

Review

Design and Evaluation of Cyclodextrin-Based Drug Formulation

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The pharmaceutically useful cyclodextrins (CyDs) are classified into hydrophilic, hydrophobic, and ionic derivatives. Because of the multi-functional characteristics and bioadaptability, these CyDs are capable of alleviating the undesirable properties of drug molecules through the formation of inclusion complexes or the form of CyD/drug conjugates. This review outlines the current application of CyDs in design and evaluation of CyD-based drug formulation, focusing on their ability to enhance the drug absorption across biological barriers, the ability to control the rate and time profiles of drug release, and the ability to deliver a drug to a targeted site.

Key words cyclodextrin derivative; inclusion complex; cyclodextrin/drug conjugate; absorption enhancement; controlled drug release; site-specific drug delivery

1. Introduction

Cyclodextrins (CyDs) were first isolated in 1891 as degradation products of starch and were characterized as cyclic oligosaccharides.¹⁾ The α -, β -, and γ -CyDs are the most common natural CyDs, consisting of six, seven, and eight glucose units, respectively (Fig. 1).²⁾ Recently, various kinds of CyD derivatives such as hydrophilic, hydrophobic and ionic derivatives have been developed to extend physicochemical properties and inclusion capacity of natural CyDs.^{3–10)}

They are applicable as a functional drug carrier to control the rate and/or time profile of drug release.^{11–22)} Hydrophilic CyDs can modify the release rate of poorly water-soluble drugs, which can be used for the enhancement of drug absorption across biological barriers, serving a potent drug carrier in the immediate release formulations.^{15,19,21–24)} Amorphous CyDs such as 2-hydroxypropyl- β -CyD (HP- β -CyD) are useful for inhibition of polymorphic transition and crystallization rates of poorly water-soluble drugs during storage, which can consequently maintain the higher dissolution characteristics and oral bioavailability of the drugs.^{25–32)} On the other hand, hydrophobic CyDs may serve as sustained release carriers for the water-soluble drugs^{33–38)} including peptide and protein drugs.^{39,40)} The delayed release formulation can be obtained by the use of enteric type CyDs such as *O*-carboxymethyl-*O*-ethyl- β -CyD.^{41,42)} A combined use of different CyDs and/or pharmaceutical additives will provide more balanced oral bioavailability with prolonged therapeutic effects.^{43–46)} The most desirable attribute for the drug carrier is its ability to deliver a drug to targeted site. The CyD/drug conjugate can survive passage through stomach and small intestine, but the drug release will be triggered by enzymatic degradation of CyD ring in colon.^{47–56)} Such CyD conjugate can be a versatile means of constructing a new class of colon-targeting prodrug. Moreover, CyD/cationic

polymer conjugates may be novel candidates for non-viral vectors to enhance the gene transfer of plasmid DNA.^{57–59)} On the basis of the above-mentioned knowledge, the advantages and limitations of CyDs in the design of advanced dosage forms will be discussed.

2. Some Characteristics of CyD Derivatives as Drug Carriers

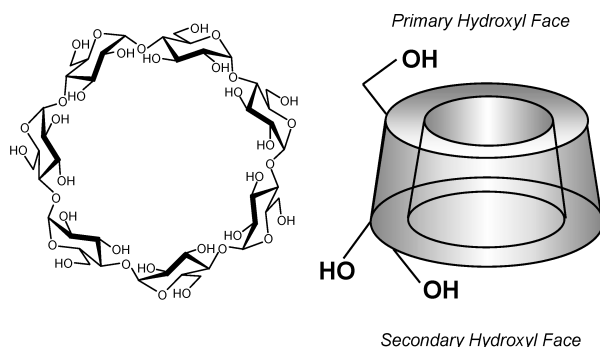
The desirable attributes of drug carriers in drug delivery system (DDS) are the multi-functional properties such as controlled-release, targeting, and absorption enhancing abilities.^{60–62)} From the safety viewpoint, bioadaptability is an important necessity, and quality and cost-performance are required for functional drug carriers. CyDs possess such characteristics: *e.g.*, they are fairly bioadaptable and hardly absorbable from gastrointestinal (GI) tracts, they interact with specific components of biomembrane such as cholesterol and lipids, their macrocyclic ring survives in stomach and small intestine, but they are biodegradable in colon and large intestine, more functional CyD derivatives are available to modify the physicochemical and inclusion properties of the host molecules.

