively, see Figure 1). The structure of CDs, involving a hydrophobic cavity and a hydrophilic exterior, enables them to form water-soluble inclusion complexes with the hydrophobic part of numerous molecules (Szejtli, 1998). They are very useful in various areas such as catalysis (Leclercq, 2005), textile (Leclercq, 2016), pharmaceutical (Jambhekar & Breen, 2016a,b, Leclercq & Nardello-Rataj, 2016; Kurkov & Loftsson, 2013; Del Valle, 2004) and food industries (Astray et al., 2009). Their biocidal mechanism is based on the lipids extraction from the cell membrane driven by complexation (Fauvelle et al., 1997; Debouzy et al. 1998). Although native CDs are biocompatible (Stella & He, 2008) and derived from biomass feedstocks, they unfortunately cannot be used directly to kill pathogens due to the high CD concentration needs to observe an effective biocidal activity (> 5 mM; Wallace et al., 2003, Leclercq et al., 2010a, Leclercq et al., 2012). Based on these considerations and on the fact that developing and marketing new biocides has become almost

impossible for industrials, one alternative is to take advantage of synergy effects, as in many domains nowadays, especially in formulation. We show indeed hereafter that combination of CDs and QACs can lead to huge synergistic effects on viruses. Some CDs associated with organic biocides have already been investigated. Two behaviors can be highlighted in respect with the aqueous solubility of organic biocides: poorly soluble biocides are significantly more active than the biocides alone due to the solubility improvement (Schmidt et al., 1996) whereas soluble ones are inactive depending on the degree of complexation (Lehner et al., 1993, 1994).

Insert Figure 1.

In order to overcome this limitation, Beck *et al.* have described systems based on chlorite and sorbic acid biocides which present reduced binding to CDs but this method is very restrictive (2002). As CD host-guest chemistry enables the implementation of self-assembly processes in aqueous environment, a simple way to avoid complexation is the dilution. For infinitely diluted solutions, free CD and biocide species prevail due to the Le Châtelier's principle (Leclercq et al., 2012). However, such a system requires highly effective biocides for which the minimum biocidal concentration (MBC) is very weak. If this is not the case (*e.g.* for [BZ] and [BA]), as QACs are relatively well soluble in water, their complexation by CDs reduces the biocidal activities (Simpson, 1992; Loftsson et al., 1992). In this context, the use of the [DiC_{10}][Cl] salt is very promising since it achieves biocide effectiveness at a relatively reduced concentration (< 1 mM). For instance, HSV-1 is deactivated within less than 1 min of contact at a dose of 0.8 mM (Argy et al., 1999). Therefore, the combination of [DiC_{10}][Cl] and CDs (active at weak and high concentrations, respectively), is expected to lead to synergies in terms of antiviral activity since both of them have the same target (*i.e.*

viral membrane, see Figure 1). This effect could be highly helpful in the fight against nosocomial infections.

2. Materials and methods

2.1. General information

All chemicals were purchased from Sigma-Aldrich Chemical Company at the highest purity available. NMR spectra were recorded in the indicated solvents. Chemical shifts are given in ppm (δ) and measured relative to the $CDCl_3$ (Sigma-Aldrich Chemical Company). The following abbreviations are used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet. All products were dried in a Freeze Dryer (Alpha 1-2 LD plus). Sterile water for injection was used in all experiments (Fresenius-Kabi, France, $\sigma = 72.0$ mN.m^{-1} at 20.0 °C).

2.2. Synthesis of di-n-decyldimethylammonium chloride

A dry flask (1 L), equipped with a magnetic stir bar, was charged with a solution of 1-decylbromide (33.17 g, 150 mmol) in acetonitrile (300 mL) and dimethyldecylamine (27.08 g, 150 mmol). The reaction mixture was stirred for 12 hours at 90 °C. The resulting solution was concentrated under vacuum to give the product. The product was dissolved in water (51 g/100 mL) and ethanol (10 mL) was added to this solution. The aqueous layer was washed with 3 × 50 mL of hexane (eliminated fractions) and 3 × 25 mL of chloroform. The chloroform layers were concentrated under vacuum to give the [DiC_{10}][Br]. An aqueous solution of [DiC_{10}][Br] was eluted on an hydroxide ion exchange resin. The aqueous solution of [DiC_{10}][OH] was neutralized with an aqueous HCl under dry argon. The [DiC_{10}][Cl] was dried and store under dry nitrogen. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 0.85 (t, 6H; CH_3, ^3J = 6.9 Hz), 1.23-1.32 (m, 28H; CH_2), 1.66 (m, 4H; N-CH_2-CH_2), 3.38 (s, 6H; NCH_3), 3.45 (m, 4H; NCH_2). ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ (ppm) = 14.1, 22.6, 22.7, 26.2, 29.2, 29.4, 31.8,

51.3, 63.5. Anal. Calc. for ($C_{22}H_{48}NCl$): C, 72.98%; H, 13.36%; N, 3.87%; Cl, 9.79%. Found: C, 72.88%; H, 13.33%; N, 3.96%; Cl, 9.83%. m.p. = 88 °C. (Yield: 95%).

2.3. Surface tension

Surface tensions were measured with the tensiometer K11 (Krüss) using the Wilhelmy plate method. A concentrated solution was prepared extemporaneously, and equilibrium surface tension was measured by dilution method in ultrapure water (σ = 72.0 mN/m at 25 °C). After each addition, the solution was gently stirred for 60s. Surface tension was recorded after equilibration for each mixture. All equilibrium surface tension values were mean quantities of at least three measurements. The precision of the force transducer of the surface tension apparatus was 0.1 mN m^{-1} and before each experiment, the platinum plate was cleaned in red/orange color flame. The temperature was stabilized at 25 ± 0.05 °C with a thermo-regulated bath Lauda RC6. The standard deviation was estimated at ± 10% of the CMC values.

2.4. [DiC_{10}]-selective electrode

The sensitive membrane was prepared using a cross-linked polymer made of a mixing of silicone gel (Rhodorsil, Rhodia) and polymethylhydromethylcyanopropylsiloxane (Petrarch system). The selectivity of the membrane is due to a "carrier" of the cationic amphiphile, di-*n*-decyldimethylammonium tetraphenylborate [DiC_{10}][BPh_4], which was prepared by mixing equimolar (1 mM) aqueous solutions of sodium tetraphenylborate ($NaBPh_4$, Aldrich) and [DiC_{10}][Cl]. The insoluble salt obtained was extracted three times by liquid–liquid extractions in dichloromethane. After evaporation of the solvent, the residual salt was recrystallized twice from methanol. The salt was then dissolved at 10 mM in THF. Before polymerization, the composition of the membrane solution is as follows: about 0.7 g of silicone gel, rhodorsil CAF3 (Rhodia), about 0.3 g of a copolymer, polymethylhydromethylcyanopropylsiloxane (Petrarch System), about 0.03 g of Aerosil300 (Degussa), 1 mL of the carrier ([DiC_{10}][BPh_4])

solution into THF and 1 mL of THF. This mix is homogenized few minutes. After evaporation of THF, the cross-linked polymer forms quickly in contact with air. To construct the selective electrode, a commercial pH-glass electrode was dipped in the membrane solution two times, allowing 15 min air-drying between each immersion. For all measurements, the sensitive electrode was used in potentiometric cells in conjunction with a reference electrode of KCl-saturated calomel protected from amphiphile diffusion by a saline agar-agar gel made from 2 M KCl solution contained in a Teflon capillary tube. Emfs were measured with a Meterlab PHM250 Ion Analyzer (Radiometer Analytical). All the measurements were carried out at constant temperature (25 ± 0.1 °C).

