# Cyclodextrins: More than Pharmaceutical Excipients

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**Abstract:** Cyclodextrins are pharmaceutical excipients used to enhance the solubility, stability, safety and bioavailability of drugs. Recent findings have shown them to display adjuvant activity in vaccine therapy and prophylactic and therapeutic activity in the treatment of several host-pathogen infections. This article focuses on their activity and mechanism of action.

**Keywords:** Cyclodextrins, protein refolding, *Bacillus anthracis*, *Cryptosporidium spp.*, Human Immunodeficiency Virus, immunomodulation, *Leishmania spp.*, *Plasmodium spp*.

## INTRODUCTION

Cyclodextrins (CDs) are useful multi-functional excipients that are known to interact with poorly water-soluble molecules *via* dynamic complex formation [1, 2]. The most common pharmaceutical application of cyclodextrin inclusion complexes derives from their capacity to alter the physicochemical properties of the guest molecules, thereby improving the aqueous solubility and dissolution rate [3-5], stability [6, 7], safety [6-8] and bioavailability [7, 9] of drugs. Cyclodextrins are often used with drugs, either as inclusion complexes or as auxiliary additives such as solubilizers, stabilizers and diluents. A further advantage of cyclodextrins is the absence of systemic toxic effects associated with oral or topical administration; some of their derivatives are safe substances approved for parenteral administration [1, 5]. These properties have positioned CDs as important enabling and functional pharmaceutical auxiliary substances.

The CDs of pharmaceutical interest are either natural or modified cyclic oligosaccharides composed of six to eight ($\alpha$-1,4)-linked D-glucopyranose units (Fig. **1**). The pharmaceutical and industrial applications of CDs, as excipients or absorption promoters, have already been discussed in several excellent reviews and books [10-15] and are not the focus of this article. Recent findings have shown that CDs display a more active role in drug formulation, and have been shown to be useful as protein refolding or renaturating agents [16, 17] or as adjuvant in vaccines [18]. Several recent studies have revealed that CDs can also act as drugs and display activity in the treatment of several host-pathogen infections. Specifically, CDs have been found to be interesting candidate microbicides for preventing HIV-1 infections [19, 20], providing protection against lethal anthrax toxin [21-23], and inhibiting the entry of Plasmodium into red blood cells [24]. Finally, CDs have also displayed effective prophylactic and therapeutic activity in the treatment of Cryptosporidiosis [25-27] and Leishmaniosis [28].

Several studies have indicated the crucial requirement of cholesterol in host–pathogen interactions. CDs have been widely reported to modulate cell membrane cholesterol levels and induce cholesterol depletion, and this appears to be the main mechanism of action involved in the important inhibitory activity of cyclodextrins and cyclodextrin derivatives during invasion by different pathogens.

The purpose of this review is to summarize and discuss some of the interesting findings of active applications of cyclodextrins (CDs) and their derivatives. This article covers some important properties of CDs related to aspects such as protein refolding, immunomodulatory activity in vaccine therapy and prophylactic and therapeutic activity in the treatment of several host-pathogen infections. In addition, we also describe the main mechanisms underlying the action of cyclodextrin at the cellular level.

## CYCLODEXTRINS AND PROTEIN REFOLDING

Protein refolding consists of the self-assembly of disordered polypeptide chains into packed protein structures that exert biological functions. It is well known that proteins in a misfolded state are not functionally relevant and in fact, many diseases are often known as misfolding or conformational diseases [29]. However, under *in vitro* denaturing conditions a protein can recover its native state by removing the denaturant, thus inhibiting the tendency of the protein to aggregate irreversibly *in vitro*. Furthermore, protein aggregation can be affected by many factors during refolding, such as temperature, pH, ionic strength, type and concentration of denaturant and protein, and by some of the steps in biopharmaceutical protein processing, such as heat treatment, filtration, shaking/shearing, spraying, drying, fermentation, reconstitution, formulation and storage [30].

A large and growing number of protein and peptides are used in therapy and biotechnology, although application in the pharmaceutical field is hampered in many cases by the tendency of these to self-aggregate, the most common manifestation of instability. Self-aggregation of proteins modifies