Table 1 contains the pharmaceutically useful β -CyD derivatives, classified into hydrophilic, hydrophobic, and ionic derivatives.^{17–20)} Among these compounds, hydrophilic CyDs such as HP- β -CyD, SBE- β -CyD, and branched β -CyD have received special attention, because their toxicity is very low and aqueous solubility is very high, promising a parenteral use.^{63–68)} The glucuronyl-glucosyl- β -CyD (GUG- β -CyD) is a new entry of branched CyDs, which contains a carboxyl group in the branched maltosyl residue.⁶⁹⁾ In fact, the hemolytic activity of GUG- β -CyD on rabbit erythrocytes is lower than that of β -CyD and G2- β -CyD (see Fig. 2). This compound shows greater affinity for the basic guest molecules, owing to the electrostatic interaction of carboxyl group

with positively charged drug molecule.

The hemolysis data are known to provide a simple and reliable measure for the estimation of CyD-induced membrane damage or cytotoxicity.^{15,19} Figure 2 shows the hemolysis curves of hydrophilic CyDs on rabbit erythrocytes. The hemolytic effects of methylated CyDs are much higher than other CyDs.^{70,71} In a series of CyD derivatives, there was found to be a positive correlation between the hemolytic activity and their capacity to solubilize cholesterol from the cell membrane. As shown in Fig. 2, it is obvious that methylated CyDs remove the cholesterol significantly from human intestinal epithelial cell monolayers.⁷² This would induce the membrane invagination through a loss of bending resistance, and consequently lead to lysis of the cells. Figure 3 shows the effects of β -CyDs on the viability of Caco-2 cells, by measuring an intracellular dehydrogenase activity.⁷¹ For the tentative evaluation of cytotoxicity, Tween 20, a typical non-ionic surfactant was used as a positive control, and eventually provided almost complete cytotoxicity even at 2 mM. In contrast to methylated CyDs, other cyclodextrins were fairly bioadaptable even in higher concentrations.

One of the drawbacks of DM- β -CyD is its membrane toxicity, causing tissue irritation and hemolysis in a concentration dependent manner. In addition, solubility in water decreases in the increasing temperature (see Fig. 4A). So, we have attempted to improve the bioadaptability and physicochemical property of DM- β -CyD, through a chemical modification, and optimally controlled their degree of substitution. When acetyl groups were introduced to the 3-position of glucose units of DM- β -CyD, the water-soluble derivative, heptakis(2,6-di-*O*-methyl-3-*O*-acetyl)- β -CyD (DMA- β -CyDs: d.s. 1–7) and evaluated their pharmaceutical properties such as solubilizing power and hemolytic activity.⁷³ The hemolytic activity of DM- β -CyD was drastically decreased by introducing acetyl groups into the secondary hydroxyl groups of DM- β -CyD, with increasing the average degree of substitution. DMA- β -CyDs are highly water-soluble and maintained certain inclusion ability comparable to TM- β -CyD, with superior bioadaptability and inclusion ability. In the case of DMA- β -CyD with degree of substitution of 7 (d.s. 7.0), no hemolysis was observed up to 100 mM. In the rabbit muscular irritation study, this compound showed negli-



| CyDs | Molecular weight | Cavity diameter (Å) | Volume of cavity (Å ³) | Solubility ^{a)} (% w/v) |
|---------------------|------------------|---------------------|------------------------------------|----------------------------------|
| α -CyD n = 1 | 972 | 4.7 ~ 5.3 | ~ 174 | 14.5 |
| β -CyD n = 2 | 1135 | 6.0 ~ 6.5 | ~ 262 | 1.85 |
| γ -CyD n = 3 | 1297 | 7.5 ~ 8.3 | ~ 427 | 23.2 |