2.5. Viruses and cells

HSV-1 (Strain Kos) and VACV (Strain Elstree) were propagated in Vero cells (ATCC® CCL-81™) in Minimum essential Medium (Gibco, Life Technologies) supplemented with 2 mM l-glutamine (Gibco, Life Technologies), 1% non-essential amino-acids (Gibco, Life Technologies) and 2% inactivated fetal calf serum (Gibco, Life Technologies). Cell-free viral suspensions of the viruses were obtained by freezing-thawing cycles followed by a low speed centrifugation to remove cell debris. RSV (local laboratory strain) was propagated in Hep-2 cells (ATCC® CCL-23™) in the same medium. Viruses titers were assayed by the cytopathic effect of serial dilutions (1:10) of virus-containing samples on Vero or Hep-2 cells. A sample (100 µL) for each dilution was used to infect four replicate wells in 96-well microtiter plates (NunclonTM Delta Surface, Thermo ScientificTM NuncTM). Virus-induced cytopathic effects were scored after 5 days of incubation at 37 °C ± 0.1 °C in a humidified 5% CO2 atmosphere. Titers were expressed as the quantity of viruses infecting 50% of the tissue culture wells (Tissue Culture Infectious Doses, TCID50) according to Spearman (1908) and Kärber (1931). The detection limit was 5.62 TCID50 mL^{-1}.Virus stocks were 2×10^7 TCID50 mL^{-1} for HSV-1, 2×10^6 TCID50 mL^{-1} for VACV, 3×10^6 TCID50 mL^{-1} for RSV.

2.6. Virus inactivation

200 µL of the viral stock solution was added to 200 µL of [DiC_{10}][Cl] or 200 µL of the different aqueous binary mixtures of CDs and [DiC_{10}][Cl] (in equimolar proportions) After incubation for 15 min (30 or 60 min) at room temperature (25 ± 0.1 °C), the mixtures were immediately filtered on MicroSpin S-400 HR columns (GE Healthcare) to separate viruses from the other components of the mixtures. Residual viruses were then titrated as described above. Each experiment was performed at least twice.

3. Results and Discussion

3.1. Virucidal activities of [DiC_{10}][Cl] alone or in combination with native CDs

As control experiments, we evaluated the virucidal activities of [DiC_{10}][Cl] alone against lipid-containing deoxyribonucleic and ribonucleic acid viruses, such as HSV-1, RSV and vaccinia viruses (VACV, as model of *Variola vera*). As expected, the initial viral population decreases with increasing [DiC_{10}][Cl] concentration after 15 min of contact time (Figure 2, blue bars). It is noteworthy that a "6-log reduction" is equivalent to 99.9999% reduction of viral titer. In this case, it appears that it is unlikely that intact viral particles are still present after the use of [DiC_{10}][Cl]. In other words, the sterilization process is very efficient. However, the [DiC_{10}][Cl] concentration needed to obtain > 6-log reduction clearly depends on the virus type: this cation is more active against HSV-1 and RSV (125 µM) than against VACV (500 µM, see Figure 2, blue bars).

Insert Figure 2.

This differential susceptibility can be correlated with the virus size. Indeed, the diameter of HSV-1 and RSV is around 200 nm (Plomp et al., 2002; Utley et al., 2008) while that of VACV is usually 320-380 by 260-340 nm (Malkin et al., 2003). As [DiC_{10}][Cl] interacts and penetrates the viral membrane, making it fragile, and leads to virus inactivation, we can

suppose that the required amount of [DiC_{10}][Cl] in order to obtain a complete membrane disorganization prior to virus inactivation is higher for VACV than HSV-1 or RSV because of the viral envelope size. Another plausible explanation can be the difference in term of cholesterol/phospholipids ratio of viral envelopes. Indeed, the viral envelopes rich in cholesterol are more stable and less flexible than those poor in cholesterol.

Before considering the mixtures, a second batch of control experiments were undertaken in the presence of natural CDs without [DiC_{10}][Cl]. In a concentration range from 20 to 1,000 µM, no activity was found after 15 min or 2 h of contact time against HSV-1, RSV and VACV. It is logical to not observe the virucidal activities of natural CDs with concentrations six times lower than those found by Wallace *et al.* (2003). Indeed, exposure of HSV-1 to β-CD (6,000 µM) resulted in "5-log reduction" of the virus titer after 24h. In addition, native CDs are well known to induce morphological changes in erythrocytes followed by hemolysis at higher concentrations. As this hemolytic activity is ascribed to the extraction of phospholipids and/or cholesterol depending on the cavity size, this information is very important because it is reliable measure for the estimation of CD-induced cytoplasmic membrane damage. Our results are also in perfect agreement with the hemolytic activities of native CDs. Indeed, hemolysis of human erythrocytes was initiated at 6,000, 3,000 and 16,000 µM for α-, β-, and γ-CD, respectively, whereas complete hemolysis was observed at 20,000, 10,000 and 41,000 µM, respectively (Irie et al., 1982). Based on this, it is reasonable that native CDs are not able to induce a virucidal activity due to their low concentrations (< 1,000 µM).

Focusing on the virucidal activity of [DiC_{10}][Cl]/CD mixtures, the minimum concentrations to achieve a "6-log reduction" have been estimated for several diluted aqueous solutions prepared from a equimolar stock solution of [DiC_{10}][Cl] and CD. In the presence of γ-CD, the biocidal activity of [DiC_{10}] is clearly significantly enhanced against the three

viruses investigated: the [DiC_{10}][Cl] concentration needed to obtain "> 6-log reduction" is reduced to 72, 40 and 85% against HSV-1, RSV and VACV, respectively (Figure 2, green bars). As the efficacy of this mixture is clearly higher than the sum effects of the single substances, these observations can be directly related to a molecular synergism between the [DiC_{10}][Cl] and the γ-CD. Indeed, their combination in an equimolar mixture increases the overall effectiveness of the formulation. Surprisingly, the mixtures based on α- and β-CD show no synergy (Figure 2, purple and orange bars). The differential behavior of the formulation with γ-CD compared to those with α-CD and β-CD on viruses are clearly not directly linked to the hemolytic activity induced by natives CDs which increases in the order γ-CD < α-CD < β-CD due to the extraction of phospholipids and/or cholesterol depending on the cavity size (Ohtani et al., 1989). Indeed, α- and β-CDs are excellently suited to solubilize phospholipids and cholesterol, respectively, whereas γ-CD is less lipid selective. Based only on the potencies of these CDs for solubilizing components of viral envelope, the most virucidal mixtures should be the α- or β-CD/[DiC_{10}][Cl] mixtures depending on the phospholipids/cholesterol ratio in the membranes: α-CD for phospholipids-rich envelopes and β-CD for cholesterol-rich membranes. In our case, the efficiency of native CDs in term of synergy with [DiC_{10}][Cl] (*i.e.* in term of virus inactivation) is clearly inconsistent with the order of magnitude of hemolytic activity. This difference could be ascribed to the presence of [DiC_{10}][Cl]. Indeed, this virucide is also membrane-active agent interacting with lipids or proteins of the viral envelope prior to its complete disorganization (Denyer, 1995; Nardello-Rataj & Leclercq, 2014). In the literature, it is reported that CDs, at low concentrations, can alleviate hemolysis induced by drugs (*e.g.* imiparine, chlorpromazine hydrochloride, propantheline bromide, *etc*.) through the formation of inclusion complexes (Funasaki et al., 2001; Funasaki et al., 1999). Nevertheless, this behavior is not just limited to drugs but also to surfactants with hemolytic activity. Indeed, the attenuation of dodecytrimethylammonium