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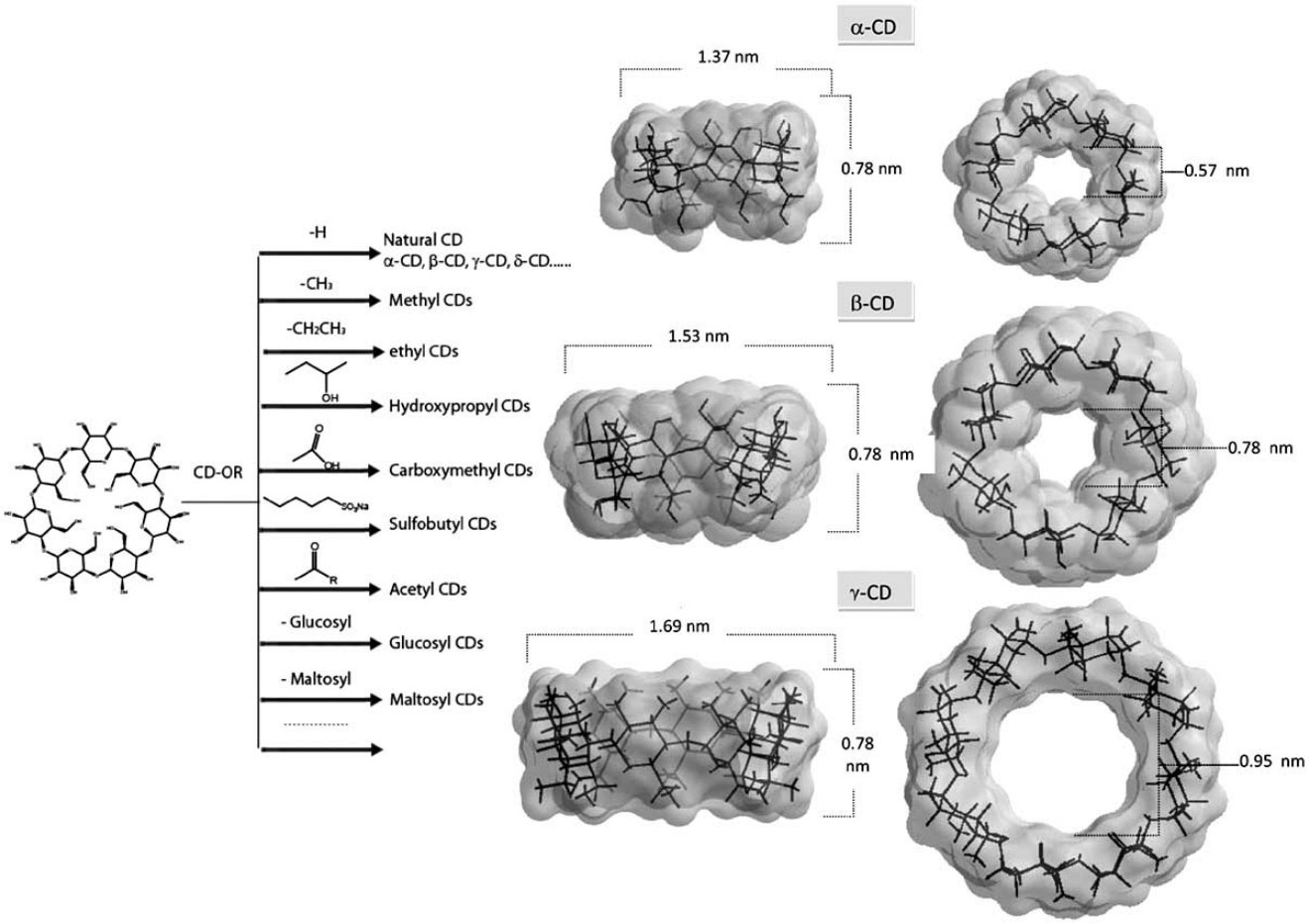


**Fig. (1).** General structure of most common cyclodextrins and their abbreviated names.

their properties; it is irreversible and leads to loss of function. In aqueous solution, the three dimensional structure of a protein in its native conformation has the more hydrophilic amino acid exposed to the aqueous solvent, and the more hydrophobic residues facing the interior. Under certain conditions, the hydrophobic residues that are normally covered will tend to interact intermolecularly in a nonspecific manner between hydrophobic regions of the partially folded polypeptide chains, thus causing aggregation of the protein. It is commonly accepted that aggregation competes with the proper folding pathways [30]. Prevention of self-aggregation is a key factor in enhancing the refolding and renaturation of proteins.

Use of CDs to prevent protein aggregation offers certain advantages such as low cost, non-toxicity, biocompatibility, availability and ease of separation from protein by for example, dialysis or gel filtration. During refolding, these clusters lead to a reduction in the active protein. A wide range of other aggregation inhibitors or folding agents have also been used, such as polyethylene glycol [31, 32], polyamino acids [33], sugars and surfactants [34-36]. The term "artificial chaperones" is usually employed to describe CDs and other non-protein additives that prevent protein aggregation. Apart from the modulation of folding and aggregation, the folding molecule must aid efficiently without interfering during protein folding and moreover, must be easily separated from the protein. Cyclodextrins have also been used in combination with other additives, as secondary removal agents for refolded protein after use of a surfactant in a first step as an anti-aggregating agent [16, 37, 38]. Protein-surfactant micelles are disrupted in the presence of CDs due to the formation of strong complexes between the CD and surfactant, and consequently refolding of the protein is allowed to be completed.

Refolding of proteins is also especially useful in the production of recombinant proteins produced in the form of inclusion bodies in transformed host cells. Many proteins of commercial importance, such as insulin, tissue plasminogen activator, etc., are purified from these inclusion bodies, which contain the misfolded protein and are thus functionally inactive. In the first step, the protein is isolated from inclusion bodies and is recovered by solubilising and finally refolding the protein. In order to solubilise these proteins and prevent their aggregation while allowing folding to occur to the native state, the inclusion of CDs in the medium may improve the recovery yield of the protein in the process. The ability of CDs to stabilize proteins is also particularly useful in protecting them against thermal denaturation and degradation. Studies of cyclodextrins that inhibit protein aggregation and refolding from the denatured state have been reported, and show how CDs can be used as agents to prolong the shelf-life of therapeutic proteins such as insulin [39] and

growth hormones [40-43]. Human growth hormone contains a large number of aromatic amino acids in the native state and is thus susceptible to aggregation under certain conditions, and it has been shown that β-CD and 2-hydroxypropyl-β-CD and to a lesser extent γ-CD and α-CD prevent protein aggregation and enhance hormone solubilisation in a concentration dependent manner [43].