a) In water at 25 °C

Fig. 1. Structures and Properties of Natural Cyclodextrins

Kaneto Uekama was bore in Makurazaki, Kagoshima on 15 March 1941. He received his B.S. from Tokyo College of Pharmacy in 1963, and M.S. in 1965 and Ph.D. in 1968 from Tohoku University. That same year he began his career as Research Associate at Tohoku University. He completed two years as a Postdoctoral Research Associate at the University of Kansas with Professor T. Higuchi from 1969 to 1971. He joined the Nagoya City University as Associate Professor in 1971 and moved to the Kumamoto University in 1974 where he was promoted to Professor of the Department of Physical Pharmaceutics in 1979. He served as a dean of the Faculty of Pharmaceutical Sciences in 1999–2003, and as a President of the Society of Cyclodextrin, Japan in 2000–2002. His research interests include the design and evaluation of cyclodextrin-based pharmaceutical formulations, and published over 450 papers including review articles. Among his recognitions are: an Academic Award from the Miyata Foundation in 1982, the Takeru & Aya Higuchi Memorial Prize, Japan, in 1996, an Academic Award from the Society of Cyclodextrin, Japan in 2000, an Award of the Pharmaceutical Society of Japan for Divisional Scientific Contributions in 2004, and he is an AAPS Fellow since 1992.



Kaneto Uekama

Table 1. Pharmaceutically Useful β -Cyclodextrin Derivatives

| Derivative | Characteristic | Possible use (dosage form) |
|---|---|---|
| Hydrophilic derivatives | | |
| Methylated β -CyD | Soluble in cold water and in organic solvents, surface active, hemolytic | Oral, dermal, mucosal ^{a)} |
| DM- β -CyD | | |
| TM- β -CyD | | |
| DMA- β -CyD | Soluble in water, low hemolytic | Parenteral |
| Hydroxyalkylated β-CyD | | |
| 2-HE- β -CyD | Amorphous mixture with different degrees of substitution, highly water-soluble (>50%), low toxicity | Oral, dermal, mucosal, parenteral (intravenous) |
| 2-HP- β -CyD | | |
| 3-HP- β -CyD | | |
| 3-HP- β -CyD | | |
| 2,3-DHP- β -CyD | | |
| Branched β-CyD | | |
| G ₁ - β -CyD | Highly water-soluble (>50%), low toxicity | Oral, mucosal, parenteral (intravenous) |
| G ₂ - β -CyD | | |
| GUG- β -CyD | | |
| Hydrophobic derivatives | | |
| Alkylated β-CyD | | |
| DE- β -CyD | Poorly water-soluble, soluble in organic solvents, surface-active | Oral, parenteral (subcutaneous) (slow-release) |
| TE- β -CyD | | |
| Acylated β-CyD (C₂—C₁₈) | | |
| TA- β -CyD | Poorly water-soluble, soluble in organic solvents | Oral, dermal (slow-release) |
| TV- β -CyD | Film formation | |
| Ionaizable derivatives | | |
| Anionic β-CyD | | |
| CME- β -CyD | pK _a =3 to 4, soluble at pH>4 | Oral, dermal, mucosal (delayed-release) |
| β -CyD·sulphate | | |
| SBE- β -CyD | pK _a >1, water-soluble | Oral, mucosal Parenteral (intravenous) |
| β -CyD·phosphate | | |
| Al· β -CyD·sulphate | | |
| | Water-insoluble | (slow-release) |

Abbreviations: DM, 2,6-di-*O*-methyl; TM, per-*O*-methyl; DMA, acetylated DM- β -CyD; 2-HE, 2-hydroxyethyl; 2-HP, 2-hydroxypropyl; 3-HP, 3-hydroxypropyl; 2,3-DHP, 2,3-dihydroxypropyl; G₁, glycosyl; G₂, maltosyl; GUG, Glucuronyl-glucosyl; DE, 2,6-di-*O*-ethyl; TE, per-*O*-ethyl; CME, *O*-carboxymethyl-*O*-ethyl; TA, per-*O*-acetyl; TV, per-*O*-valeryl; SBE, sulfobutyl ether; β -CyD·sul, β -CyD·sulphate;

a) Mucosal: nasal, sublingual, ophthalmic, pulmonary, rectal, vaginal, etc.