bromide-induced hemolysis increases in the order γ-CD < α-CD ≈ β-CD (Funasaki et al., 2000). This effect is directly correlated with the magnitude of the binding constants between CDs and cationic surfactant that increases in the order γ-CD < α-CD ≈ β-CD. Therefore, the formation of complexes between the dodecytrimethylammonium cation and α- or β-CD suppresses the hemolysis-induced by the surfactant whereas this effect is kept with γ-CD due to the weak binding constant (Funasaki et al., 2000). It could be argued that CDs in some manner have a similar effect in our case.

3.2. Thermodynamics of [DiC$_{10}$][Cl]/[CD] mixtures

In the case of a binary mixture based on a single surfactant (S) and CD, it is assumed that two inclusion complexes can be observed depending on the cavity size (Leclercq et al., 2010a). Furthermore, according to its concentration, the surfactants can self-assemble to form micelles. Additional equilibria occur in the presence of viruses such as lipids extraction from the viral envelope by CD or the insertion of the surfactant in the membrane. Figure 3 shows a schematic model of the equilibria occurring in the [DiC$_{10}$][Cl]/CD mixtures.

Insert Figure 3.

As it is well known that addition of CD to a micellar surfactant solution leads to the dissociation of the micelle, we have determined the apparent CMC of [DiC$_{10}$][Cl]/[CD] mixtures. Consequently, the effect of the natural CDs on the micellization process of the [DiC$_{10}$][Cl] itself has been studied by measuring the surface tension, σ, at 25 °C by the dilution method. In this method, as the dilution occurs for the surfactant as well as for the CD, the results are directly comparable with the virucidal tests (see experimental section). In the absence of CDs, the surface tension isotherm is typical of true surfactants and the critical micelle concentration (CMC) values is in good agreement with the data previously reported (1 200 μM, Leclercq et al., 2013). As expected, the presence of CDs leads to significant

changes in surface tension isotherms: the surface tension deviates from that observed for the $[DiC_{10}][Cl]$ alone reflecting the formation of inclusion complexes (Figure 4).

Insert Figure 4.

Unfortunately, the complete rationalization of the isotherms obtained with CDs is very difficult because of the ability of inclusion complexes to participate in the surface monolayer stabilization. Actually, our previous results supports that the α- or β-CD/$[DiC_{10}][Cl]$ 1:1 inclusion complexes participate in the surface-adsorbed monolayer (Leclercq et al., 2013). However, in the presence of CD, the apparent CMCs are in the order: β-CD (5,140 μM) ≈ α-CD (5,550 μM) ⋘ γ-CD (9,710 μM). Therefore, the micellization equilibrium is not relevant in this case: the virucidal activities are obtained in the premicellar region for all mixtures (see discussion above).

Next, in order to explicit the equilibria involved in the initial steady state, we have experimentally determined the free $[DiC_{10}]$ concentration, $[DiC_{10}]_{free}$, in the $[DiC_{10}][Cl]$/CD mixtures using a $[DiC_{10}]$-selective electrode. In the absence of CD and viruses and below its CMC (1,200 μM), the electromotive force (emf) of an aqueous solution of $[DiC_{10}][Cl]$ presents a pseudo-Nernstian behavior which is a unique function of $[DiC_{10}]_{free}$ (eq. 1, Figure 5, Leclercq et al., 2010b).

$$emf = 59.2 + 55.1 \times \log[DiC_{10}]_{free} \quad (1)$$

In contrast, for $[DiC_{10}][Cl]$/CD mixtures, the emf curves, recorded by the dilution method, deviates from the Nernstian behavior in the negative direction. These deviations are assigned to the formation of inclusion complexes which reduce the free $[DiC_{10}]$ concentration. The extent of deviation is in the order β-CD ≈ γ-CD < α-CD.

Insert Figure 5.

The free [DiC_{10}] concentrations can be calculated from the emf values since equation 1 provides accurate and reproducible measurements of these concentrations (Rauwel et al., 2012). The evolution of $[DiC_{10}]_{free}$ concentration as a function of the total concentration of [DiC_{10}][Cl] in the binary [DiC_{10}][Cl]/CD mixture is presented in Figure 6.

Insert Figure 6.

As the binding constants (K_1 and K_2) have been previously estimated from the [DiC_{10}]-selective electrodes by the titration method applied to a fixed concentration of [DiC_{10}][Cl] with varying concentrations of native CDs, we can easily estimate the initial composition of our systems (Leclercq et al., 2010a). Indeed, below the CMC of the surfactant (S) and in the presence of CD, it is assumed that the molarity of 1:1 and 1:2 complexes is expressed as

$$[S \bullet CD] = K_1[S][CD] \qquad (2)$$

$$[S \bullet CD_2] = K_2 K_1[S][CD]^2 \qquad (3)$$

where [S] and [CD] are the free concentrations of S and CD, respectively. In eqs 2 and 3, [S•CD] and [S•CD_2] denote the concentrations of the complexes with 1:1 and 1:2 stoichiometries respectively. The mass balance equations are written as

$$C_{CD} = [CD] + K_1[S][CD] + 2K_1K_2[S][CD]^2 \qquad (4)$$

$$C_S = [S] + K_1[S][CD] + K_1K_2[S][CD]^2 \qquad (5)$$

Eliminating [S] from eqs. 4 and 5 gives the cubic equation:

$$K_1K_2[CD]^3 + K_1\left(1 - K_2\left(C_{CD} - 2C_S\right)\right)[CD]^2 + \left(1 + K_1C_S - K_1C_{CD}\right)[CD] - C_{CD} = 0 \qquad (6)$$

The free CD concentration ([CD]) can be calculated from eq. 6 and substituting in eq. 5 to obtain the free surfactant concentration ([S]). Finally, the complexes concentrations ([CD•S] and [CD_2•S]) can be calculated with eqs. 2 and 3. All results are presented in Figure 6. It is

clear that the association constants, previously determined, describe very well the free [DiC_{10}][Cl] concentrations (Figure 6, compare the continuous lines and the experimental values). The goodness of fits is given in Table 1. As these values tend to 1, the binding constants values, previously determined from titration method, fits very well with our present results obtained with the dilution method. Therefore, it is not necessary to fit values again.

Insert Table 1.

