

Cyclodextrins in peptide and protein delivery

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Abstract

The objective of this contribution is to summarize recent findings on the potential of cyclodextrins (CDs) and their derivatives as carriers for therapeutically important peptides, proteins and oligonucleotides. As one of the indices relevant to bioadaptability of CDs in pharmaceutical uses, their interaction with cellular membranes *in vitro* is outlined. CDs enable the creation of advanced dosage forms for the next generation of drugs that are difficult to formulate and deliver with the existing pharmaceutical excipients. Furthermore, the diagnostic uses of CDs for the direct measurement of cholesterol in high-density and low-density lipoproteins in serum are discussed on the basis of their ability to recognize the surface properties of each lipoprotein particle. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Advances in biotechnology have allowed the

economical and large-scale production of therapeutically important complex polymers of amino acids (peptides and proteins) and nucleosides (antisense molecules) to be used to combat poorly controlled diseases. This rapid progress in molecular biology, however, has not been matched by the progress in

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the formulation and development of delivery systems for the next generation of drugs. Rational drug design does not necessarily mean rational drug delivery, which strives to incorporate into a molecule the properties necessary for optimal transfer between the site of administration and the pharmacological target site in the body [1].

There are considerable hurdles to be overcome before practical use can be made of therapeutic peptides and proteins due to chemical and enzymatic instability, poor absorption through biological membranes, rapid plasma clearance, peculiar dose–response curves, and immunogenicity. Many attempts have addressed these problems by chemical modifications or by coadministration of adjuvants to eliminate undesirable properties of peptides and proteins [2]. Cyclodextrin (CD) complexation seems to be an attractive alternative to these approaches [3].

In this contribution, we focus primarily on recent findings on the potential of CDs and their derivatives as novel bioadaptable carriers for peptides, proteins and oligonucleotides with emphasis on the interaction between CDs, these drug candidates and various endogenous molecules. In addition, the diagnostic uses of CDs for measuring lipoprotein cholesterol in serum without the need for prior separation are described on the basis of their ability to recognize differences in surface properties of each lipoprotein fraction.

2. Bioadaptability of cyclodextrins

Since safety is a primary concern when considering new excipients intended for use in pharmaceutical formulations, the toxicological issues together with their biological fates must be thoroughly investigated prior to their practical use. The safety status of pharmaceutically useful CDs, including pharmacokinetics and toxicological profiles following administration by a variety of routes, has been recently reviewed [4,5]. This contribution focuses on the interaction of CDs with biological membranes and discusses its toxicological implications.

One of the most substantial requirements for drug carriers is that they have either no or acceptably low levels of intrinsic cytotoxicity. Studies using isolated

erythrocytes, which have no nucleus, mitochondria, endoplasmic reticulum and other organelles, may provide a simple and reliable vehicle to classify the CDs in regard to their cytotoxicity, because the interaction of CDs with plasma membranes must be the initial step of cell damage. CDs are known to induce shape changes of membrane invagination on human erythrocytes, and at higher concentrations induce lysis [6,7]. The hemolytic activity of the parent CDs is reported to be in the order of β -CD > α -CD > γ -CD > δ -CD [8]. These differences are ascribed to the differential solubilization rates of membrane components by each CD. The acyl chain of phospholipid fits tightly into the hydrophobic cavity of the smallest α -CD and more loosely into the larger inner space of β - and γ -CDs, whereas the side chain of cholesterol is preferably included in the β -CD cavity. Of the three parent CDs tested, γ -CD has the least lipid selectivity.

The process of solubilization occurs without entry of CDs into the membranes, a mechanism of solubilization/lysis different from that of detergents, which first incorporate themselves into the membranes then extract membrane components into micelles. CDs form a new lipid-containing compartment (or pool) in the aqueous phase into which components of the erythrocyte surface are extracted. This new pool equilibrates freely and reversibly with the cell surface. The removal of cholesterol from the cells may result in an increase in membrane fluidity, which would induce the membrane invagination through a loss of bending resistance, and consequently lead to the lysis of the cells. In addition, our previous studies have shown that CDs remove phospholipids especially phosphatidylcholine and sphingomyelin from the outer half of the membrane bilayer, leading to imbalance of the bilayer and which may contribute in part to formation of stomatocytes through an inward bending of the membranes. A similar solubilization process was found for the CD-induced lysis of the artificial membranes composed of lecithin and cholesterol [9].

β -CD also removes proteins from erythrocyte membranes [7]. Analysis of extracts by electrophoresis indicated that polypeptides of bands V and VI were preferentially extracted. β -CD also extracts bands III and IV to a lesser extent. This indicates that β -CD causes profound changes in membranes,

since bands III and IV are tightly bound and cannot be eluted using simple procedures. Band III is an integral protein (ectoprotein), which interacts with the hydrocarbon core of the lipid bilayer and is exposed to aqueous medium on both extracytoplasmic and cytoplasmic sides.

When the character of the lipophilic cavity of CDs is modified by chemical derivatization, the effects on cell membranes can be dramatically changed [10–17]. The hemolytic activity of CDs correlates with their inclusion ability toward membrane lipids rather than their intrinsic solubility or surface activity. This view is supported by the fact that a positive correlation is found between the hemolytic activity of several CDs and their capacity to solubilize cholesterol, which acts as the main rigidifier in lipid bilayers, in spite of their quite different physico-chemical properties. It should be noted that CDs lose their abilities to interact with the membranes, when their cavities are occupied with the guest molecules. This fact further supports the aforementioned mechanism for CD-induced hemolysis. On the other hand, some efforts have been made for designing CD derivatives with enhanced inclusion ability and lower toxicity, including methyl ethers, glycerol cyclic ethers and tertiary butyl ethers of CDs [18]. Recently, we prepared heptakis(2,6-di-*O*-methyl-3-*O*-

acetyl)- β -CD (DMA- β -CD (7.0)) and evaluated its pharmaceutical properties such as solubilizing power and hemolytic activity [19]. As shown in Fig. 1-A, the hemolytic activity of DMA- β -CD (7.0) was significantly lower than those of β -CD, heptakis(2,6-di-*O*-methyl)- β -CD (DM- β -CD), heptakis(2,3,6-tri-*O*-methyl)- β -CD (TM- β -CD), 2-hydroxypropyl- β -CD (HP β -CD) and sulfobutyl ether of β -CD (SBE- β -CD). The hemolytic activity of DM- β -CD was drastically decreased by introducing acetyl groups into the secondary hydroxyl groups at the 3-position of DM- β -CD, with increasing the average degree of substitution of the acetyl groups (Fig. 1-B). DMA- β -CD showed neither measurable hemolysis nor morphological changes of erythrocytes even at higher concentrations of more than 100 mM, while it maintained certain inclusion ability comparable to TM- β -CD.

Of the CDs tested, the effects of sulfated CDs (S-CDs) on the cell membranes differ from those of the other hydrophilic CDs [15–17]. For instance, S- β -CD shows biphasic effect on the shape of erythrocytes; i.e. the crenation at relatively low concentrations and the invagination at higher concentrations. The S- β -CD-induced membrane crenation arises from a direct action on the membranes rather than cell metabolism-mediated effects. Unlike

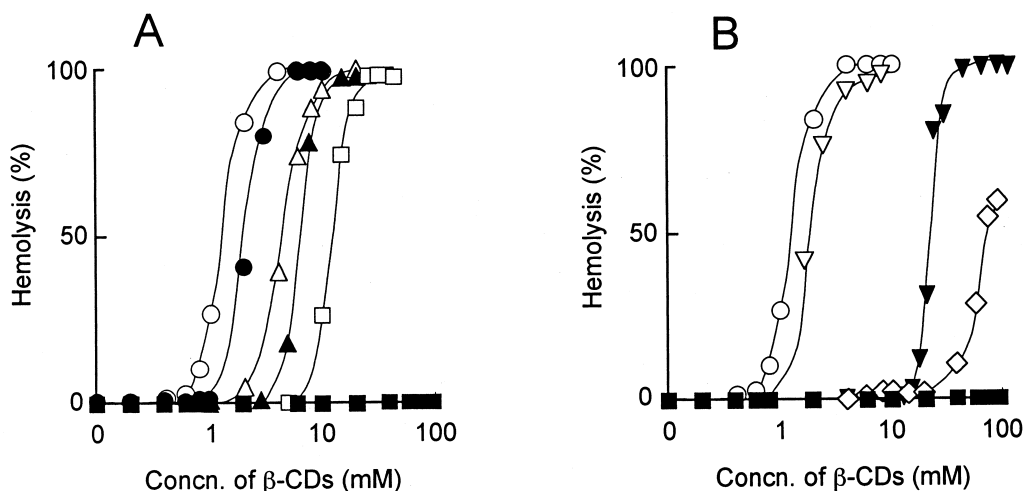


Fig. 1. Hemolytic effects of β -CDs on rabbit erythrocytes in phosphate buffered saline (pH 7.4) at 37°C (see [19]). $\circ$: DM- β -CD, $\bullet$: TM- β -CD, $\triangle$: β -CD, $\blacktriangle$: HP- β -CD^a, $\square$: SBE- β -CD^b, $\blacksquare$: DMA- β -CD (7.0), ∇ : DMA- β -CD (1.5), $\blacktriangledown$: DMA- β -CD (3.8), $\diamond$: DMA- β -CD (6.3). Each value in parenthesis represents the average degree of substitution of acetyl groups in DMA- β -CD. ^aThe average degree of substitution of 2-hydroxypropyl groups was 4.8.; ^bThe average degree of substitution of sulfobutyl groups was 3.5.