CDs have been found to serve as aggregation suppressors for a wide range of proteins, most of them enzymes, while maintaining their functions. For example, α-CD, γ-CD and 2-hydroxypropyl-β-CD have been used to prevent aggregation of carbonic anhydrase II (or carbonic anhydrase B) [44] after denaturation by incubation in guanidine hydrochloride at pH 8.5. The aggregation inhibition was dependent on the concentration of cyclodextrin, and the recovery of the active enzyme was about 90%. However, refolding of carbonic anhydrase B was decreased at protein concentrations higher than 100 mM in the presence of varying amounts of CD (0-100mM) owing to increased protein aggregation. In the case of carbonic anhydrase, neutral or cationic CDs and those with small inner cavities were demonstrated to be more efficient for folding than anionic CDs or those with larger cavities. Some others, such as carboxymethyl-α-CD, were found to promote aggregation during protein refolding, resulting in even lower yields obtained during carbonic anhydrase refolding without the use of any other substances [45]. The ability of CDs to act as artificial "chaperones" by enhancing refolding of proteins from a denatured or aggregation state has been also shown with a large variety of proteins. In the case of lysozyme, α-CD was more effective than γ-CD as a refolding agent at similar molar CD concentrations. However, the order of efficacy was inverted when a different denaturing process was used for lysozyme [46]. Other neutral CDs also enhanced lysozyme folding, thus preventing protein aggregation [47, 48]. In general, the presence of anionic ring substituents (carboxyl of phosphate groups) diminished the renaturation ability of CDs. This effect has also been observed with carbonic anhydrase [44]. CDs have also been found to have similar folding aid effects with denatured phosphofructokinase [49], cellulase [50], alkaline phosphatase [51], citrate synthase [52] and amylase [53, 54]. Denatured proteins were also treated with oligosaccharides without a cavity such as maltohexaose or glucose, and no renaturation effects were observed. Recently, methyl-β-CD and 2-hydroxypropyl-β-CD have been shown to be good aggregation agents for mink and porcine growth hormones during the renaturation process, when expressed in *E. coli* as inclusion bodies [55]. Furthermore, CDs have also been used to suppress the temperature related aggregation of both polypeptides. Temperature was also an important factor in aggregate suppression during refolding of human growth hormone with α-CD [56].

Elucidation of the mechanism by which CD acts as a refolding agent is still controversial. Refolding of proteins into their three dimensional structure (native state) from a linear chain usually takes place through intermediate species that contain hydrophobic aggregates. The aromatic residues of the protein are involved in the formation of nuclei regions and irreversible aggregates. These hydrophobic sites in the folding intermediate can form inclusion complexes with CDs. It is known that hydrophobic aromatic amino acids such as tryptophan and phenylalanine are able to form weak inclusion complexes with CDs [57]. The internal hydrophobic cavity of CD is bound to these hydrophobic regions of the protein acting as a hydrophilic layer, which increases the solubility of the protein [53]. These studies of protein refolding with CDs were carried out in the presence of competitors (tryptophan, phenylalanine, leucine) and the role of the CD cavity was clear and explains the reduction in undesirable protein aggregation. Several studies have demonstrated the specific interaction between cyclodextrins and specific protein sites (hydrophobic amino acids) by different techniques, e.g. Cooper used differential scanning calorimetry (DSC) for this [58], and Horsky and Pitha [59] used competitive spectrophotometry with p-nitrophenol as a competing reagent to demonstrate the interaction between peptides containing L-phenylalanine. Matsubara [60] used DSC to characterize of α-chymotrypsin with maltosyl-β-CD. Maletic [61] demonstrated the complex formation between β-CD and phenylalanine residues by use of calorimetric titration and capillary eletrophoresis, and Koushik [62] demonstrated the interaction between 2-hydroxypropyl-β-CD and deslorelin by use of UV and fluorescence spectroscopy. The inclusion complexes between CDs and aromatic amino acids have been demonstrated in NMR studies by Otzen [43] (human growth hormone), and by Aachman [63], for β-CD and ubiquitin, chymotrypsin inhibitor 2 (CI2), S6 and insulin. Qin [54] showed how a peptide involved in Alzheimer's disease (amyloid- β peptide) was included in the β-CD cavity by its phenyl rings, resulting in lower toxicity in *in vitro* cultured cells.

However, other studies have demonstrated that the CD cavity is not essential for inhibiting aggregation and enhancing protein refolding. In the case of lysozyme, the denatured enzyme was refolded in the presence of CD and a competitor, which formed strong inclusion complexes with the CD cavity. The recovery yield of protein was similar to that obtained without competitors in the medium [37, 47]. In summary, many aspects, such as CD cavity, protein structure, denaturation conditions, ring substituents and other features play a role in the important protein refolding activity of cyclodextrins. Efficient refolding of proteins requires better understanding of the factors involved in the process and different cyclodextrins are probably required for refolding different proteins.

## ANTIVIRAL ACTIVITY OF CYCLODEXTRINS

Raft membrane microdomains are very important in the process of virus infection. Cholesterol in particular plays an important role in different aspects of the life cycle of several viruses [64]. The importance of cholesterol in the entry of nonenveloped viruses has been demonstrated for simian virus 40 (SV40), rotavirus, enterovirus, echovirus, adenovirus, reoviridae, and rhinovirus [65, 66]. Entry of enveloped viruses into cells involves binding to specific receptors and fusion of the viral membrane with a cellular membrane. These processes require cholesterol in the target cellular membrane and/or in the viral envelope membrane [67]. For example, influenza virus [68] and canine distemper virus infection [64] are sensitive to cholesterol depletion from the viral membrane, but murine leukemia virus [69], Ebola virus, and Marburg virus [70] are sensitive to cholesterol de-

pletion from the target cellular membrane. In other cases, such as human immunodeficiency virus (HIV), herpes simplex virus, hepatitis C virus and gastroenteritis virus, cholesterol is required in both membranes for effective viral infection [68, 71-73].