As depicted in Figure 6, infinitely diluted solutions ([DiC_{10}][Cl]$_T$ ≤ 30 µM) show the prevalence of free species ([DiC_{10}] and CD). When [DiC_{10}][Cl]$_T$ ≥ 30 µM, [DiC_{10}]•CD and [DiC_{10}]•CD_2 inclusion complexes are gradually formed leading to a rapid divergence of the free CD concentration compare to the free [DiC_{10}][Cl]. As this behavior is directly related to the formation of 1:2 inclusion complexes, the evolution of free species ([DiC_{10}] and CD) are similar for γ-CD. For [DiC_{10}][Cl]$_T$ = 35 µM, it is clear from these results that the relative ratio of inclusion complexes are marginal and that the free [DiC_{10}] and CD) concentrations are quite similar for all native CDs (see Figure 6). Moreover, at this concentration, negligible fractions of lipids (phospholipids or cholesterol) bound to native CDs in steady state can be also invoked because the magnitude of binding constants with lipids are quite similar to the constants observed with [DiC_{10}][Cl] (*e.g.* ~1.7×10^4 for β-CD/cholesterol; Frijlink et al., 1991; Shiotani et al., 1995). However, we can reasonably suppose that the inclusion complexes can be seen as a reservoir of [DiC_{10}] and CD molecules which are readily available for interaction with the viral envelope (i.e. complexation and insertion, for CDs and [DiC_{10}], respectively). Consequently, the thermodynamic in initial steady state fails to explain the differential effects of CDs especially for [DiC_{10}][Cl]/CD mixtures (35 µM each) against HSV-1 (see Figure 2).

3.3. Kinetics of the virus inactivation

It is known that virus inactivation is a kinetic process wherein the "viability" of viruses varies as a function of time (Weavers & Wickramanayake, 2001). As the apparent differential effect of native CDs, observed after 15 min of exposure, can be related to dissimilarities in term of inactivation rates, we have determined the virus titers after 15, 30 and 60 min of exposure to $[DiC_{10}][Cl]$/CD mixtures (35 µM each) against HSV-1 (Figure 7).

Insert Figure 7.

As expected, virus inactivation varies as a function of time. The time needed to obtain "> 6-log reduction" (i.e. 99.9999% reduction of viral titer) for $[DiC_{10}][Cl]$/CD mixtures are γ-CD much greater than β-CD greater than α-CD. It is noteworthy that this time is always smaller than the $[DiC_{10}][Cl]$ alone. Therefore, the synergism occurs for all native CDs in term of virus inactivation rates. However, the fast kinetic process is obtained with the $[DiC_{10}][Cl]$/ γ-CD mixtures. The analysis and interpretation of inactivation kinetics are extremely complex. Based on the hemolytic activity of native CDs, we can suppose that the mechanism of action is quite different for α-CD on the one hand and β- and γ-CDs on the other. Indeed, the α-CD cavity is perfect for the extraction of phospholipids whereas β-CD and γ-CD extract preferentially cholesterol although γ-CD is less active than β-CD (Ohtani et al., 1989). However, it is known that the rate of cholesterol transfer between vesicles is considerably accelerated in the presence of submillimolar concentrations of γ- and β-CD (64- and 63-fold, respectively) compared to that observed in the absence of CD (Leventis & Silvius, 2001). In contrast, α-CD is able to accelerate the transfer of phospholipids (Leventis & Silvius, 2001). Therefore, even if the fractions of cholesterol bound to γ-CD are initially marginal, we can reasonably suppose that the insertion of $[DiC_{10}][Cl]$ is faster in the presence of γ-CD because the concentrations of free $[DiC_{10}][Cl]$ and γ-CD are identical due to the presence of 1:1 complex (see Figure 6). In these conditions, the insertion of $[DiC_{10}][Cl]$ associated to the complexation of cholesterol by γ-CD allows an easy displacement of equilibria and accelerate the apparent rate of virus inactivation. In contrast, with α- and β-CD, due to the presence of 1:2 inclusion complexes, the free CD concentration is weaker than $[DiC_{10}][Cl]$ leading to a slower $[DiC_{10}][Cl]$ insertion than with γ-CD. In line with the experimental contact time, the

α- and β-CD/[DiC_{10}][Cl] mixtures act slower than the γ-CD/[DiC_{10}][Cl] mixture resulting in an apparent loss of activity at given time and concentration. It is noteworthy that similar results are obtained with the other enveloped viruses investigated here.

4. Conclusion

According to the old adage of strength through unity, we have demonstrated that [DiC_{10}] cations associated with CDs act as a much more virucidal agent than each compound alone due to the lipid and/or cholesterol extraction from the viral envelope. As a consequence, the [DiC_{10}][Cl] concentration is reduced up to 85% for the same virucidal activity with the γ-CD. As depicted in Table 2, the best compromise in terms of toxicity (hemolysis, cytotoxicity and degradability) and virucidal activity (concentration and time to obtain sterilization) is obtained with the equimolar [DiC_{10}][Cl]/γ-CD mixture.

Insert Table 2.

Moreover, such antiviral mixtures taking advantage of the synergy between native CDs and classical virucides allow the partial replacement of a significant amount of biocides by eco- and bio-compatible CDs. This result is particularly interesting both for the formulator and the user as it allows to decrease the toxicological and ecotoxicological drawbacks of classical biocides while complying with the regulation. The eco-compatible and biocide terms, largely antithetical, may be combined for the first time under the name of eco-biocide. These results support the idea that the supramolecular assistance by the CDs is a phenomenon of general importance. This synergistic effect provides elements valuable for further developments and will probably become the new Holy Grail in the coming years particularly in the fight against viruses and other nosocomial infections.

Acknowledgements

Chevreul Institute (FR 2638), Ministère de l'Enseignement Supérieur et de la Recherche, Région Nord - Pas de Calais and Fonds Européen de Développement Régional (FEDER) are acknowledged for supporting and funding partially this work. We are grateful to Pr. Didier Hober (Université de Lille) for supplying us the vaccinia virus and Dr. Gaétan Rauwel (Laboratoires Anios, France) for fruitful discussions.

References

Aitken, C., Jeffries, D. J., 2001. Nosocomial spread of viral disease. Clin Microbiol Rev. 14, 528-546.

Antlsperger, G., Schmid, G., Toxicological comparison of cyclodextrins. In: Szejtli, J., Szente, L. (Eds.) Proceedings of the Eighth International Symposium on Cyclodextrins, Springer-Science+Business Media, B.V., Dordrecht, Netherland, 1996, pp. 149-155.

Argy, G., Bricout, F., d'Hermies, F., Cheymol, A., 1999. Study of prophylaxis by didecyl dimethyl ammonium chloride against herpes simplex virus infection in nude mice. C. R. Acad. Sci. III, Sci. Vie, 322, 863-870.

Arima, H., Motoyama, K., Irie, T. Recent findings on safety profiles of cyclodextrins, cyclodextrin conjugates, and polypseudorotaxanes. In: Bilensoy, E. (Ed.) Cyclodextrins in pharmaceutics, cosmetics, and biomedicine: current and future industrial applications, Wiley, Hoboken, NJ, U.S.A., 2011, pp. 91-122.

Astray, G., Gonzalez-Barreiro, C., Mejuto, J. C., Rial-Otero, R., Simal-Gándara, J., 2009. A review on the use of cyclodextrins in foods. Food Hydrocolloid, 23, 1631–1640.