β -CyD, S- β -CD binds to the erythrocytes and may be confined to the outer surface of the membrane bilayer, which may expand the exterior layer relative to the cytoplasmic half, and thereby induce the cells to crenate. The lack of hemolytic activity of S- β -CD may be due to the minimal capacity to solubilize the membrane lipids, together with a protective interaction with the membranes and an increase in osmotic pressure in the medium.

Furthermore, the effects of CDs on other types of cells have been studied using human skin fibroblasts [20,21], intestinal cells [21,22] and brush border membrane vesicles [23], P388 murine leukaemic cells [24], and *E. coli* bacterial cells [25]. All these studies suggest that phenomena involved in the CD-induced cytotoxicity are not specific to the cell type, and are determined primarily by a loss of vital cellular components through solubilization by CDs [24].

3. Interaction of cyclodextrins with amino acids and polypeptides

CDs can recognize not only the size and shape but also the chirality of amino acids [26,27]. However, molecules of many peptides and proteins are too hydrophilic and bulky to be wholly included in the CD cavity and the topological constraints of the peptide backbone may reduce the formation of inclusion complexes, thus their interaction with CDs could be only local, that is, accessible hydrophobic side chains may form inclusion complexes with CDs [28–30]. Such interaction possibly affects the overall three-dimensional structure of peptides and proteins or inhibits their intermolecular association and thus changes their chemical and biological properties.

A synthetic nonapeptide buserelin acetate, defined as pyroGlu-His-Trp-SerTyr-D-Ser(*tert*-butyl)-Leu-Arg-PrO-ethylamide, is a highly potent agonist of luteinizing hormone-releasing hormone. Ultraviolet absorption and circular dichroism spectroscopies indicate that the aromatic side chains of buserelin acetate, L-tryptophan and L-tyrosine residues, are incorporated into the hydrophobic environment of the DM- β -CD cavity [31].

Fig. 2 shows the effects of DM- β -CD at various concentrations up to 100 mM on the carbon-13

nuclear magnetic resonance (^{13}C -NMR) chemical shifts of buserelin acetate (20 mM) in D_2O , where an absolute value for the chemical shift displacement (ppm) for each carbon of the peptide was plotted against the concentration of DM- β -CD added. The ^{13}C -NMR chemical shift displacements indicate that in addition to the two aromatic side chains, a tertiary butyl D-serine residue is inserted into the DM- β -CD cavity. Based on the two-dimensional rotating frame nuclear Overhauser effect spectroscopy and other spectral data, the most probable location of the three possible binding sites of buserelin acetate within the DM- β -CD cavity is shown in Fig. 3. Furthermore, upon the addition of DM- β -CD at concentrations of more than 40 mM, most of the carbonyl carbons in the peptide backbone were shifted upfield, indicating global conformational changes of the peptide.

On the other hand, the continuous variation plots for the buserelin acetate DM- β -CD system showed a 1:1 stoichiometry of the complex. Therefore, the complexation should be initiated by the inclusion of one of the three binding sites on the buserelin molecule into DM- β -CD, which may in turn prevent further access of the second CD to the other binding sites, probably due to steric hindrance and/or conformational changes of the peptide. Consequently, the three buserelin acetate complexes with DM- β -CD at a 1:1 molar ratio with a difference in the binding site may coexist in the solution. This heterogeneity in the structure of the complex formed is consistent with the complexity in the near-ultraviolet circular dichroism spectrum, indicating that upon binding to DM- β -CD, the local environment around the aromatic groups in the peptide differs distinctly.

Hydrophilic CDs affect the tertiary structure of recombinant human growth hormone (hGH) in aqueous solution [32]. The electrospray ionization mass spectrum of hGH obtained in an acidic medium gave a broad charge distribution. The higher charge state distribution was observed for hGH with 6-O-maltosyl- β -CD (G_2 - β -CD), suggesting less compact conformation of the protein (Fig. 4). Furthermore, in the presence of G_2 - β -CD, new signals were observed, which correspond to the 1:1 and 1:2 adducts of ionized hGH with the CD. Fluorescence and circular dichroism spectroscopies revealed that CDs changed the tertiary structure of hGH but had no effect on the secondary structure of the protein,

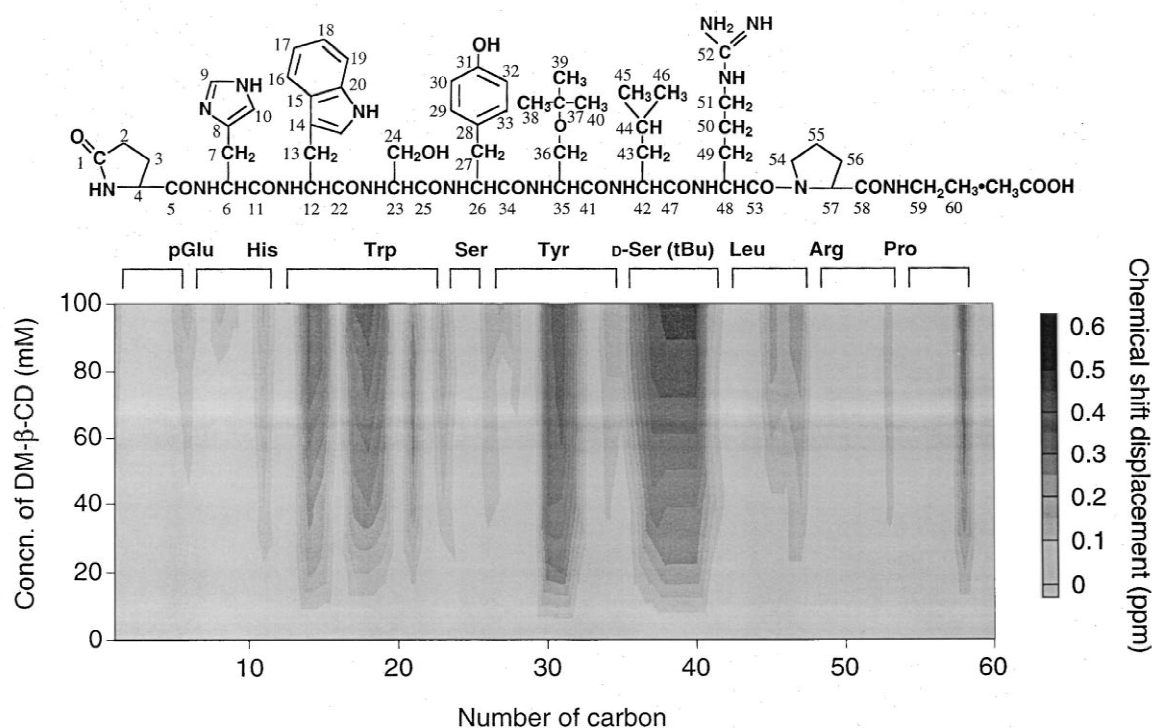


Fig. 2. ^{13}C -NMR chemical shift displacements of busserlin acetate (20 mM) as a function of DM- β -CD concentrations in D_2O (see [31]).

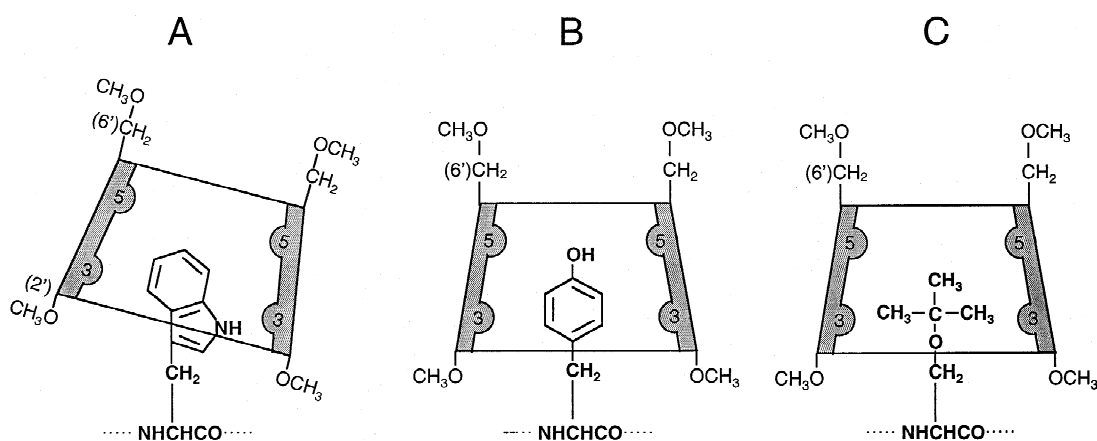


Fig. 3. Proposed structures of inclusion complexes for three side chains in busserlin acetate with DM- β -CD in solution (see [31]). A: L-tryptophan residue, B: L-tyrosine residue, C: tertiary butyl-D-serine residue.

showing a molten globule-like state. The proton (^1H -) NMR signals of tyrosine residues in the hGH molecule were largely shifted with a rise in the G_2 - β -CD concentrations, indicating that the CD

reduces the intramolecular hydrophobic interaction of the protein.