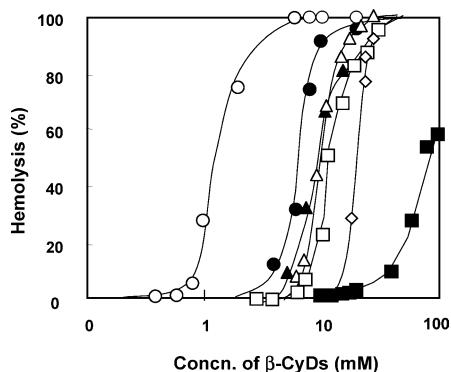


Fig. 2. Hemolysis Curves of Hydrophilic β -CyDs on Rabbit Erythrocytes in 0.1 M Phosphate Buffer Solution (pH 7.4) at 37 °C

○: DM- β -CyD, ●: β -CyD, △: HP- β -CyD (d.s. 4.8), ▲: G₂- β -CyD, ◇: SBE- β -CyD (d.s. 6.2), □: GUG- β -CyD, ■: DMA- β -CyD (d.s. 6.3).

gible damage to the muscle tissue, giving the same irritation score of the saline injection. Interestingly, DMA- β -CyD attenuated nitric oxide (NO) production in macrophages stimulated with lipopolysaccharide (LPS) and lipoteichoic acid (LTA), probably due to the suppression of the LPS and LTA binding to their receptors on the cells.⁷⁴⁾ Moreover, DMA- β -CyD (d.s. 7.0) significantly suppressed the septic shock induced by LPS and D-galactosamine in mice.⁷⁵⁾

Figure 4A shows the temperature dependency of the solubility of three β -CyDs in water.⁷⁶⁾ With increasing temperature, solubility of β -CyD gradually increased, while that of

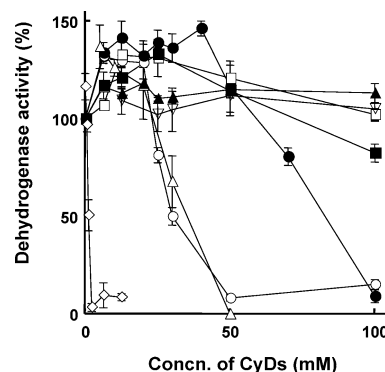


Fig. 3. Effects of β -CyDs on Viability of Caco-2 Cells, by Measuring an Intracellular Dehydrogenase Activity in HBSS at 37 °C

Caco-2 cells were washed three times with HBSS (pH 7.4) and then incubated for 60 min with 100 ml of HBSS containing CyDs (○, DM- β -CyD; ●, M- β -CyD; △: TM- β -CyD, ▲: HP- β -CyD, ◇: SBE4- β -CyD, □: SBE7- β -CyD, ■: GUG- β -CyD) and Tween 20 (◇) at various concentrations at 37 °C. After three washes with HBSS to remove CyDs and Tween 20 again, cell viability was assayed by the WST-1 method. Each point represents the mean \pm S.E. of 6–8 experiments.

DM- β -CyD decreased remarkably, showing a feature of non-ionic surfactant. In the case of HP- β -CyD, however, very high solubility was maintained in a wide range of temperatures. In Fig. 4B, HP- β -CyD showed the highest solubility in all the ethanol/water solvent systems, compared to other CyDs. This kind of property is advantageous to many purposes of pharmaceutical formulation.

When all the hydroxyl groups of CyDs were substituted by

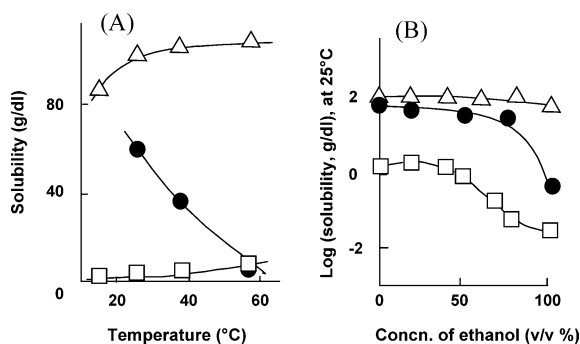


Fig. 4. Effect of Temperature (A) and Solvent Polarity (B) on Solubility of Three β -CyDs in Water

□: β -CyD, ●: DM- β -CyD, △: HP- β -CyD (D.S. 5.8).