Beck, G. J., Kerslake, E. D. S., Olejnik, O., 2002. Preserved cyclodextrin-containing compositions. U.S. Patent 0,076,449.

Chepurnov, A. A., Bakulina, L. F., Dadaeva, A. A., Ustinova, E. N., Chepurnova, T. S., Baker, J. R. Jr., 2003, Inactivation of Ebola virus with a surfactant nanoemulsion. Acta Trop. 87, 315-320.

Debouzy, J.C., Fauvelle, F., Crouzy, S., Girault, L., Chapron, Y., Göschl, M., Gadelle, A., 1998. Mechanism of α-cyclodextrin induced hemolysis: a study of the factors controlling the association with serine-, ethanolamine-, and choline-phospholipids. J Pharm Sci. 87, 59-66.

Del Valle, E. M. M., 2004. Cyclodextrins and their uses: a review. Process Biochem. 39, 1033-1046.

Denyer, S. P., 1995. Mechanisms of action of antibacterial biocides. Int. Biodeter. Biodegr. 36, 227-245.

Ebola situation report. World Health Organisation. [March 30, 2016].

Fauvelle, F., Debouzy, J. C., Crouzy, S., Göschl, M., Chapron, Y., 1997. Mechanism of α–cyclodextrin–induced hemolysis: the twostep extraction of phosphatidylinositol from themembrane. J. Pharm. Sci. 86, 935-943.

Frijlink, H. W., Eissens, A. C., Hefting, N. R., Poelstra, K., Lerk, C. F., Meijer, D. K. F., 1991. The effect of parenterally administrated cyclodextrins on cholesterol levels in the rat. Pharmaceut. Res. 8, 9-16.

Funasaki, N. Ohigashi, M., Hada, S., Neya, S., 2000. Surface tensiometric study of multiple complexation and hemolysis by mixed surfactants and cyclodextrins. Langmuir, 16, 283-388.

Funasaki, N., Ohigashi, M., Hada, S., Neya, S., 1999. Quantitative Prediction of the Suppression of Drug-Induced Hemolysis by Cyclodextrins from Surface Tension Data. Langmuir, 15, 594–599.

Funasaki, N., Okuda, T., Neya S., 2001. Mechanisms and surface chemical prediction of imipramine-induced hemolysis suppressed by modified cyclodextrins. J. Pharm. Sci. 90, 1056-1065.

Gerba, C. P., 2015. Quaternary ammonium biocides: efficacy in application. Appl. Environ. Microbiol. 81, 464-469.

Gilbert, P., McBain, A. J., 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin. Microbiol. Rev. 16, 189-208.

Hall, C. B., 1977. The shedding and spreading of respiratory syncytial virus. Pediatr. Res. 11, 236-239.

Hauthal, H. G., 2004. Dynamic surfactants and nanostructured surfaces for an innovative industry. SÖFW-Journal, 130, 3-17.

Irie, T., Otagiri, M., Sunada, M., Uekama, K., Ohtani, Y., Yamada, Y., Sugiyama, Y. 1982. Cyclodextrin-induced hemolysis and shape changes of human erythrocytes in vitro; J. Pharm. Dyn. 5, 741-744.

Jambhekar, S. S., Breen, P., 2016a. Cyclodextrins in pharmaceutical formulations: structure and physicochemical properties, formation of complexes, and types of complex. Drug Discov. Today, 21, 356-362.

Jambhekar, S. S., Breen, P., 2016b. Cyclodextrins in pharmaceutical formulations: solubilization, binding constant, and complexation efficiency. Drug Discov. Today, 21, 363-368.

Kärber, G., 1931. Beitrag zur kollectiven Behandlung pharmakologischer Reihenversuche. Arch. Exp. Path. Pharmak. 162, 480-487.

Kurkov, S. V., Loftsson, T., 2013. Cyclodextrins. Int. J. Pharm. 453, 167-180.

Leclercq, L., Lubart, Q., Aubry, J.-M., Nardello-Rataj, V., 2013. Modeling of multiple equilibria in the self-aggregation of di-*n*-decyldimethylammonium chloride/octaethylene glycol monododecyl ether/cyclodextrin ternary systems. Langmuir, 29, 6242-6252.

Leclercq, L., Lubart, Q., Dewilde, A., Aubry, J.-M., Nardello-Rataj, V., 2012. Supramolecular effects on the antifungal activity of cyclodextrin/di-n-decyldimethylammonium chloride mixtures. Eur. J. Pharm. Sci. 46, 336-345.

Leclercq, L., Nardello-Rataj, V., 2016. Pickering emulsions based on cyclodextrins: A smart solution for antifungal azole derivatives topical delivery. Eur. J. Pharm. Sci. 82, 126-137.

Leclercq, L., Nardello-Rataj, V., Rauwel, G., Aubry, J.-M., 2010a. Structure-activity relationship of cyclodextrin/biocidal double-tailed ammonium surfactant host-guest complexes: towards a delivery molecular mechanism? Eur. J. Pharm. Sci. 41, 265-75.

Leclercq, L., Nardello-Rataj, V., Turmine, M., Azaroual, N., Aubry, J.-M., 2010b. Stepwise aggregation of dimethyl-di-n-octylammonium chloride in aqueous solutions: from dimers to vesicles. Langmuir 26, 1716-1723.

Leclercq, L., Sauthier, M., Castenet, Y., Mortreux, A., Bricout, H., Monflier, E., 2005. Two-phase hydroformylation of higher olefins using randomly methylated α-cyclodextrin as mass transfer promoter: a smart solution for preserving the intrinsic properties of the rhodium/trisulfonated triphenylphosphine catalytic system. Adv. Synth. Catal. 347, 55-59.

Leclercq, L., Smart medical textiles based on cyclodextrins for curative or preventive patient care. In: Hu, J. (Ed.) Active coatings for smart textiles, Woodhead Publishing, Duxford, U.K. 2016, pp. 391-427.

Lehner, S. J., Müller, B. W., Seydel, J. K., 1993. Interactions between p-hydroxybenzoic acid esters and hydroxypropyl-β-cyclodextrin and their antimicrobial effect against Candida albicans. Int. J. Pharm. 93, 201-208.

Lehner, S. J., Müller, B. W., Seydel, J. K., 1994. Effect of hydroxylpropyl-β-cyclodextrin on the antimicrobial action of preservatives. J. Pharm. Phamacol. 46, 186-191.

Leventis, R., Silvius, J. R. 2001. Use of cyclodextrins to monitor transbilayer movement and differential lipid affinities of cholesterol. Biophys. J. 81, 2257-2267.

Loftsson, T., Stefánsdóttir, Ó., Friòriksdóttir, H., Guòmundsson, Õ., 1992. Interactions between preservatives and 2-hydroxypropyl-β-cyclodextrin. Drug Dev. Ind. Pharm. 18, 1477-1484.

Malkin, A. J., McPherson, A., Gershon, P. D., 2003. Structure of intracellular mature vaccinia virus visualized by in situ atomic force microscopy. J. Virol. 77, 6332-6340.

Moore, L. E., Ledder, R. G., Gilbert, P., McBain, A. J., 2008. In vitro study oft he effect of cationic biocides on bacterial population dynamics and susceptibility. Appl. Environ. Microbiol. 74, 4825-4834.