α -CD is known to be a competitive inhibitor for starch hydrolysis by β -amylase, a fact which sup-

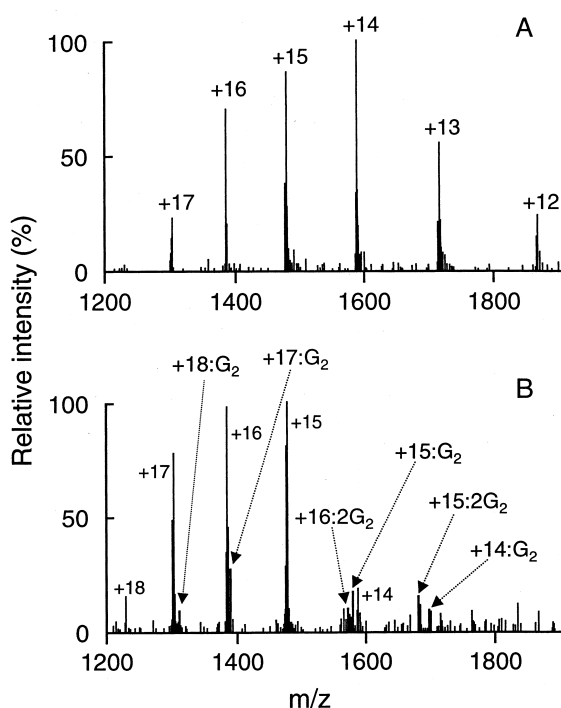


Fig. 4. Electrospray mass spectra of hGH (0.1 mM) in positive ion mode in the absence (A) and presence (B) of G_2 - β -CD (10 mM) in water-methanol-acetic acid (49:49:2) solution (pH 3.0) (see [32]).

ports the induced fit theory of catalysis. On the other hand, a crystallographic study has shown that α -CD binds to δ -methyl groups of leucine 383 at a cleft entrance of soybean β -amylase and occupies much of the entrance [33]. Since α -CD does not extend deeply enough into the cleft to make contact with the enzyme's catalytic center, it should inhibit catalysis by physically blocking the access of substrates to the reaction center. This finding would provide a novel mechanism whereby α -CD inhibits β -amylase, differing from the induced fit theory.

β -CD is known to bind to the substrate binding domain of maltodextrin binding protein from *Escherichia coli* with the same stability order as those of the linear maltodextrins, although it is not a physiological ligand for that protein [34]. An electron spin resonance study has revealed that the binding of physiological ligands (e.g., maltose and maltotetraose) induces a considerable closure of the N- and C-terminal lobes of the protein, while little closure occurs upon the binding of β -CD [35]. The

resulting β -CD-protein complex is incapable of interacting properly with the inner membrane-associated transporter complex. Other studies have shown that β -CD binds to the starch-binding domain of *Aspergillus niger* glucoamylase, but does not inhibit the enzyme activity, indicating that there is no interaction between the catalytic and the starch-binding domains [36,37]

4. Hydrophilic cyclodextrins as solubilizers and stabilizers

CDs can be used to solubilize and stabilize various biomedically-important peptides and proteins including growth hormones [38,39], interleukin-2 [38], monoclonal antibody MN12 [40], aspartame [41], tumor necrosis factor [42], albumin [43], γ -globulin [43], lactate dehydrogenase [44], etc. For instance, α -CD increases the solubility of cyclosporin A, an immunosuppressive agent, in eyedrop form, and helps the drug to penetrate into the cornea with the least local toxicity [45,46]. Cyclosporin A exhibits a low therapeutic index and a poor oral bioavailability with large intra- and inter-individual variations, because of the limited solubility, the influence of foods and bile flow, the poor intestinal membrane permeability, the intestinal and hepatic first-pass metabolism and P-glycoprotein counter-transport processes. Of the hydrophilic CDs tested, DM- α -CD is the most potent solubilizer for cyclosporin A [47,48]. DM- α - and DM- β -CDs enhance the extent of oral bioavailability of cyclosporin A about 5-fold, reaching ~25% of that of the intravenous administration in rats. On the other hand, both DM-CDs do not affect the lymphatic transfer of cyclosporin A (Fig. 5). It is noteworthy that DM-CDs decrease the inter-individual variability in plasma levels of cyclosporin A after oral administration, as the decrease in the coefficient of variation for the plasma drug level from 63% to 22% [49].

Interactions of CDs with side chains on oligomeric peptides can dissociate the oligomers, especially if the complexation occurs at sites in the peptide-peptide interface. The propensity of insulin to form both reversible and irreversible aggregates in solution leads to complications in the development of long-term insulin therapeutic systems and limits the

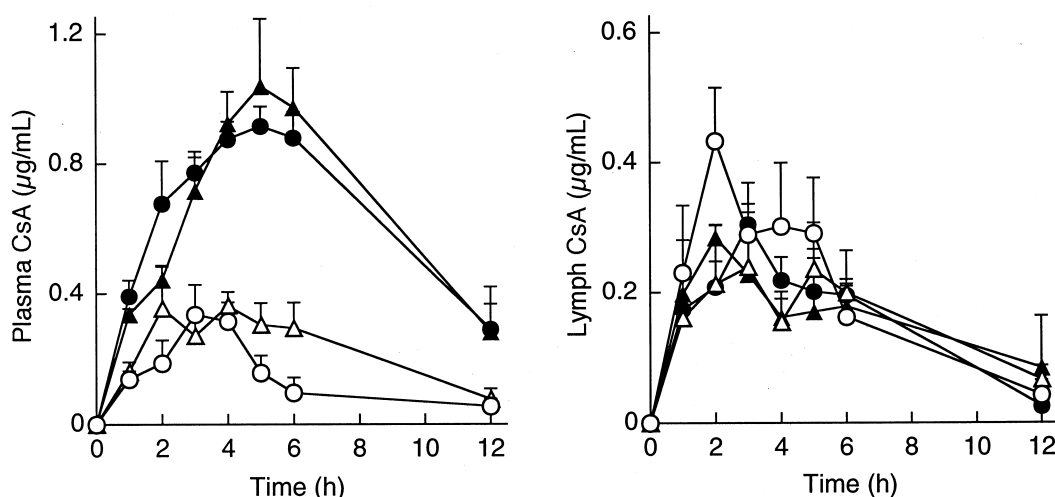


Fig. 5. Plasma and lymph levels of cyclosporin A (CsA) after oral administration of CsA (10 mg/kg) suspensions with or without CDs (molar ratio of 1:10 (CsA:CD)) to rats (see [49]). ○: CsA alone, △: with α -CD, ▲: with DM- α -CD, ●: with DM- β -CD.

rate of subcutaneous absorptions, a process which is too slow to mimic the physiological plasma insulin profile at the time of meal consumption. These problems are further complicated by the tendency for insulin to adsorb on to the surfaces of containers and devices, perhaps by mechanisms similar to those inducing aggregation [50].

Some hydrophilic CDs, including HP- β -CD and G_2 - β -CD, significantly inhibit the adsorption of insulin to hydrophobic surfaces of containers and its aggregation in neutral solutions [51]. HP- β -CD is also found to prevent the shaking-induced formation of insoluble aggregates of insulin in neutral solutions [52,53]. Both β -CDs facilitate the permeation of insulin through the ultrafiltration membranes, and increase the surface tension of insulin solutions. In the circular dichroism spectrum of insulin, the β -CDs increase the negative band intensity around 208 nm assigned to α -helix structure of insulin, while decrease that around 275 nm assigned to the antiparallel β -structure of insulin oligomers. These spectral changes are in close agreement with those observed when insulin aggregates are dissociated to monomer or lower-order aggregates.

Hydrogen–deuterium exchange measurements coupled with electrospray ionization mass spectrometry have shown that the exchange rate of insulin was rapid in 30% v/v acetic acid solution

where the peptide is predominantly in a monomer state, and the rate was unchanged by the addition of G_2 - β -CD. However, the exchange rate significantly slowed down in pH 2.0 solution where insulin is predominantly in a dimer state, and the rate increased with increasing G_2 - β -CD concentrations, indicating that G_2 - β -CD shifts the monomer–dimer equilibrium of insulin in favour of the dissociated form (Fig. 6) [54].

Dilution microcalorimetric study indicates a sequential binding of CDs to at least two possible sites on the insulin monomer at acidic condition [55]. Based on the two-dimensional ^1H -NMR measurements, G_2 - β -CD may include accessible hydrophobic amino acid residues of insulin such as phenylalanine and tyrosine at the N-terminal end (B1) and in the C-terminal region (B25 and B26) of the B-chain, these side chains having a high motional freedom, while the side chains in the α -helices are not significantly perturbed in the presence of G_2 - β -CD [56]. Thus, G_2 - β -CD should perturb the intermolecular hydrophobic contacts between aromatic side chains across the monomer–monomer interfaces, eventually leading to the inhibition of self-association of the peptide.