Numerous studies have demonstrated that interaction with CDs and consequent depletion of cell membranes and/or viral envelope components somehow interfere in the infection processes of many viruses. In fact, methyl-β-CD, one of the CD derivatives that interacts most strongly with cholesterol, is commonly used by scientists as a useful tool for demonstrating the role of cholesterol in the process of entry of virus into the cells. These studies demonstrate the ability of cyclodextrins to reduce viral infections through cholesterol depletion, suggesting the potential use of these sugars in the prevention and treatment of some infectious diseases.

One of the best studied antiviral activities of CDs is their possible use in the prevention of HIV infection. As reported by Moriya *et al.* [19], some of the first reports regarding the anti HIV-1 activity of CDs was presented at the 37$^{th}$ meeting Society of Japanese Virologists in November 1989. These early studies on the anti-HIV activity of cyclodextrins were carried out with sulphated CDs derivatives that proved to be potent inhibitors of human immunodeficiency virus (HIV), cytomegalovirus (CMV) and herpes simplex virus (HSV) [19, 20] in which CD activity was attributed to inhibition of the binding of HIV-1 virions to the cells. Among the sulphate derivatives studied, the molecule in which the C-2 position was modified with a lipophilic benzyloxy group exhibited the best anti-HIV activity. Nevertheless, although this CD derivative was absorbed after oral administration in animal experiments, the absorption rate after oral administration was not sufficient for clinical use [74]. The authors proposed the use of these derivatives in vaginal pessaries, combined with the use of condoms, to inhibit transmission of free infectious virions to the cells as well as cell-to-cell transmission.

Along the same line, in the 1990s, Hildreth *et al*. [75-77] performed studies of how the cholesterol membrane affects the infectivity of the HIV virus. These authors discovered that removing cholesterol from cell membranes with 2-hydroxypropyl-β-CD as depleting agent inhibits the infectious ability of HIV [78]. These findings raise some hope that β-CDs may have the potential to act as a microbicide, which could be applied topically to the vagina or rectum to prevent HIV transmission. Hildreth and colleagues tested 2-hydroxypropyl-β-CD in mice carrying human immune cells, and reported that CDs were effective in reducing HIV infection *via* the vagina by 95%, with no side effects, compared with the microbiocidal compound nonoxynol-9 used as a spermicidal. These natural sugars can be safely used in humans, as they are classified as GRAS excipients, and at the moment studies are being carried out to evaluate the potential of this molecule as a chemical condom to block the sexual transmission of HIV, herpes simplex virus, and other STD pathogens. Whether or not β-CD formulations in topical microbiocides will be clinically effective at slowing or stopping HIV infection remains to be demonstrated.

The important role that cholesterol plays in entry and infection of cells by HIV has been directly related to the anti-HIV activity of CD. Infection with HIV begins when the virus binds to the CD4 receptor, located in a lipid raft, and to a co-receptor (chemokine receptor sites CCR5 or CXCR4) on the host-cell surface. Attachment to the receptor and co-receptor enables HIV to fuse with the host-cell membrane and to empty its contents into the cell so that it can replicate. Percherancier *et al.* [79] suggested that the presence of HIV-1 receptors in rafts was not required for viral infection and even when the majority of CD4 was forced to locate in non-raft regions of the plasma membrane, HIV entry could still proceed. Nevertheless, this is in direct contrast with the opinion that HIV requires CD4 to be located in a raft to gain entry [80-83] In a study with the CD4 cell mutant RA5, Popik and Alce [83] concluded that raft localization of CD4 is not required for virus entry, however, post-binding fusion/entry steps may require lipid raft assembly, thus depletion of plasma membrane cholesterol inhibits HIV-1 entry. Cholesterol extraction with methyl-β-CD reduced signalling through CCR5 and completely abolished the inhibition of forskolin-stimulated cAMP accumulation, with no effect on internalization [84]. Recently, Cardaba *et al.* [84] demonstrated that depletion of cholesterol destroyed microdomains in the membrane, and concluded that membrane cholesterol was essential for CCR5 signalling and that integrity of lipid rafts is not essential for effective CCR5 internalization.

Virion-associated cholesterol is also critical for HIV-1 infection [85]. Different studies have demonstrated that depletion of virion-associated cholesterol leads to an increase in density of virion particles, de-stabilization of virion structure, and suppression of virion infectivity [77, 86]. The most probable role of cholesterol is to facilitate the entry process, as cholesterol depleted HIV-1 virions are able to bind to the cell membrane, but unable to enter the cells [87]. The correlation between infectivity and incorporation of raft-promoting sterols into virion particles suggests that the lipid order plays a crucial role in viral infectivity. The inclusion of raft-promoting sterols may provide the rigidity required to form a stable hemifusion stalk structure with the target cell membrane, which enables subsequent viral entry through formation of the fusion pore [85].

Therefore, the cholesterol depletion mediated by CDs may act by preventing the entry of the virus in the cells at two levels, thus modifying the conditions of the target cells membranes and the viral membrane.