Moore, S. L., Payne, D. N. Types of antimicrobial agents. In: Fraise, A. P. Lambert, P. A. Maillard J.-Y. (Eds.) Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization (4th Ed.), Blackwell Science, Oxford, U.K. 2004, pp. 8-97.

Nardello-Rataj, V., Leclercq, L., 2014. Encapsulation of biocides by cyclodextrins: toward synergistic effects against pathogens. Beilstein J. Org. Chem. 10, 2603-2622.

Ohtani, M., Irie, T., Uekama, K., Fukunaga, K. Pitha, J. 1989. Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes. Eur. J. Biochem. 186, 17-22.

Plomp, M., Rice, M. K., Wagner, E. K., McPherson, A., Malkin, A. J., 2002. Rapid visualization at high resolution of pathogens by atomic force microscopy. Am. J. Pathol. 160, 1959-1966.

Rauwel, G., Leclercq, L., Criquelion, J., Aubry, J.-M., Nardello-Rataj, V., 2012. Aqueous mixtures of di-n-decyldimethylammonium chloride/polyoxyethylene alkyl ether: dramatic

influence of tail/tail and head/head interactions on co-micellization and biocidal activity. J. Colloid Interface Sci. 374, 176-186.

Schmidt, A., von der Eltz, H., Kaluza, K., 1996. Cyclodextrin-biocide complex. U.S. Patent 5,506,216.

Shiotani, K., Uehata, K., Irie, T., Uekama, K., Thompson, D. O., Stella, V., 1995. Differential effects of sulfate and sulfobutyl ether of beta-cyclodextrin on erythrocyte membranes in vitro. J. Pharm. Res. 12, 78−84.

Simpson, W. J., 1992. Neutralisation of the antibacterial action of quaternary ammonium compounds with cyclodextrins. Microbiol. Lett. 90, 197-200.

Spearman, C., 1908. The method of 'right and wrong cases' ('constant stimuli') without Gauss' formulae. Br. J. Psychol. 2, 227-242.

Stella, V. J., He, Q., 2008. Cyclodextrins. Toxicol. Pathol. 36, 30-42.

Szejtli, J., 1998. Introduction and general overview of cyclodextrin chemistry. Chem Rev. 98, 1743-1754.

Utley, T. J., Ducharme, N. A., Varthakavi, V., Shepherd, B. E., Santangelo, P. J., Lindquist, M. E., Goldenring, J. R., Crowe, Jr. J. E., 2008. Respiratory syncytial virus uses a Vps4-independent budding mechanism controlled by Rab11-FIP2. Proc. Natl. Acad. Sci. USA 105, 10209-10214.

Valenti, W. M., Menegus, M. A., Hall, C. B., Pincus, P. H., Douglas, R. G. Jr., 1980. Nosocomial viral infections: epidemiology and significance. Infect Control., 1, 33-7.

Wallace, K. B., Khan, M. A., Carlson, R. M., Rice, S., Froberg, M. K., 2003. Cyclodextrin compositions and methods of treating viral infections. U.S. Patent 0,220,294.

Weavers, L. K., Wickramanayake, L. K. Kinetics of inactivation of microorganisms. In: Block, S. S. (Ed.) Disinfection, Sterilization and Preservation (5th Ed.), Lippincott Williams & Wilkins, Philadelphia, U.S.A. 2001, pp. 65-78.

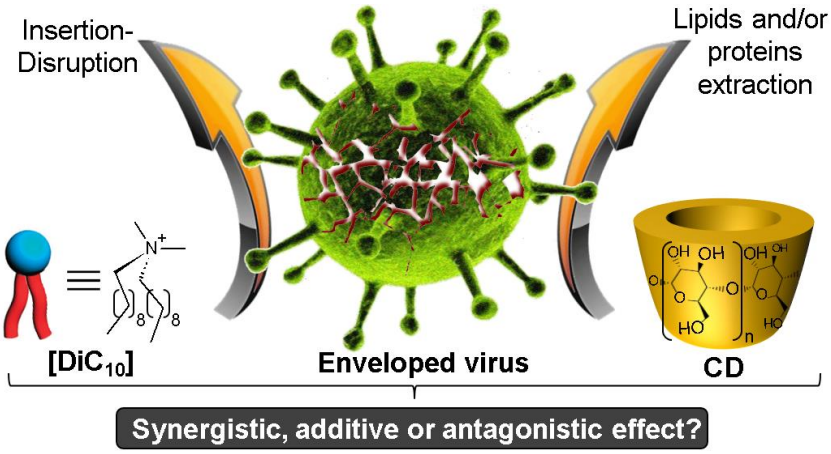


Figure 1. Effects of di-*n*-decyldimethylammonium cation, [DiC_{10}], and natural cyclodextrins, CDs, on enveloped viruses.