By contrast, SBE- β -CD shows varying effects on insulin aggregation, depending on the degree of substitution of the sulfobutyl group, i.e., the inhibi-

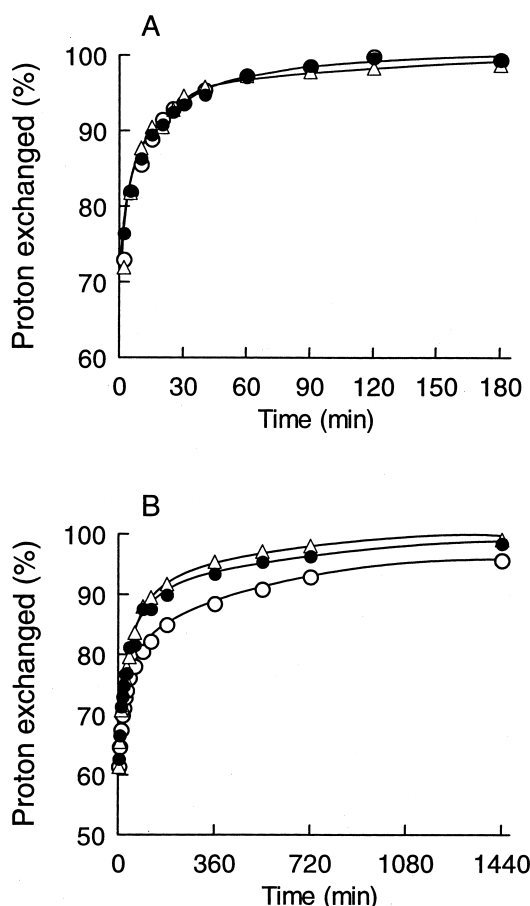


Fig. 6. Deuterated ratio of insulin versus time of exposure to D_2O in the absence and presence of G_2 - β -CD in 30% v/v acetic acid (A) and pH 2.0 solution (B) at 25°C (see [54]). $\circ$: insulin alone, $\bullet$: with 0.05 M G_2 - β -CD, $\triangle$: with 0.1 M G_2 - β -CD.

tion at relatively low substitution and acceleration at higher substitution. In such cases, the electrostatic interaction between the positive charges of insulin and the negative charges of the sulfonate group of SBE- β -CD seems to be more of a factor than the inclusion effects. These results suggest that proper use of hydrophilic CDs could be effective in designing rapid or long-acting insulin preparations [57].

Alzheimer's disease is characterized pathologically by extracellular amyloid deposits in the brain, containing a β -A4 amyloid peptide, derived from a larger amyloid precursor protein. Although a principal morphological marker of the disease, the role of

the amyloid deposits has been controversial. Studies using electrospray ionization mass spectrometry have revealed that β -CD interacts with a synthetic 40 amino acid β -amyloid peptide, presumably with the hydrophobic aromatic residues on the peptide. β -CD inhibits the fibrillization of the amyloid peptide and reduces its neurotoxic effects on rat pheochromocytoma cells [58]. However, the available data are still too preliminary to be applied to prevent amyloid accumulation in vivo. On the other hand, HP- β -CD can be used to inject high concentrations of the amyloid peptide into the brain without marked acute toxicity and thereby may provide a physiologically relevant model for the amyloid deposits observed in the human brain [59].

5. Hydrophilic cyclodextrins as artificial chaperones

The process by which protein molecules achieve their native compact conformations is a subject of both fundamental and practical importance. In particular, the practical interest in the protein refolding problem stems from the fact that proteins are over-produced by genetically engineered cells in the form of cytoplasmic aggregates or inclusion bodies, in which the proteins are misfolded and therefore functionally inactive. Weak interactions of CDs with unfolded proteins may enhance the solubility of denatured proteins by masking the exposed hydrophobic residues, thereby possibly assisting the refolding of the proteins. In this way CDs might act as small chaperone-mimics in the protein folding process in cases where refolding is inhibited by poorly-reversible aggregation or entanglement [60,61].

Calorimetric studies for thermal unfolding of hGH at a neutral pH have demonstrated that the ratio of the van't Hoff enthalpy to the calorimetric enthalpy ($\Delta H_v/\Delta H_c$) is more than one, indicating the aggregation of the protein during the unfolding process. The hydrophilic CDs increase the unfolding temperature for hGH and decrease the $\Delta H_v/\Delta H_c$ ratio. These results suggest that the interaction of the hydrophilic CDs with accessible hydrophobic side chains in the hGH molecule leads to the less compact conformation of the protein and reduces its aggregation during the unfolding process [62].

When hGH is refolded from its molten globule-like intermediates produced by a denaturant guanidium hydrochloride, insoluble aggregates are formed, as indicated by an increase in absorbance of the solution at 350 nm. As shown in Fig. 7, 6-*O*-glucosyl- β -CD (G_1 - β -CD) and G_2 - β -CD, as well as non-ionic surfactant Tweens, significantly reduced the aggregation of hGH during refolding from the intermediates, while G_2 - α -CD, HP-CDs and linear saccharides showed no noticeable inhibitory effect. Furthermore, the size exclusion chromatographic analysis revealed that the concentration of the hGH monomer remaining in solution after refolding was highest in the presence of G_2 - β -CD. This indicates that the β -CD cavity with a branched sugar moiety is most preferable to prevent the aggregation of hGH [62].

More efficient protein refolding is established when CDs are used in combination with detergents [63–66]. In the first step, the non-native target protein is captured by a detergent under conditions that would normally lead to irreversible protein aggregation, in which the substrate protein cannot spontaneously refold from the detergent-complexed

state. In the second step, removal of the detergent from the protein is triggered by the addition of a CD, allowing the protein to refold.

6. Hydrophilic cyclodextrins as absorption enhancers

The systemic delivery of peptide- and protein-based drugs via various mucosal routes is receiving extensive scrutiny as an alternative to the oral and parenteral routes. The transmucosal delivery has advantages of being noninvasive and of bypassing gastrointestinal and hepatic clearances. Among them the peptide delivery through nasal mucosa seems to be most successful and practical; nasal sprays for some therapeutic peptides are already available commercially [2]. However, even with the intranasal route of delivery, the nasal epithelium presents both a physical and a metabolic barrier to the absorption of peptides and proteins. Therefore, the use of absorption-promoting agents is necessary to achieve sufficient intranasal absorption of most peptides and proteins. The potential of CDs, especially the methylated CDs, as nasal absorption enhancers has been demonstrated for luteinizing hormone-releasing hormone agonists [67,68], insulin [69–72], adrenocorticotrophic hormone analogue [73], calcitonin [74], granulocyte colony-stimulating factor [75], insulin-like growth factor-I [76], etc. The absorption enhancement afforded by CDs can be attributed primarily to their ability to reduce the physical and/or metabolic barriers to these peptides and proteins. Further details on the uses of CDs in nasal drug delivery have been described in chapter 6.

The limited systemic bioavailability of peptides and proteins is partly due to the existence of a substantial enzymatic barrier in the epithelial cells. CDs can protect peptides and proteins against enzymatic as well as chemical degradation [77,78]. For example, CDs, especially G_2 - β -CD, significantly inhibited the enzymatic degradation of buserelin acetate in rat nasal mucosa [68]. On the basis of the inclusion mode of buserelin acetate with CDs as described in Section 2, they may protect buserelin acetate sterically from proteolytic enzymes, by including the hydrophobic side chains of the peptide within the CD cavity, because these binding sites are

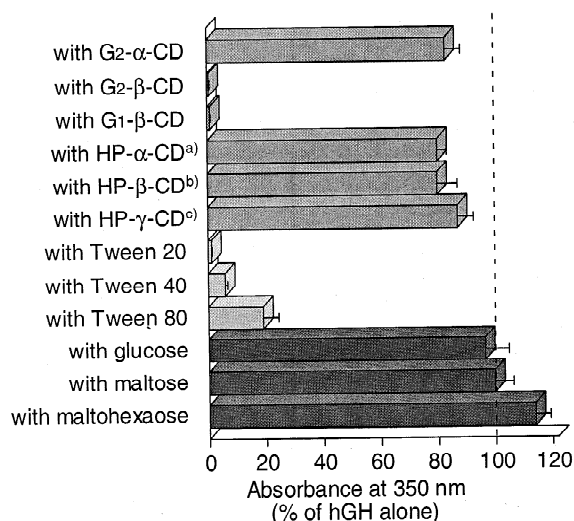


Fig. 7. Effects of additives (50 mM) on aggregation of hGH during refolding from molten globule-like intermediates (the initial concentration of hGH: 500 μ M) in phosphate buffer (pH 5.0) containing 4.5 M guanidium hydrochloride at 25°C (see [62]).

^aThe average degree of substitution was 4.1; ^bThe average degree of substitution was 4.8; ^cThe average degree of substitution was 4.8.

located near the enzymatic cleavage sites of the peptide [68].

The possibility for the CDs to directly deactivate the proteolytic enzymes, however, should not be totally dismissed [71,79,80]. For example, G_2 - β -CD decelerated the hydrolysis of busserelin acetate catalyzed by α -chymotrypsin, a typical serine protease. Based on the kinetic studies, this deceleration can be explained solely by a non-productive encounter between a complex of the substrate with G_2 - β -CD and the protease at relatively low CD concentrations, while the direct inhibitory effect of G_2 - β -CD on the proteolytic activity made a considerable contribution to the overall deceleration of the hydrolysis at higher CD concentrations [80].