## ANTIBACTERIAL ACTIVITY OF CYCLODEXTRINS

As described above, viruses require the integrity of lipid rafts for internalization. Entry *via* lipid rafts has also been documented for bacterial toxins such as the cholera toxin, anthrax toxin and the Listeria toxin (LLO). Among other bacteria, *Shigella*, *Salmonella*, the FimH-expressing *E. coli* strain, *Chlamydia* and *Listeria* require lipid rafts for their entry or the presence of their target receptors within lipid rafts [88]. Despite the large amount of research and development on CDs, very little information on the antimicrobial activity of CDs against pathogen bacteria has been published to date.

The cholera toxin is an enterotoxin produced by *Vibrio cholerae* and is composed of two subunits, A and B. The A subunit comprises two peptides, A1 and A2, connected by a disulphide bond. The A2 peptide binds to a pentamer of B subunits that have a high affinity for the cell surface GM1 ganglioside predominantly clustered in membrane rafts. Binding of the B subunit to GM1 enables endocytosis and subsequent transport to the endoplasmic reticulum [89]. Prevention of cholera toxin internalization is possible with CDs by cholesterol depletion and inhibition of dynamic function, which suggests that raft domains play a role in this process [89].

Anthrax is a deadly disease caused by *Bacillus anthracis*, a bacteria considered to be one of the most dangerous biological weapons. Currently, there is no effective treatment for inhalational of anthrax and the only approved therapy is the administration of antibiotics after exposure. However, antibiotic administration is ineffective as a therapy against anthrax if exposure to the bacterium has led to the production of levels of toxins and other virulence factors sufficient to kill the host [23]. The two toxins playing an essential role in anthrax pathogenesis are formed by three polypeptides: protective antigen, lethal factor and oedema factor. Protective antigen combines either with lethal and/or oedema factor to form lethal and oedema toxin respectively [90]. In 2005 Karginov *et al*. [21] demonstrated an approach to disabling the toxins based on the high-affinity blockage of the protective antigen pore by designing a low-molecular weight compound that prevented entry of lethal and oedema toxin into cells. Guided by the geometrical structure, polysymmetry and the negative charge net of the protective antigen pore (Fig. **2**), a modified positively charged β-cyclodextrin (per-substituted 6-aminoalkyl (per-6-aminoalkyl) derivatives of β-cyclodextrin), which effectively blocked protective antigen channel conductance *in vitro*, was synthesized. This β-CD derivative protected against lethal toxin mediated macrophage killing, and was able to prevent the death of Fischer F344 rats administered lethal or oedema toxin [90]. These CDs interacted strongly with the protective antigen pore lumen, blocking Protective Antigen-induced transport. In a recent study, the same research group [91] demonstrated that one of these βCDs derivatives administered in combination of the antibiotic ciprofloxacin was significantly more effective at protecting mice against *B. anthracis* infection than antibiotic treatment alone. The study showed a survival rate of up to 90% among animals treated with this CD derivative and ciprofloxacin. Other authors have pointed out the possibility of sequestering unknown lipids components, other than cholesterol, with molecular masses of 650-690 Da from the cell membrane of *Bacillus sp*. and related strains [92].

In addition, it has been proposed that entry of bacterial toxins into the cells *via* raft microdomains is fundamental in determining the fate of intracellular bacterial pathogens such as *Shigella*, *Salmonella*, the FimH-expressing *E. coli* strain, *Chlamydia* and *Listeria*. The process of entry appears to be cholesterol dependent, regardless of the specific molecules in lipid rafts targeted by microorganisms [93]. Pharmacological depletion or sequestration of plasma membrane cholesterol with CDs significantly decreases internalization and /or reduction of binding to surface cell receptors. Treatment of targets cells with methyl-β-CD resulted in the inhibition of the internalization of *E. coli* producing Dr fimbriae [93], of the entry of *Brucella* [94], *Mycobacterium* [95], *Listeria* [96] and resulted in a decrease in the total number of cell-associated *chlamydiae* and in significant (90%) inhibition of internalized *chlamydiae* [97]. In addition, cholesterol depletion with methyl-β-CD impaired the entry of *Shigella* [98] by decreasing bacterial binding to the surface cell receptor. Another example of antimicrobial activity of CDs is the inhibition of growth of *Mycobacterium sp*. in such a way that the