Table 2. Some Characteristics of Per-*O*-acylated β -CyDs

| Compound | R | Melting point (°C) | [M] _D ^{a)} | Solubility (mg/dl) ^{b)} |
|---|-----------------------------------|--------------------|--------------------------------|----------------------------------|
| β -CyD | H | 280 | +1850 ^{d)} | 119.0 |
| TA-; Per- <i>O</i> -acetyl- β -CyD | COCH ₃ | 201—202 | +2522 | 823.0 |
| TP-; Per- <i>O</i> -propionyl- β -CyD | COC ₂ H ₅ | 168—169 | +2450 | 423.5 |
| TB-; Per- <i>O</i> -butyryl- β -CyD | COC ₃ H ₇ | 126—127 | +2607 | 219.8 |
| TV-; Per- <i>O</i> -valeryl- β -CyD | COC ₄ H ₉ | 54—56 | +2640 | 283.0 |
| TH-; Per- <i>O</i> -hexanoyl- β -CyD | COC ₅ H ₁₁ | — ^{c)} | +2620 | 3.7 |
| TO-; Per- <i>O</i> -octanoyl- β -CyD | COC ₇ H ₁₅ | — ^{c)} | +2763 | — ^{e)} |
| TD-; Per- <i>O</i> -decanoyl- β -CyD | COC ₉ H ₁₉ | — ^{c)} | +2668 | — ^{e)} |
| TL-; Per- <i>O</i> -lauroyl- β -CyD | COC ₁₁ H ₂₃ | — ^{c)} | +2829 | — ^{e)} |

a) In chloroform at 25 °C. b) In 80% v/v ethanol/water at 25 °C. c) Oily substance. d) In water. e) Could not be determined because of the low solubility.

acetyl or longer acyl groups, the solubility of these CyDs in water decreased proportionally to their degree of substitution or the length of the alkyl chains (Table 2).⁷⁷⁾ The concentrated solutions of per-*O*-acylated β -CyDs in organic solvents were highly viscous and sticky and gelation took place upon evaporation of the solvents. These properties are thought to be particularly useful for a slow-release carrier of water-soluble drugs. The longer acyl side chains, however, severely interfere with the ability of per-*O*-acylated β -CyDs to form inclusion complexes.⁷⁸⁾ TB- β -CyD has the prominent retarding effect for water-soluble drugs, owing to the mucoadhesive property and appropriate hydrophobicity that differ from other derivatives having shorter or longer chains. The guest molecule may be monomolecularly dispersed in the interstices of the alkyl side chain networks of per-*O*-acylated β -CyDs. The X-ray crystallographic study revealed that the self-inclusion of acyl groups occurred with increasing the chain length, in particular, one butanoyl chain intramolecularly penetrate the cavity. Therefore, the peracylated CyDs will prevent the inclusion of any other guest molecule into the central cavity, and the guest molecules can only be accommodated in the matrix between the acyl chain.

When an ethanol solution containing per-*O*-valeryl- β -CyD (TV- β -CyD) and drug was spread on a polyethylene backing sheet, an adhesive film was obtainable by means of the ordi-

nary casting method.⁷⁹⁾ The detaching force of TV- β -CyD film (3.7 N), a tentative measure of adhesion *in vitro*, was much higher than those of the adhesive polymers (0.7, 1.0 and 0.5 N for hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC) and poly-2-hydroxyethylmethacrylate (PEHMA), respectively). This adhesive film seems to be a candidate for a functional drug reservoir, because the water-soluble drugs were slowly released from the film.⁸⁰⁾