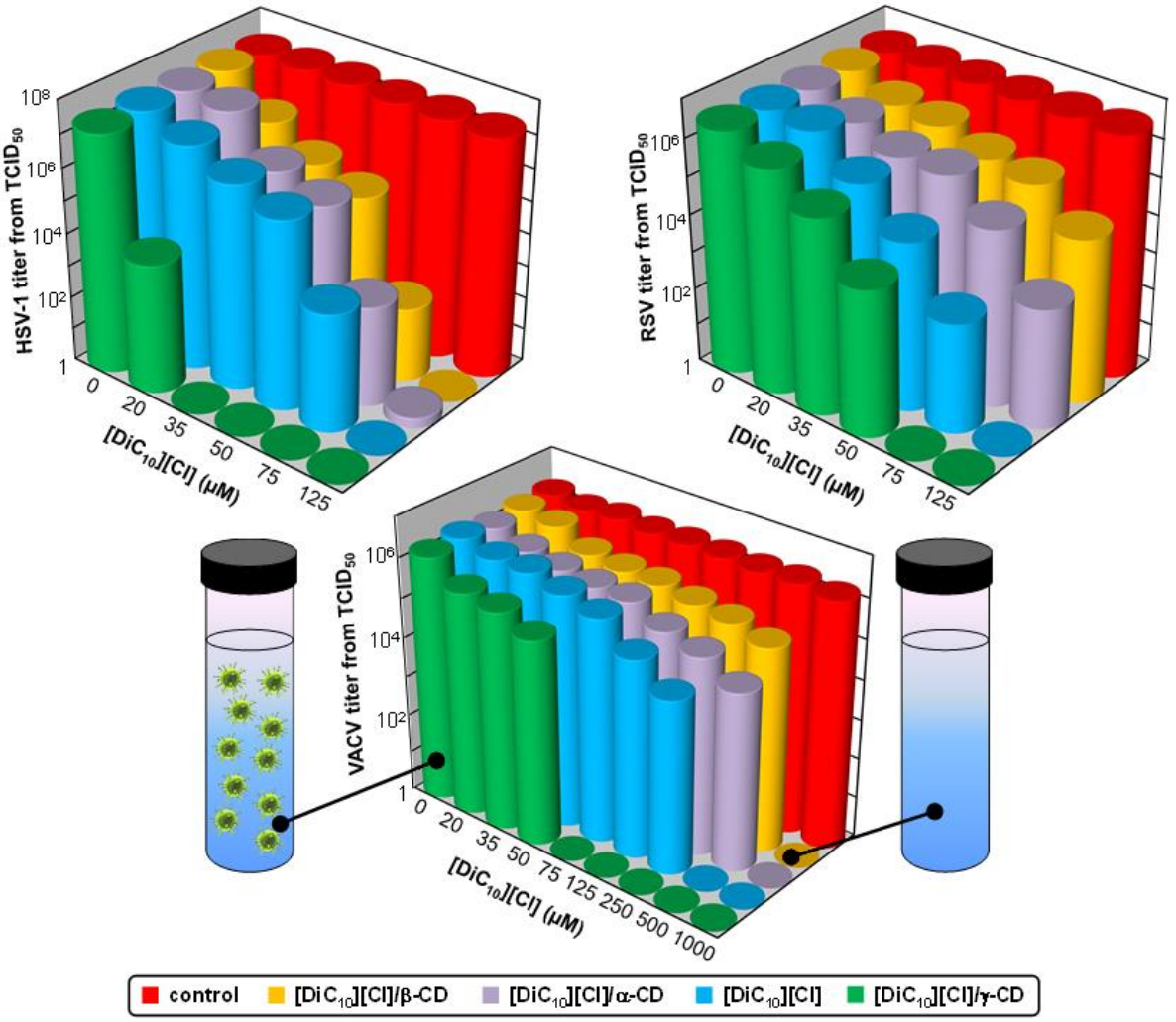


Figure 2. Dose-dependent virucidal action of $[DiC_{10}][Cl]$ with or without natural CDs from herpes simplex (HSV-1), respiratory syncytial (RSV) and vaccinia virus (VACV) (NB. the control experiments correspond to the viral titer in the absence of CDs and $[DiC_{10}][Cl]$; the standard deviation of virus titer is $\pm$ 10%).

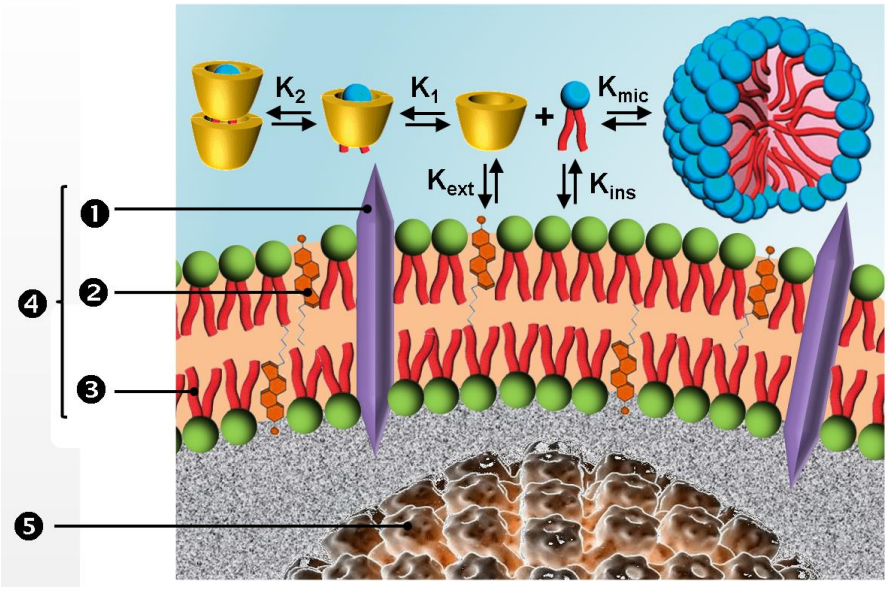


Figure 3. Schematic interrelation among the equilibria of complexation (K_1 and K_2), micellization (K_{mic}), CD-induced lipids extraction (K_{ext}), and [DiC_{10}] insertion (K_{ins}) for a binary mixture of [DiC_{10}][Cl] and CD in the presence of viruses (1. protein, 2. cholesterol, 3. phospholipid, 4. envelope, and 5. nucleocapsid).

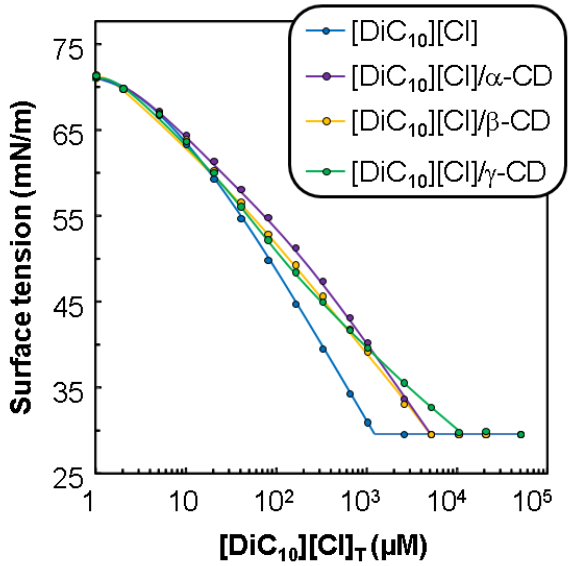


Figure 4. Surface tension (σ) plotted against total $[DiC_{10}][Cl]$ concentration for equimolar $[DiC_{10}][Cl]$/CD binary mixtures at 25.0 °C compare to $[DiC_{10}][Cl]$ alone.

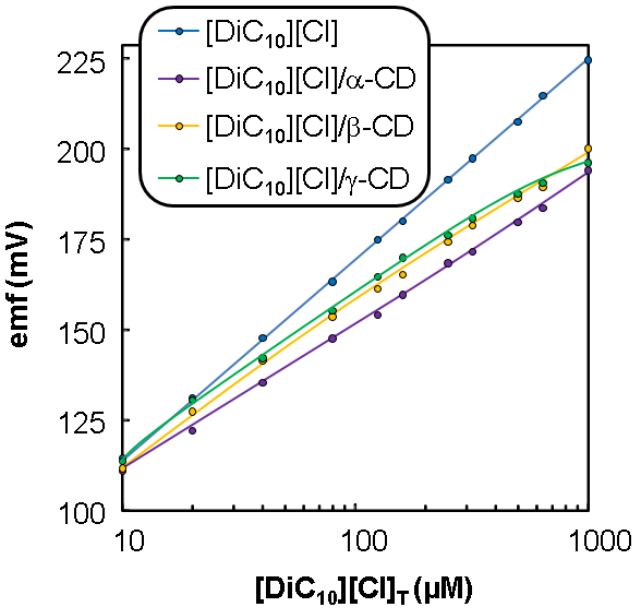


Figure 5. Electromotive force (emf) plotted against total $[DiC_{10}][Cl]$ concentration for equimolar $[DiC_{10}][Cl]$/CD binary mixtures at 25.0 °C compare to $[DiC_{10}][Cl]$ alone.