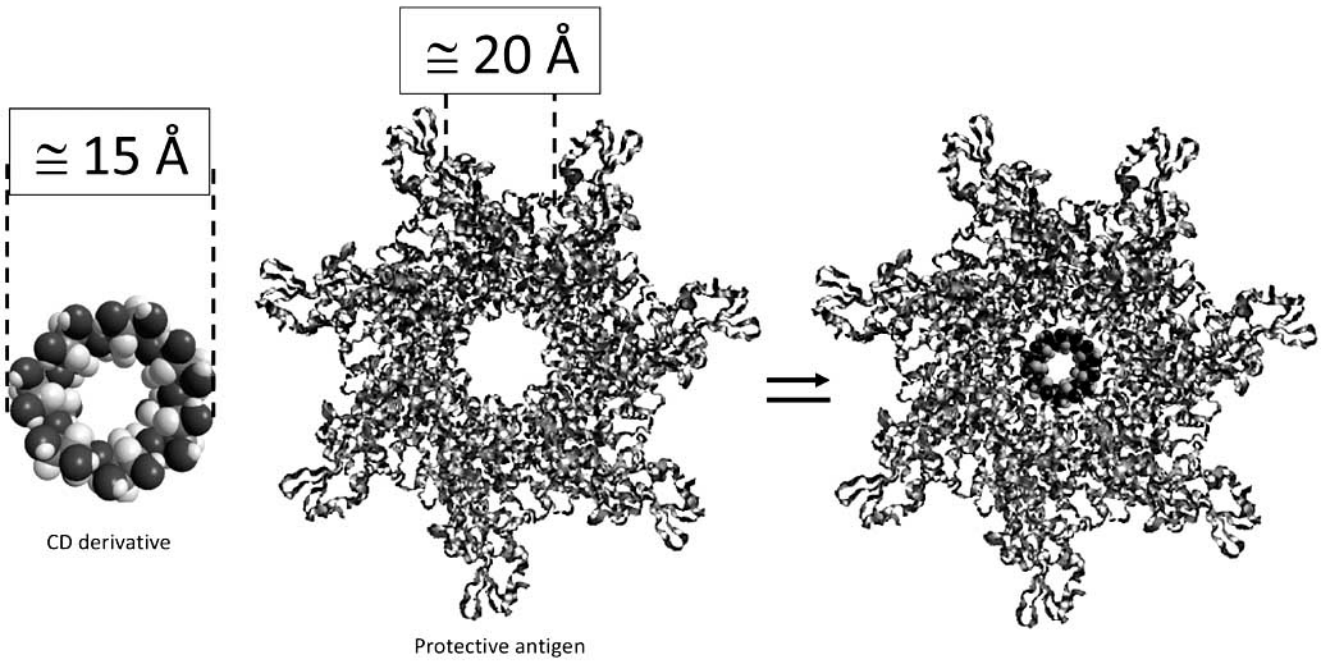


**Fig. (2).** Schematic illustration of a β-CD derivative (left) in comparison with the PA channel (right). Modified from [90].

microorganism was not able to grow in the presence of 180 mM of methyl-β-CD [99].

## ANTIPARASITIC ACTIVITY OF CYCLODEXTRINS

In addition to the effects on bacterial toxin and pathogens by CDs, cholesterol depletion by CDs has been found to inhibit the entry of and sustained infection by some intracellular parasites such as the protozoans *Cryptosporidium* [100], *Leishmania* [101, 102], *Trypanosoma* and the malaria parasite *Plasmodium* [24] . Some interesting studies have indicated the crucial requirement of cholesterol in these host–pathogen interactions. In case of some parasites such as the protozoans *Toxoplasma gondii* and *Trypanosoma cruzi*, an intricate relationship that has evolved between host cholesterol metabolism and infection by intracellular parasites has been demonstrated. The ability to manipulate levels of cholesterol in the membrane with a reasonable degree of specificity with cyclodextrins and/or cholesterol-sequestering agents like nystatin and filipin, has contributed to the understanding of the role of cholesterol in parasitological infection.

Several *in vitro* and *in vivo* studies with different animal species (mice, cows, sheep and goats) have shown that oral administration of cyclodextrins is one of the best and most effective palliative and preventive treatments in the treatment of cryptosporidiosis [27, 100, 103-109]. The first studies on *in vitro* anti-cryptosporidium activity of CDs showed that the viability and infectivity of purified *C. parvum* oocysts, exposed for 24 hours to β-CD [103] and α-CD [109] (2.5% suspension) were drastically reduced. These studies showed a high proportion of nonviable oocysts (81.5% and 81% respectively). Inoculation of mice with the treated oocysts showed a significantly lower intensity of experimental infection, determined 7 days post-inoculation, than in the control litters. In a more recent i*n vitro* study comparing the anti-cryptosporidium activity of CDs and some already described anti-cryptosporidium drugs in *C. parvum* oocysts [107] it was demonstrated that a high proportion of nonviable oocysts ranging from 30 to 40 % were present when in contact with α, β or both natural cyclodextrins. In contrast, the viability of oocysts surprisingly did not appear significantly affected by some of anticryptosporidial drugs described i.e., paromomycin, azithromycin, halofuginone lactate, toltrazuril and nitazoxanide did not show significant activity against the oocysts. Similarly, Castro-Hermida *et al.* [100, 110] have shown that oral administration of β-CD to suckling mice produced more *in vivo* anticryptosporidial activity than two antimicrobial, antiparasitic drugs, diloxanide furoate and G1, in prophylactic and therapeutic treatment. Finally, the results obtained in different *in vivo* studies have suggested that both β-CD and α-CD may be suitable for use in the early control of cryptosporidiosis.

The specific prophylactic and therapeutic anticryptosporidial activity mechanisms of CDs are still unknown, although it is suspected that CDs may act at three different stages of the parasite cycle.

In the first stage, cyclodextrins can interact with the components of the cell wall of *Cryptosporidium* oocysts, thereby modifying its permeability. It has been demonstrated that oocysts treated with cyclodextrins incorporated two fluorogenic vital dyes (4′ ,6-diamidino-2-phenylindole and propidium iodide) in a greater proportion that untreated oocysts. Mitschler *et al.* [111] analyzed the membrane lipid composition of *C. parvum* and described the presence of cholesterol and also indicated that phosphatidylcholine was the predominant lipid, comprising 65% of the total phospholipids. It is therefore likely that depletion of phospholipids and cholesterol at the oocyst cell wall level may occur by treatment with α and β-CD respectively, causing structural and diffusion alterations and consequently, a reduction in oocyst viability.