Further insight into the direct interaction between G_2 - β -CD and α -chymotrypsin was gained by differential scanning calorimetry (Table 1). In the case of α -chymotrypsin alone, the ΔH_v value was about half the ΔH_c value, suggesting the presence of intermediate states in the unfolding process, presumably due to the autolysis [80]. By contrast, these quantities for the unfolding of α -chymotrypsin with G_2 - β -CD were nearly the same, indicating that the denaturation is very close to a two-state process. A similar two-state denaturation of α -chymotrypsin has been observed in the pH range 2–4, in which the protease is no longer reactive. It is well known that maltose stabilizes proteins against thermal denaturation through its structure-making effect on the surrounding water molecules. In fact, maltose increased the T_m value of α -chymotrypsin by approximately 5°C, but did not affect the $\Delta H_v/\Delta H_c$ ratio.

In the case of G_2 - β -CD, the conformational energy of the unfolded α -chymotrypsin is likely to be reduced by incorporating the exposed hydrophobic groups in the unfolding protein into the CD cavity [28], which may compensate fully for the

thermal stabilization arising from the branched sugar moiety. Rather small ΔH_c value of α -chymotrypsin with G_2 - β -CD may be ascribable to the binding of G_2 - β -CD to aromatic amino acid residues of the protease, a process which is known to be endothermic. These results indicate that G_2 - β -CD reduces the catalytic activity of α -chymotrypsin in such a way that the accessible hydrophobic side chains of the protease may be incorporated into the CD cavity, a situation which should produce some localized distortion and/or steric hindrance near the catalytic site of the protease.

Another potential barrier to the nasal absorption of peptides and proteins is the limitation in the size of hydrophilic pores through which they are thought to pass. The hydrophilic CDs can solubilize some specific lipids from biological membranes through the rapid and reversible formation of inclusion complexes, leading to an increase in the membrane permeability [7]. CDs may affect nasal mucosal membranes in the same manner, thus allowing their extended use as adjuvants to improve the nasal absorption of poorly absorbable peptide and protein drugs. The lipid solubilization mediated by CDs may cause changes in transcellular processes, and these changes are believed to be transmitted to the paracellular region, which appears to be the most likely route for the transport of polypeptides [71,81].

Nasal preparations must be critically evaluated for their possible effect on the nasal mucociliary functions, which are known to defend the respiratory tract against noxious inhaled materials such as dust, allergens and bacteria. Since most of the enhancers including CDs may promote the systemic absorption of peptides and proteins by perturbing membrane integrity in a rather non-specific manner, it is inevitable that varying extents of insult would occur to the mucosal tissue in intimate contact with the enhan-

Table 1

Thermodynamic parameters of α -chymotrypsin (80 μ M) in the absence and presence of G_2 - β -CD (0.1 M) and maltose (1 M) in isotonic phosphate buffer (pH 7.4) (see [80])

System	T_m (°C)	ΔH_c (kcal/mol)	ΔH_v (kcal/mol)	$\Delta H_v/\Delta H_c$
α -Chymotrypsin alone	48.9 $\pm$ 0.2	167 $\pm$ 2	81 $\pm$ 1	0.49 $\pm$ 0.02
With G_2 - β -CD	48.7 $\pm$ 0.1	126 $\pm$ 6	116 $\pm$ 2	0.93 $\pm$ 0.06
With maltose	54.2 $\pm$ 0.4	188 $\pm$ 14	86 $\pm$ 3	0.46 $\pm$ 0.05

Each value represents the mean $\pm$ S.D. of three experiments.

cers. When compared with other absorption-promoting agents and preservatives used commonly in nasal formulations, CDs exert a rather mild and reversible effect on the surface morphology of nasal mucosa and the ciliary beating [82–84]. To evaluate the nasal tissue tolerability to CDs, the release behaviors of several biochemical markers from nasal mucosa were measured using an in-situ recirculating perfusion technique in rats [85]. Five percent solutions of CDs released the biochemical markers from the nasal mucosa with the efficacy increasing in the order: SBE- β -CD $\leq$ 2-hydroxyethyl- β -CD < HP- β -CD < heptakis(3-mono-*O*-methyl)- β -CD < DM- β -CD, a sequence which is almost proportional to their hemolytic activity or ciliotoxicity. Other studies using an in-vivo lavage technique have shown that the rat nasal mucotoxicity increased in the order: HP- β -CD < randomly methylated β -CD < DM- β -CD < sodium glycocholate < sodium taurodihydrofusidate < L- α -lysophosphatidylcholine < laurith-9 [86]. This rank order correlates well with those observed in morphological as well as ciliotoxicity studies. Based on the results of these studies, the minimal concentration of DM- β -CD necessary to achieve substantial absorption enhancement in rats is considered to be $\sim 2\%$, showing only a mild effect on the nasal ciliary function. It should be also noted that the efficacy and safety of CDs differ largely between species [87–89] and is also greatly dependent upon the dosage form [90–93].

In an attempt to design a more effective and less irritating nasal formulation, combinations of CDs with absorption enhancers have received increasing attention [94–98]. For instance, HP- β -CD solubilized a lipophilic absorption enhancer, HPE-101 and potentiated its action at an appropriate combination ratio without causing severe local irritation on the nasal application [99–101]. When the concentration of HPE-101 was kept constant at 1% (w/v), the nasal membrane permeability of fluorescein isothiocyanate dextran with an average molecular mass of 4400 Da (FD-4) increased with a rise in the HP- β -CD concentration, reaching a maximum when just enough HP- β -CD ($\sim 15\%$, w/v) was used to keep all the enhancer in solution. HP- β -CD may potentiate the activity of HPE-101 by solubilizing, thus making it more available at the mucosal surface for subsequent penetration into the nasal epithelium, a site of action.

Upon further addition of HP- β -CD beyond the critical concentration necessary for complete solubilization of HPE-101, the activity of the enhancer decreased. The excess amount of HP- β -CD in solution may reduce the free fraction of HPE-101, which is in an equilibrium with the complexed form, and thereby reduce the thermodynamic activity of the enhancer at the mucosal surface.

The sole use of HPE-101 in emulsion showed the maximal enhancing effect on the nasal absorption of FD with an average molecular mass of ~ 40000 . On the other hand, the combination of HPE-101 with HP- β -CD provided the prominent enhancement of the nasal absorption of FDs with average molecular masses of less than 20 000, compared with that obtained for the sole use of the enhancer (Fig. 8). With increasing molecular masses of FDs, the permeation enhancing effect of this combination decreased steeply. The rate of overall processes involving the dissociation of HPE-101 from its HP- β -CD complex and subsequent uptake of the free form of the enhancer into the nasal mucosa was much faster than that of the enhancer from oil droplets into the mucosa. Therefore, the rapid onset and short duration of the enhancing effect of this combination would

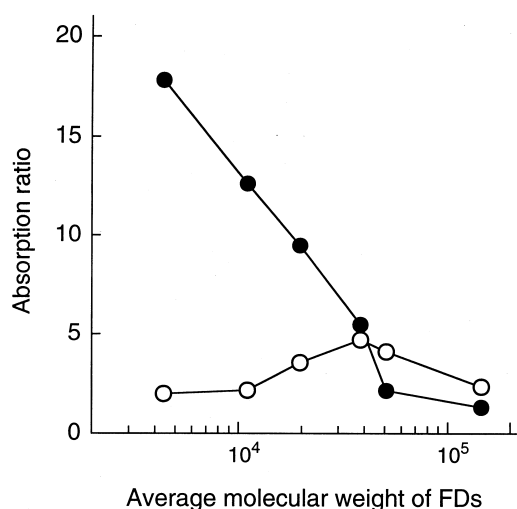


Fig. 8. Enhancing effects of HPE-101 (1% w/v) and HP- β -CD (15% w/v) on nasal absorption of FDs (7.5 mg/kg) with various molecular weights to rats, where the extent of nasal absorption of FDs without the enhancer up to 3 h post-administration was taken as unit (see [102]). ○: with HPE-101, ●: with HPE-101 and HP- β -CD (the average degree of substitution of 5.8).

not be enough to allow the slow diffusion of FDs with higher molecular masses through the nasal mucosa [102]. These results indicate that HPE-101 solubilized in HP- β -CD at the appropriate combination ratio could cause just enough and transient perturbation of the nasal mucosa to allow the absorption of the permeation marker, without widespread damage of the epithelium. The approach described here would be extended to the optimal use of other lipophilic absorption enhancers particularly in the environment of the mucosal absorption site.

As described in chapter 8, CDs have been applied to optimize the transdermal delivery of drugs intended either for local or systemic use. CDs may interact with some components of the skin. For instance, DM- β -CD is known to extract cholesterol and triglyceride from powdered hide [103] and from rabbit skin *in vitro* [104], a process which may reduce the function of skin as a barrier and eventually may contribute in part to the absorption enhancement of poorly absorbable drugs, including peptide- and protein-based drugs. In such a case, particular attention should be directed toward the possible irritation effects of CDs on the skin. Our previous studies have demonstrated that the parent CDs at sufficiently higher concentrations caused skin irritation in guinea pigs in the order of γ - < α - < β -CD, a result which depends largely on their abilities to extract lipids from the skin [105].