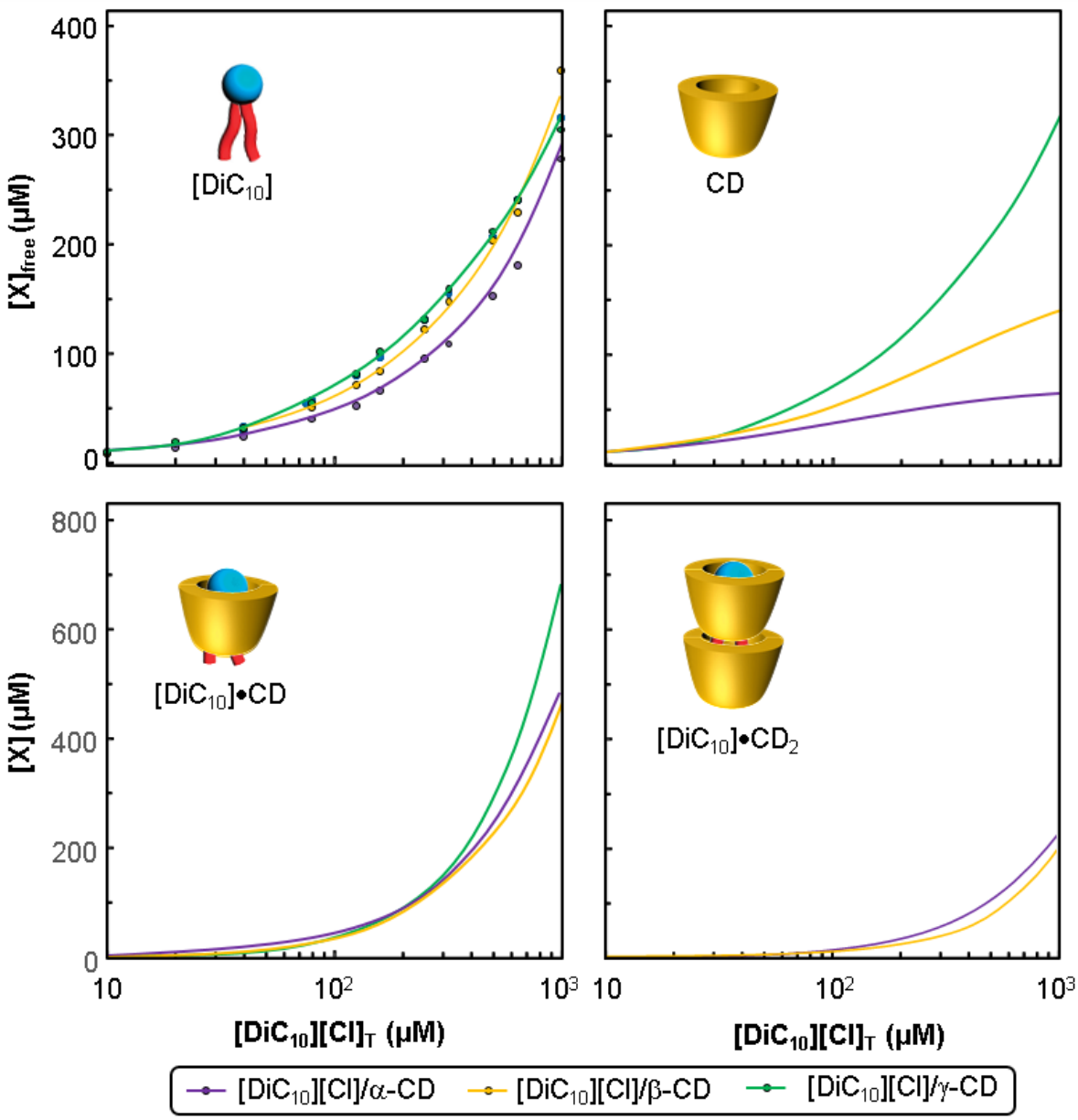


Figure 6. Evolution of free [DiC_{10}], free CD, [DiC_{10}]•CD, and [DiC_{10}]•CD_2 concentrations as a function of the total [DiC_{10}][Cl] concentration for equimolar [DiC_{10}][Cl]/CD binary mixtures at 25.0 °C in steady state. Circles correspond to experimental values whereas continuous lines are calculated from the binding constants (see equations. 2-6).

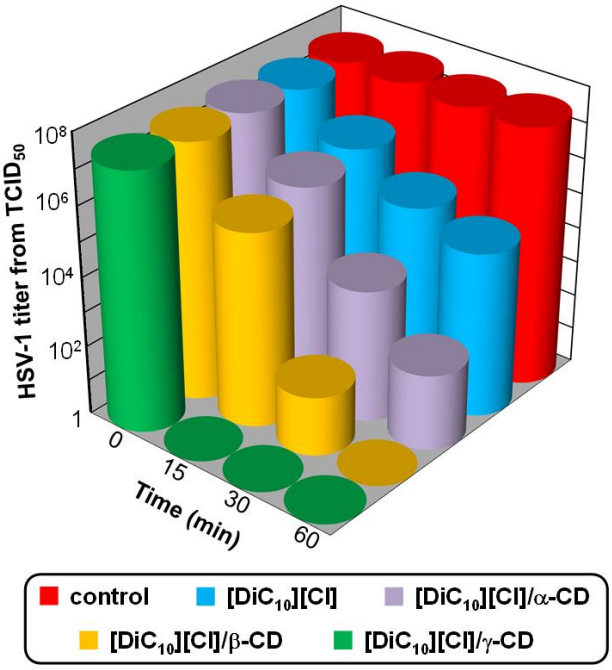


Figure 7. Time-dependent virucidal action of $[DiC_{10}][Cl]$ with or without natural CDs (35 μM each) from herpes simplex (HSV-1) (NB. the standard deviation of virus titer is ± 10%).

Table 1. Binding constants values between [DiC_{10}][Cl] and native CDs.[a]

CD	K_1 (M^{-1})[a]	K_2 (M^{-1})[a]	R^{2b}
α-CD	26 000	7 500	0.9929
β-CD	9 700	2 900	0.9945
γ-CD	7 600	0	0.9979

[a] Determined from [DiC_{10}]-selective electrode at 25 °C (Leclercq et al., 2010a). [b] obtained in this work from equations 2-6 and binding constants (K_1 and K_2).

Table 2. Toxicity and affinity of native CDs and thermodynamic parameters and virucidal activity of $[DiC_{10}][Cl]$/CD mixtures.

		α-CD	β-CD	γ-CD
Toxicity of native CDs	Hemolysis[a]	++	+++	+
	Cytotoxicity[b]	+++	++	+
	Degradability[c]	+++	+++	+++
CD-affinity for	Phospholipids[d]	+++	++	+
	Cholesterol[d]	+	+++	++
	$[DiC_{10}][Cl]$/CD mixtures			
Steady state	Complexes[e]	1:1 + 1:2	1:1 + 1:2	1:1
	$[CD]_{free}$[e]	$\ll [DiC_{10}]_{free}$	$< [DiC_{10}]_{free}$	$= [DiC_{10}]_{free}$
Virucidal activity	Efficiency compared to $[DiC_{10}][Cl]$ alone[e]	$\approx [DiC_{10}][Cl]$	$\approx [DiC_{10}][Cl]$	$\ll [DiC_{10}][Cl]$
	Inactivation rate[e]	+	++	+++

[a] From Irie et al., 1982. [b] From Arima et al., 2011. [c] From Antlsperger & Schmid, 1996 (according to OECD 302B). [d] From Ohtani et al., 1989. [e] This work.