In the second stage, although the molecular and biochemical mechanisms involved in oocyst excystation step are poorly understood, it is believed that host environmental factors such as bile salts, proteases, temperature and pH, trigger the excystation of ingested oocysts [112]. Feng *et al.* [113] studied the effect of a bile salt routinely used to induce excystation of *Cryptosporidium* oocysts (sodium taurocholate), and showed that it significantly enhanced the invasion of several cultured cell lines by freshly excysted *Cryptosporidium parvum* and *Cryptosporidium hominis* sporozoites. The presence of bile salts in the intestinal area probably plays a crucial role in the excystation of ingested oocysts and therefore in cryptosporidium infectivity. It is well known that β-CDs interact strongly with bile salts to form stable inclusion complexes with high stability constants, and that competitive inclusion complexation with components like bile acid, cholesterol or lipids, etc. is one of the major forces that favour release of the drugs in the intestinal lumen, especially those that form complexes with high stability constant [7]. Cyclodextrins probably decrease the concentration of free bile salts by complexation in the intestinal lumen thereby inhibiting oocyst excystation and the subsequent release of sporozoites, and thus reducing the infectivity of the parasites.

Finally, it has been demonstrated that lipid rafts play an important role in attachment of *C. parvum* and entry to host epithelial cells, and thus depletion of phopholipids and cholesterol by cyclodextrins would apparently reduce the attachment and entry of sporozoites. *Cryptosporidium parvum* sporozoites attach to the intestinal and biliary epithelial cells *via* specific molecules on host-cell surface membranes including host galactose- and *N*-acetyl-D-galactosamine- containing cell surface receptors [114]. Nelson *et al.* [114] found that *C. parvum* infection in an *in vitro* model of human biliary cryptosporidiosis activated acid-sphingomyelinase, an enzyme involved in lipid raft membrane aggregation, to recruit selective lipid raft components to infection sites, and also that disruption of known lipid raft components and associated lipid raft membrane aggregation decreased *C. parvum* attachment to and entry into cholangiocytes. Recruitment of lipid raft components is also associated with accumulation of galactose- and *N*-acetyl-D-galactosamine- associated membrane-binding molecules for parasite attachment at infection sites and is involved in *C. parvum* induced activation of the phosphatidylinositol 3-kinase/Cdc42 pathway and subsequent actin remodelling at the infection sites. Thus, lipid rafts are required for *C. parvum* attachment to and entry into host cells. Disruption of lipid rafts and knockdown of acid-sphingomyelinase significantly decreased *C. parvum* attachment and cellular invasion.

*Leishmania* causes leishmaniosis, a disease that is now considered endemic in 88 countries in Africa, Asia, Europe, North America and South America. Leishmaniosis is transmitted by the infected female sand fly *Phlebotomous spp.* Once transmitted in the bloodstream, promastigotes are efficiently phagocytosed by host macrophages. Entry of promastigotes into macrophages involves recognition of specific ligands on the parasite cell surface by macrophage receptors and subsequent internalization of the promastigotes. In *Leishmania amazonensis* the amastigotes have glycosphingolipids present on their surface with the structure Galβ1–3Galα, which is recognized by 30 kDa macrophage receptors. Furthermore, other *Leishmania* species, such as *Leishmania major* and *Leishmania (Viannia) braziliensis* contain glycosylinositolphospholipids, which are involved in parasite-macrophage interaction [115]. Lipid rafts have also been found to be important in *Leishmania* infectivity and requirement of host membrane cholesterol in the binding, and internalization of *Leishmania donovani* in macrophage cells has been demonstrated [101, 102]. Treatment of *Leishmania* parasites with methyl-β-CD [115] caused a significant reduction in parasite sterol levels (40% or 70% at a CD concentration of 20 mM or 40 mM, respectively) and disruption of membrane microdomains. Although this treatment did not affect parasite viability, macrophage infectivity was significantly lower (about 50%) than infectivity by control parasites, probably because binding of the promastigotes to the cell surface was affected. For this reason Pucadyil *et al.* [101] proposed the exploration of cyclodextrin-based compounds, which modulate host membrane cholesterol levels, as a possible therapeutic strategy against leishmaniosis in addition to other intracellular parasites.

Another parasitic infection that has been demonstrated to be sensitive to treatment with CDs is malaria. Antimalarial activity of sulphate-CD derivatives has been described [24]. Sulphated cyclodextrins inhibit the invasion of *Plasmodium* merozoites by interacting with receptors present on the surface of erythrocytes and may thus disrupt the invasion process.

## IMMUNOMODULATORY EFFECTS OF CYCLODEXTRINS

Natural cyclodextrins (CDs) and their derivatives, with their hydrophobic inner cavities and hydrophilic outer surfaces have been applied in many areas as drug delivery systems. However, the capability of CDs to enhance the immune response has scarcely been investigated. Some studies have explored the immunomodulatory effects of adjuvant formulations including cyclodextrin and cyclodextrin analogs both *in vitro* and *in vivo.*

These attempts were mainly focused on including hydrophobic cyclodextrin modification in vaccine formulations incorporated in a squalene-in-water emulsion [116, 117]. The use of these adjuvants clearly induced a more intense antibody response in live animals than the usual W/O-based vaccines with the additional technological advantage of producing stable emulsions between the antigen suspension and the adjuvant formulation.

The properties of vaccine delivery systems based on the use of biodegradable polymers and cyclodextrins to encapsulate antigenic extracts have also been widely employed. These antigenic materials often present low encapsulation loading due to the formation of irreversible aggregates in aqueous solutions. Some approaches for formulations containing γ- and β-CD have demonstrated an increase in antigen loading and therefore, enhanced immunological performance [118, 119].