Differential scanning calorimetric studies have shown that DM- β -CD affected the endothermic transition of an isolated human stratum corneum, while no noticeable changes were observed for the stratum corneum treated with HP- β -CD [106]. Other studies have shown that randomly methylated β -CD extracted all the major lipid classes from an isolated stratum corneum of hairless rats and reduced the barrier function of the skin, while HP- β -CD had the limited specificity for cholesterol and triglycerides and to a small extent, cholesterol esters. Nevertheless, neither CD induces any major modification of the differential scanning calorimetric profile and the Fourier-transformed infrared spectrum of the stratum corneum, suggesting the CDs do not penetrate the lipid structure of the stratum corneum [107].

The practical use of CDs to increase the drug solubility and stability and to improve the drug delivery from the rectal formulations has been extensively reviewed in chapter 8. In contrast, the

use of CDs as rectal absorption enhancers needs great care as to their irritating effects on rectal mucosa and also the potential for pathogenic substances and the CDs themselves to be systemically absorbed. *In vitro* permeation studies using an isolated rectal mucosal preparation of rabbits have revealed that α -CD at higher concentrations (~ 100 mM) reduced the basal transepithelial potential difference and short-circuit current and increased transepithelial conductance, a circumstance which facilitated its own mucosal permeation as well as the drug permeation in a rather non-specific manner [108]. DM- β -CD is reported to enhance the rectal absorption of insulin from the hollow-type oleaginous suppository in rabbits, in which both the peptide and the CD are dissolved in citrate buffered solution (pH 3.0) and loaded into a cavity of the suppository. This formulation seems to be less irritating to the rectal mucosa, as indicated by the fact that the hyperpermeable state of the rectal mucosa mediated by DM- β -CD returned to a normal physiological level within 24 h after rectal administration [109].

7. Hydrophobic cyclodextrins as sustained-release carriers

Chronic treatment with peptide and protein drugs has disadvantages; the short biological half-lives of the drugs require long-term daily injection or frequent nasal application in order to maintain a therapeutic concentration of the drugs. Therefore, attention has been directed toward the development of drug delivery systems with controlled-release features so as to realize their potential and efficacy. Several approaches have been proposed including the use of implants or injectable microcapsules of biodegradable copolymers or gel-forming agents.

Injectable oily suspensions of buserelin acetate with sustained-release feature can be obtained by using hydrophobic CDs such as heptakis(2,6-di-*O*-ethyl)- β -CD (DE- β -CD) [110,111] and heptakis(2,3,6-tri-*O*-acetyl)- β -CD (TA- β -CD) and octakis(2,3,6-tri-*O*-acetyl)- γ -CD (TA- γ -CD) [112]. The interfacial transfer of buserelin from the peanut oil suspension into the aqueous phase was significantly retarded by the hydrophobic CDs in the order of TA- β -CD < TAY-CD < DE- β -CD. The drug release from a vehicle is influenced by various factors

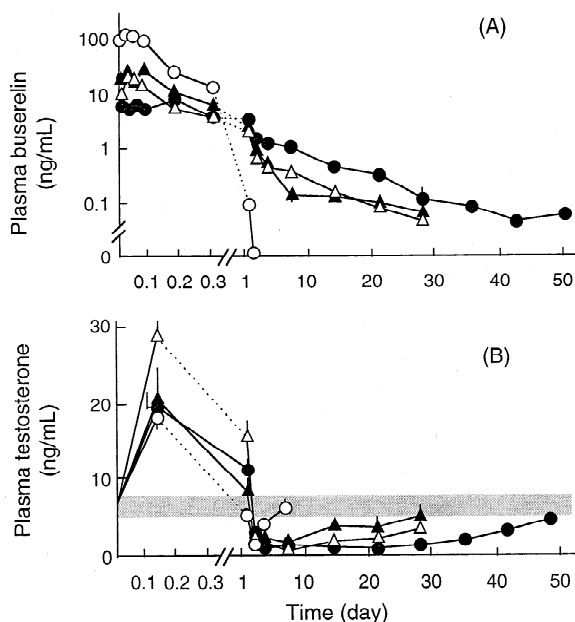


Fig. 9. Plasma levels of buserelin (A) and testosterone (B) after subcutaneous administrations of buserelin acetate and its CD complexes in oily suspension (1 mg/kg as buserelin acetate) to rats (see [110–112]). ○: buserelin acetate alone, ●: DE- β -CD complex, ▲: TA- β -CD complex, △: TA- γ -CD complex.

including drug–vehicle interactions, solubility, partition coefficient and particle size of the drug in the vehicle. Buserelin acetate was practically insoluble in peanut oil and the solubility was only slightly

increased by the complexation with DE- β -CD or TA-CDs. In contrast, the solubility of the CDs in the vehicle increased in the order of TA- β -CD < TA- γ -CD < DE- β -CD corresponding with the retardation order of buserelin release. The above results suggest that the drug might be dispersed within an oily matrix through a weak interaction of the drug with the CDs.

A single subcutaneous injection of the oily suspension of buserelin acetate containing TA- β -CD, TA- γ -CD and DE- β -CD in rats provided retardation of plasma levels of buserelin, with giving 25, 39 and 70 times longer mean residence time, respectively, than that of the drug alone (Fig. 9). Simultaneously with the suppression of plasma testosterone to castrate level, the antigonadal effect of buserelin continued for 1, 2, and 4 weeks and a significant weight reduction on genital organs was observed (Fig. 10). For example, when the DE- β -CD complex was administered subcutaneously to rats, the weight of genital organs decreased on week 1 due to an antigonadal effect, and the weight reduction was maintained for 8 weeks, while the drug alone showed no significant effect.

Since TA-CDs are ester-type derivatives, they are susceptible to alkaline hydrolysis resulting in reformation of corresponding parent CDs and acetic acid in a 1:3 molar ratio in a fashion of the first-order kinetics. TA-CDs are also degraded enzymatically

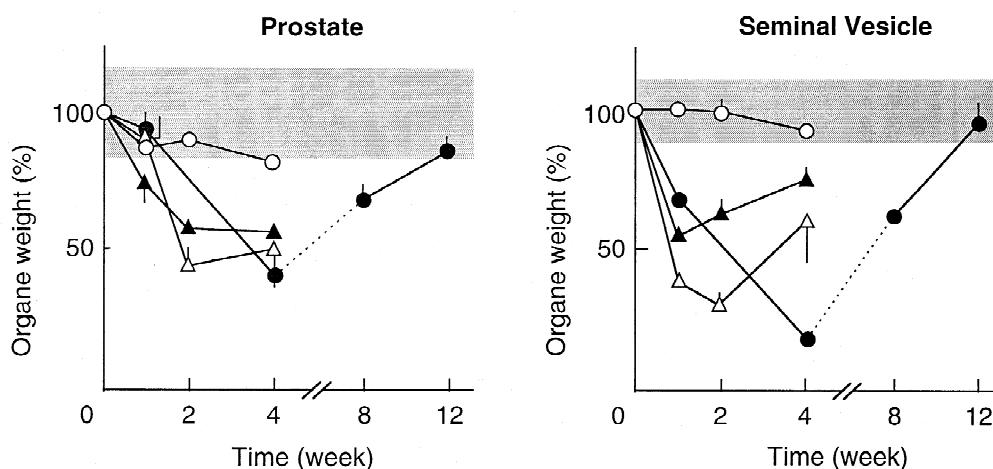


Fig. 10. Weight changes of genital organs in rats after subcutaneous administrations of buserelin acetate and its CD complexes in oily suspension (1 mg/kg as buserelin acetate) (see [110–112]). ○: buserelin acetate alone, ●: DE- β -CD complex, ▲: TA- β -CD complex, △: TA- γ -CD complex.

with the rat skin homogenates. For example, the residual amounts of TA- β -CD and TA- γ -CD were 72% and 60% after the 8 h incubation, respectively, while DE- β -CD remained intact under the experimental conditions because of the ether-type derivative. Although there is no in-vivo kinetic evidence on TA-CDs subcutaneously administered, once they are hydrolyzed into the respective parent CDs at the injection site, the resulting CDs are supposed to be easily absorbed and excreted into urine. Since the enzymatic hydrolysis of TA-CDs at the injection site may proceed gradually as described above, it may be free from nephrotoxicity. In fact, no abnormality was found in the hemodiagnosis during the experiments through week 0 to week 4 after the subcutaneous administration of oily suspension containing TA-CDs. Furthermore, it is likely that TA-CDs, in addition to controlling the release of buserelin acetate from the oil vehicle, may also act as stabilizers for the peptide against the enzymatic degradation at the site of administration. These facts suggest that TA-CDs are preferable drug carriers for subcutaneous injection of peptides and proteins than DE- β -CD due to their possible bioabsorbable characteristics.

8. Sulfated cyclodextrins as heparinoids

The introduction of sulfate groups onto the hydroxyl groups of CDs confer biological activities, such as anti-inflammatory and antilipemic activities, similar and sometimes superior to those of heparin on such derivatives [113]. Recently, sulfated CDs (S-CDs) have been found to be effective in inhibiting cellular invasion by human immunodeficiency retrovirus [114–117] and to have antiangiogenic activity in combination with appropriate angiostatic steroids [118–120].

In our previous study a single intravenous administration of S- α -, S- β - or S- γ -CD at a dose of 1 g/kg was tolerated well in rats without conspicuous changes in blood chemistry values, while several parameters in rats receiving other polyanions including heparin, dextran sulfate and poly-L-aspartic acid at the same doses provoked renal or hepatic disorders [121]. In clinical practice, higher doses of heparinoids are sometimes associated with untoward reactions such as bleeding episodes due to their

anticoagulant activity. The anticoagulant activity of S-CDs was about 100 times weaker than that of heparin on a weight basis and was comparable to that of dextran sulfate with a similar sulfur content.