Recently, methyl-β-CD treatment of macrophages was found to enhance macrophage activation by cholesterol depletion of the plasma membrane [120]. Phosphorylation of mitogen-activated protein kinases (MAPKs), TNF-α and cyclooxygenase-2 (COX-2) expression were induced in response to phagocytosis of chitin microparticles and enhanced when cells were treated with methyl-β-CD, which in this case suggested a Th1 adjuvant effect of the cyclodextrin. Other authors have demonstrated that administration of hydroxypropyl-CDs, hydroxyethyl-CDs by themselves did not produce any immunological response in mice. However, these compounds exerted an immunosuppressive effect when administrated with oligonucleotide immune stimulators [121] suggesting that cyclodextrins reduced the non-specific binding of oligonucleotides with proteins since the complex between the two components was not strong enough.

Although CD themselves apparently did not affect NO production and iNOs expression, and it is clear that is not cell-specific, it was demonstrated that by disrupting lipid raft integrity by use of methyl-β-CD, it was possible to inhibit LPS-induced cell activation [122]. Furthermore, inhibitory effects of some cyclodextrin analogs on NO production, iNOs expression and TNF-α production in a concentration dependent manner has been demonstrated by *in vitro* experiments with macrophages previously stimulated with LPS, poly I:C of CpG-DNA [123, 124].

The mechanism of cyclodextrin and analogues on suppression of the innate immune response is still uncertain, mainly because very few studies have been performed *in vivo*. Depending on the cavity size and other physicochemical features (solubility, hydrophobicity etc.) of each CD, in some cases, the attenuated effect on macrophage activation may be caused by suppression of the uptake of the stimulating compound into the cells; in other cases, this effect would be more likely to be due the capability of CD to extract and remove not only cholesterol and phospholipids but also membrane proteins from cell surface, therebye disrupting the structures and distribution of TLR in the lipid rafts of the plasma membrane.

Together these results lead us to conclude that the presence and accumulation of receptor molecules within lipid rafts in specific zones of plasma membrane serve to facilitate cell activation, bacterial recognition (innate immune response) and signalling. The membrane microdomains of lipid rafts can be disrupted by cyclodextrins inhibiting induced TNFα- secretion or inhibiting the activation of previously stimulated macrophages.

## MISCELLANEOUS ACTIVITY

In addition to the effects of cyclodextrins on cell membrane and on a variety of microorganisms, as well as on drug delivery, CDs have been found to display other important

abilities in particular therapies. Some 20 years ago hydroxypropyl-CDs was used successfully in the treatment of a 2-year-old boy with several signs and symptoms of chronic hypervitaminosis A. Hydroxypropyl-CDs was infused into the patient resulting in release of liver-stored retinoids into serum in high quantities. Circulating retinyl esters transiently increased during the infusion (from 407 to 4791 micrograms/dL), and urinary total vitamin A excretion increased to 23 micrograms/dL after infusion, thus saving the patient's life [125].

A sulphate derivative of cyclodextrin has been also investigated as a chondroprotective agent in experiments performed in rabbits with induced osteoarthritis [126]. Non toxic concentrations of 1mg/Kg of sulphate-CD administered three times a week induced an observable reduction in osteophytes and a restoration of chondrocyte homeostasis. The therapy did not affect the animals' condition (measured as weight difference). Apparently, the mechanism involved in the efficacy of the derivative is a possible tendency for it to be accumulated in articular cartilage, like other sulphated polysaccharides, and the reduction in proinflammatory IL-1 and TNF-α expression as previously described for *in vitro* experiments with cultured chondrocytes [127]. Since the present therapy for ostheoarthritis patients is based only on pain relief, treatment with CDs offers an interesting new alternative, although further examination *in vivo* is required.

Recently, treatment with CDs has been tested as a potential therapy in Niemann-Pick Type C disease (NPC). NPC disease is a multisystem disorder attributable to inactivation of NPC1 protein caused primarily by a mutation. The mutation in the NPC1 gene is characterized by sequestration of LDL-derived cholesterol from the endosomal/lysosomal compartment to the cytosolic compartment of cells. This defect results in activation of macrophages in many tissues, progressive liver disease, and neurodegeneration. Camargo *et al*. [128] reported that intraperitoneal delivery of cholesterol mobilized with cyclodextrins decreased liver cholesterol storage in NPC1 mice. These authors observed that hydroxypropyl-CDs lowered liver unesterified cholesterol levels and delayed neurological symptoms in the npc1 mouse. However, intraperitoneal or intrathecal delivery of cyclodextrins only slightly delayed the onset of neurological symptoms. Recently Liu *et al.* [129] established that a single dose of hydroxypropyl-β-CDs administered at 7 days of mice life transiently overcame the transport defect in the late endosomal/lysosomal compartment, temporarily suppressing further synthesis of sterol and allowing a major portion to be metabolized and excreted from the body. The single dose of hydroxypropyl-β-CDs caused the flow of the sequestered LDL-derived cholesterol in many organs from lysosomes to the cytosolic pool. Activation and influx of macrophages into the liver and brain were also reduced and expression of proinflammatory proteins in these organs was decreased. These researchers demonstrated that by 49 days, the single injection of hydroxypropyl-β-CDs resulted in a reduction in whole-body cholesterol levels, improvement in liver function, less neuro-degeneration and significant prolongation of life. These findings suggested that CDs acutely reversed the lysosomal transport defect observed in NPC disease and completely overcame the transport defect caused by the NPC1 mutation [129]. The FDA recently approved the "compassionate use" of intravenous infusions of hydroxypropyl-β-CDs for treatment of two NPC patients.

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