Basic fibroblast growth factor (bFGF) is a potent mitogen that stimulates the proliferation of a wide variety of cells and could play a crucial role in wound healing processes. The therapeutic potential of bFGF, however, has not been fully realized because of its susceptibility to proteolytic inactivation and short duration of retention at the site of action. Recent studies have demonstrated that sulfated oligosaccharides, including a sodium salt of S- β -CD (Na·S- β -CD), have a high affinity for bFGF and protect it from heat, acid, and proteolytic degradation. These sulfated oligosaccharides may bind close to the putative heparin binding domain, a cluster of several basic amino acid residues, on the surface of the bFGF molecule, probably through an electrostatic interaction [122,123]. Unfortunately, the highly hydrophilic nature of Na·S- β -CD is not suited to the design of bFGF formulations with controlled-release features.

A water-insoluble aluminium salt of S- β -CD (Al·S- β -CD) was prepared, and its possible utility as a stabilizer and sustained-release carrier for bFGF was evaluated [124,125]. An adsorbate of bFGF with Al·S- β -CD was prepared by incubating the protein with a suspension of Al·S- β -CD in water. The mitogenic activity of bFGF released from the adsorbate, as indicated by the proliferation of kidney cells of baby hamsters (BHK-21), was almost comparable with that of the intact protein. Al·S- β -CD significantly protected bFGF from the proteolytic degradation by pepsin or α -chymotrypsin compared with their sodium salts and other oligosaccharides. The in-vitro release of bFGF from the adsorbate was sustained in proportion to a rise in the ratio of Al·S- β -CD to the protein.

Of the bFGF preparations tested, the adsorbate of bFGF with Al·S- β -CD, when given subcutaneously to rats, showed the most prominent increase in the formation of granulation tissues, probably due to the stabilization and sustained delivery of the mitogen (Fig. 11). These results suggest that the adsorbate of bFGF with Al·S- β -CD has a potent therapeutic efficacy for wound healing, and can be applicable to oral protein formulation for the treatment of intestinal mucosal erosions.

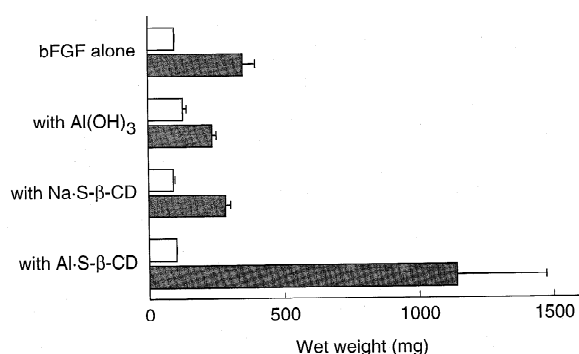


Fig. 11. Effects of bFGF preparations (10 $\mu\text{g}/\text{disc}$ as bFGF) on granulation tissue formation in rats (see [124]). Empty bars: without bFGF, filled bars: with bFGF.

An hypothesis is proposed to provide a common mechanism for conventional antiulcer therapy, in which endogenous growth factors such as basic fibroblast growth factor (bFGF) play a central role [126]. An aluminium salt of sucrose sulfate (sucralfate) has a high affinity for bFGF and protects it from acid degradation and inactivation. Oral administration of sucralfate elevates local levels of bFGF in the ulcer bed, indicating that sucralfate acts as a potent angiogenesis stimulator, primarily on the basis of its ability to stabilize and slowly release locally available bFGF. As described previously, Al·S-β-CD is a more potent stabilizer and sustained-release carrier

for bFGF than sucralfate and other sulfated saccharides (Table 2) [125]. The rigid macrocyclic structure of S-β-CD imposes spatial constraints on the sulfate groups, which enhances the charge density and the affinity for bFGF. The oral administration of Al·S-β-CD tended to enhance the healing rate of acetic acid-induced gastric ulcers and cysteamine-induced duodenal ulcers, probably in a similar manner to sucralfate. In particular, the oral administration of Al·S-β-CD loaded with bFGF had the most prominent healing effects on the ulcers [125]. In addition, the heparin-mimicking activity of S-CDs has been successfully applied to the inhibition of restenosis after the surgical approaches to the treatment of atherosclerosis [127–129], the chromatographic separation of heparin binding proteins [130,131].

9. Cyclodextrins as oligonucleotide carriers

Antisense oligonucleotides are widely used as research tools for inhibiting specific gene expression and are under investigation for possible use as therapeutic agents for the treatment of diseases caused by several viruses, including herpes simplex virus, cytomegalovirus and especially human immunodeficiency virus [132]. Cellular uptake, internalization and nuclease resistance of oligonucleotides are important factors in determining the effectiveness of antisense therapies. To improve the cellular uptake of oligonucleotides, several approaches have been proposed, including the encapsulation into liposomes or nanoparticles, the use of membrane-perturbing agents and the conjugation with cholesterol or poly-L-lysine [133–135]. Although these strategies have resulted in increases in cellular uptake of oligonucleotides and/or increases in their stability against nucleases, cytotoxicity and non-sequence specific activities may also increase.

A recent study using charge-transfer chromatography indicates that HP-β-CD interacts with nucleoside derivatives bearing different alkyl chains in such a manner as both sterical and hydrophobicity parameters of the nucleosides affect the stability of the complexes [136]. Hydroxyalkylated β-CDs can increase the uptake of phosphorothioate oligodeoxynucleotide in human T cell leukemia H9 cell line by two- to three-fold [137]. Confocal microscopic ob-

Table 2

Effects of additives (25 mg/ml) on stability of bFGF (250 $\mu\text{g}/\text{ml}$) in the presence of pepsin (50 $\mu\text{g}/\text{ml}$, pH 1.6) for 3 h at 37°C (see [125]).

CDs	pH	Remaining bFGF (%) ^a
Without additives	1.67	N.D.
α-CD	1.67	N.D.
β-CD	1.60	N.D.
γ-CD	1.66	N.D.
HP-β-CD	1.65	N.D.
Na·S-α-CD	1.74	1.6±1.3
Na·S-β-CD	1.74	3.3±1.4
Na·S-γ-CD	1.73	5.1±1.0
Na·S-HP-β-CD	1.63	4.5±3.2
Al·S-α-CD	3.81	96.4±1.2
Al·S-β-CD	3.88	99.2±2.6
Al·S-γ-CD	3.82	96.5±3.1
Al·S-HP-β-CD	3.86	93.6±2.1
Dextran sulfate	1.84	20.2±9.6
Sucralfate	3.78	59.6±6.1

^a, Each value represents the mean±S.D. of three determinations.

servations confirmed that the increase in the oligonucleotide uptake observed with the CDs was indeed due to an increase in intracellular uptake, rather than an increase in surface binding of the oligonucleotide. Since the CDs neither affected the efflux of the oligonucleotide from the cells nor stabilized it against endogenous cellular nucleases, they should mediate its influx into the cells, probably through the complexation or the direct effect on the cellular surface. Furthermore, the CDs can suppress the immune stimulation induced with the oligonucleotides *in vitro* and *in vivo*. It is possible that the complexation of the oligonucleotides with the CDs reduces the non-specific binding of the oligonucleotides with proteins, thereby reducing their immune stimulation. Additional advantages of the CDs are their abilities to reduce certain other undesirable side-effects of oligonucleotides such as reduction of platelet counts *in vivo* [138].

Introduction of a thiogalactose residue onto the primary hydroxyl group of β -CD significantly improves the cellular uptake of phosphodiester oligonucleotide probably through a ligand-carbohydrate cell receptor interaction. In addition, the complexation could modify the intracellular distribution of the antisense oligonucleotide, leading to the enhanced antiviral activity [139]. A recent study has shown that CDs enhance adenoviral-mediated gene delivery to the intestinal epithelium with minimal cytotoxicity. A tertiary amino derivative of β -CD is the most effective in improving the adenoviral transduction efficiency in differentiated Caco-2 cells [140].

Conjugates of nucleotides with adamantane at the 3'-end of host nucleotides have increased nuclease resistance compared to their parent oligonucleotides. HP- β -CD can enhance the cellular uptake of the oligonucleotide-adamantane conjugates [141]. In a recent study a series of short peptides derived from the basic region of the basic leucine zipper protein GCN4 was synthesized to examine the cooperative DNA binding to direct repeat sequences. A modified lysine residue bearing an adamantyl group at the ϵ -amino group was incorporated at the N-terminal position, and β -CD was attached at the C-terminal cysteine residue of the parent basic region peptide. The resulting peptide formed an intramolecular inclusion complex in the absence of a specific DNA sequence, while it formed an α -helical trimer-DNA

complex with T3 DNA. This strategy could be useful in designing novel sequence-specific DNA binding peptides [142].

10. Use of cyclodextrins for lipoprotein measurements

In diagnostic preparations, CDs have been successfully utilized as substrates, stabilizers, solubilizers and scavengers of interfering substances [143]. CDs are reported to interact with serum lipoproteins, the efficacy increasing in the order: very-low-density lipoprotein (VLDL) < high-density lipoprotein (HDL) < low-density lipoprotein (LDL) [144]. CDs may form complexes with accessible hydrophobic regions in the lipoproteins, and consequently precipitate them in a manner different from those described for polyanion-metal combinations [145].

Of the CDs tested, DM- β -CD shows the most prominent effect on the electrophoretic pattern of the serum lipoproteins [146]. In particular, an anodic mobility of the band assigned to HDL is significantly decreased with increasing concentrations of DM- β -CD. The interaction of DM- β -CD with the four major lipoprotein classes in the serum (chylomicron, VLDL, LDL and HDL) was investigated by combining two methods: separation by high-performance liquid chromatography with a gel permeation column and selective detection of lipid constituents of each lipoprotein fraction including cholesterol, phospholipids, and triglycerides by enzymatic reaction. When the relatively low concentrations of DM- β -CD (< 10 mM) were added to each lipoprotein fraction, it reduced the elution volume of the HDL fraction, with a small change in the lipid composition, suggesting a change in the density or surface charge of the HDL particles and/or its aggregation to macroparticles. On the other hand, DM- β -CD at the same concentration range only slightly affected the elution volume of the other lipoprotein fraction. Furthermore, DM- β -CD at higher concentrations (> 60 mM) increased the elution volume of all the lipoprotein fractions in a non-selective way, probably due to the partial solubilization and/or collapse of the particles. When DM- β -CD was added to each lipoprotein fraction, an increase in turbidity of the mixture is observed only for the HDL fraction. Additionally, the increment of turbidity in the whole

serum treated with DM- β -CD was well correlated to the concentration of HDL in the serum determined by the conventional precipitation-based method, suggesting the potential application of DM- β -CD to the direct measurement of HDL particle in the serum without any separation procedures.

Recently, we have developed an automated method for measuring HDL-cholesterol in serum without prior separation, using polyethylene glycol (PEG)-modified enzymes and S- α -CD [147]. When cholesterol esterase and cholesterol oxidase are modified with PEG, they show selective catalytic activities towards lipoprotein fractions, with the reactivity increasing in the order: LDL < VLDL $\approx$ chylomicron < HDL. In the presence of magnesium ions, S- α -CD reduced the reactivity of cholesterol, especially in chylomicron and VLDL, without need for precipitation of those lipoprotein fractions (Fig. 12). A combination of PEG-modified enzymes with S- α -CD provides selectivity for the direct measurement of HDL-cholesterol in serum.

It is well known that polyanions such as heparin and dextran sulfate interact primarily with positive charges of the protein moiety and, in the presence of the divalent metal ions associated with the zwitterionic polar heads of the phospholipids, may also contribute to the interaction by forming insoluble complexes [145]. As described in Section 8 in this chapter, the heparin-like activity of S- α -CD may also enable lipoproteins to interact in a manner similar to that reported for polyanions. S- α -CD used in this method has an average molecular mass of 2194 Da, which is ~ 100 times smaller than those of the polyanions used in precipitation-based methods. Furthermore, the charge density of S- α -CD appears to be higher than those of the polyanions. These differences between S- α -CD and the polyanions may explain the differential effects on lipoproteins. Unlike the polyanions, S- α -CD forms water-soluble complexes selectively with chylomicron and VLDL, which are resistant to the PEG-modified enzymes, and thus exhibit reduced reactivities towards cholesterol in those lipoprotein fractions. The method described here is simple and reliable for measuring HDL-cholesterol in serum without the need for prior separation of other fractions and has been used clinically as a first-line test to monitor HDL-cholesterol in serum [148–150].

Many of the current techniques used in the

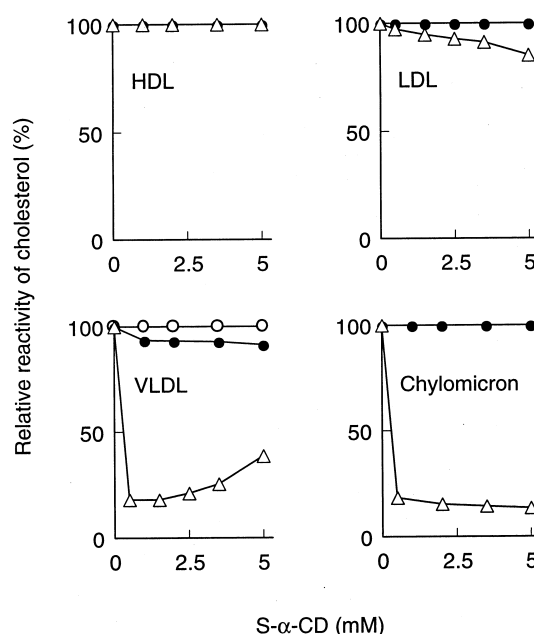


Fig. 12. Effects of S- α -CD and MgCl₂ on relative reactivities of cholesterol in various lipoprotein fractions with PEG-modified cholesterol esterase (1 kU/l) and cholesterol oxidase (5 kU/l) in 3-(*N*-morpholino)propanesulfonic acid buffer (pH 7.0) at 37°C (see [147]). ○: 0.5 mM MgCl₂, ●: 1 mM MgCl₂, △: 2 mM MgCl₂.

determination of LDL-cholesterol in serum are cumbersome, time consuming and require specialized instrumentation, which limits their use in the clinical laboratory. We have established a direct method for determining the concentration of LDL-cholesterol in serum in a convenient format with the combined use of a non-ionic surfactant, polyoxyethylene-polyoxypropylene triblock copolymer (POE-POP) and S- α -CD [151]. Only a very small sample volume (4 μ l) is needed for this method, without the need for isolation of LDL. LDL-cholesterol can be determined in a short time (10 min) by this homogenous method, which can be easily automated. The strategy used for direct measurement of LDL-cholesterol in serum is based on the cooperative actions of POE-POP as a quencher for HDL-cholesterol and S- α -CD as a quencher for chylomicron and VLDL-cholesterol. Correlation between the proposed method and other methods (Friedewald formula, immunoseparation, high-performance liquid chromatography (HPLC) and beta-quantification methods) are summarized in Table 3. In the serum samples from

Table 3

Correlation between the proposed LDL assay and other methods with sera from healthy volunteers (I), patients with hyperlipemia (triglycerides < 4.5 mmol/l) (II), and with hyperlipemia (triglycerides $\geq$ 4.5 mmol/l) (III)^{a)} (see [151]).

Method	Group	Slope (a)	y-intercept (b)	r	n	Mean $\pm$ SD (mmol/l)
Proposed method	I				86	2.752 $\pm$ 0.954
	II				41	5.131 $\pm$ 2.333 ^b
	III				34	2.979 $\pm$ 1.378
Friedewald formula	I	0.973	0.137	0.987	86	2.715 $\pm$ 0.975
	II	0.933	0.468	0.990	41	4.994 $\pm$ 2.483 ^b
	III	0.395	2.338	0.552	34	2.020 $\pm$ 1.771 ^{b,c}
Immunoseparation	I	0.936	0.140	0.972	86	2.718 $\pm$ 0.918
	II	0.981	0.047	0.980	41	5.076 $\pm$ 2.312 ^b
	III	0.988	−0.044	0.974	34	2.907 $\pm$ 1.384
HPLC	I	0.995	0.003	0.987	86	2.741 $\pm$ 0.962
	II	1.023	−0.202	0.989	41	5.048 $\pm$ 2.397 ^b
	III	0.932	0.059	0.980	34	2.834 $\pm$ 1.309
Beta quantification	I	1.001	0.010	0.989	86	2.764 $\pm$ 0.965
	II	1.008	−0.065	0.992	41	5.164 $\pm$ 2.361 ^b
	III	1.010	−0.052	0.992	34	2.964 $\pm$ 1.381

^a In the form $y = ax + b$, y = the proposed method, x = other method.

^b $P < 0.01$ vs group I.

^c $P < 0.01$ vs the proposed method.

healthy volunteers, the results obtained by the proposed method are well correlated with other methods, but in those from patients with hyperlipemia (triglycerides $\geq$ 4.5 mmol/l), the correlation coefficient increased in the order: Friedewald formula < immunoseparation < beta-quantification method. The proposed method for measuring LDL-cholesterol would include the contribution of intermediate density lipoprotein and lipoprotein (a), as the Friedewald equation or beta-quantification method does. Because all the particles of this wide-density LDL population are atherogenic, the proposed method might be a more sensitive indicator of risk for premature coronary artery diseases than a method that has the limited specificity for the narrow-density LDL population.

11. Conclusions

Modern drug discovery processes appear to have resulted in a higher incidence of more complex, often more insoluble, unstable and/or poorly absorbable polymers as drug candidates. Solubility, stability and membrane permeability issues continue to be major formulation obstacles hindering the development of advanced dosage forms for the next generation of drugs. CDs are able to eliminate some of

these undesirable properties of the drug candidates through inclusion complex formation, which consequently improves drug delivery via various routes of administration. In this contribution, particular attention was also paid to the potentials of S-CDs as heparin-mimicking modulators for growth factors involved in repairing processes and as diagnostic agents for the direct measurement of lipoprotein cholesterol in serum. Although the toxicological issues together with biological fates should be investigated in detail, the CDs described here have many advantages as novel tools for the delivery of peptides, proteins and oligonucleotides and should be pursued.

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