6-*O*-(Carboxymethyl)-*O*-ethyl- β -cyclodextrin (CME- β -CyD) was developed to exhibit pH dependent solubility for use in selective dissolution of the drug/CyD complex as well as a stabilizer for acid-susceptible drugs.^{41,81)} CME- β -CyD displays limited solubility under acidic conditions such as stomach with the solubility of the complex increasing as pH pass through the pKa (ca. 3.7) of CME- β -CyD. Then, the enteric type CME- β -CyD complex has been used in *in-vitro* and *in-vivo* studies with diltiazem, a calcium channel antagonist, and molsidomine, a peripheral vasodilator.⁴²⁾

3. Enhancement of Drug Absorption by Hydrophilic CyDs

The possible enhancing mechanisms of CyDs on the bioavailability of drugs in various administration routes are summarized as follows: 1) hydrophilic CyDs increase the solubility, dissolution rate, and wettability of poorly water-soluble drugs,^{82—101)} 2) CyDs prevent the degradation or disposition of chemically unstable drugs in gastrointestinal tracts as well as during storage,^{102—111)} 3) CyDs perturb the membrane fluidity to lower the barrier function, which consequently enhances the absorption of drugs including peptide and protein drugs through the nasal and rectal mucosa,^{112—121)} 4) competitive inclusion complexation with third components (bile acid, cholesterol, lipids, etc.) to release the included drug,^{122—126)} 5) inhibition of the P-gp-mediated efflux of drug from intestinal epithelial cells.^{71,72)} In this section, factors affecting the drug absorption by hydrophilic CyDs will be discussed.

3.1. Control of Solid-State Properties of Drugs by Amorphous CyDs

Crystal modifications significantly affect various pharmaceutical properties such as solubility, dissolution rate, stability and bioavailability of poorly water-soluble drugs. As a consequence, the rational control of crystal growth, habit and polymorphic transition, using pharmaceutical additives, becomes an attractive and interesting area of drug research and development. Many reports have shown that crystalline drugs such as nifedipine,^{127,128)} chloramphenicol palmitate,³¹⁾ and tolbutamide,^{129,130)} can be converted to an amorphous form by complexation with amorphous HP- β -CyD. Therefore, the effects of aging on the crystallization, dissolution and absorption of tolbutamide, an oral hypoglycemic agent, from its HP- β -CyD complex were investigated, in comparison with those of polyvinylpyrrolidone (PVP) solid dispersion.¹³¹⁾ All amorphous powders were prepared by a spray-drying method. During the accelerated storage condition at 60 °C and 75% R.H., a stable form of tolbutamide (Form I) was crystallized from the amorphous PVP dispersion, whereas a metastable form of tolbutamide (Form II) was crystallized from the HP- β -CyD complex. The dissolution rate of tolbutamide from both HP- β -CyD complex and PVP dispersion was significantly faster than that of drug alone. However, the dissolution rate of the drug

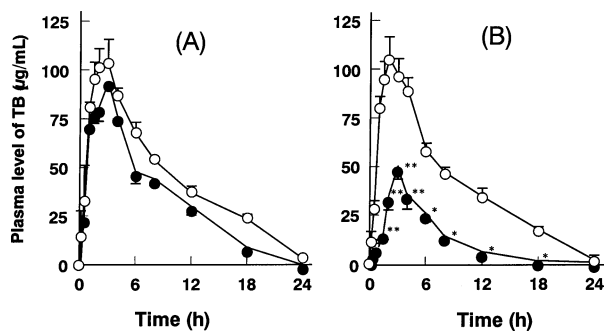


Fig. 5. Effect of Storage on Plasma Levels of Tolbutamide (TB) after Oral Administrations of TB/HP- β -CyD Complex (A) or TB/PVP Solid Dispersion (B) (equivalent to 100 mg/body TB) in Dogs

○: initial, ●: after storage for 1 week at 60°C, 75% R.H. Each point represents mean \pm S.E. of 3–4 experiments. Significant differences from HP- β -CyD complex or PVP solid dispersion before storage were noted as follows: * $p < 0.05$, ** $p < 0.1$.

from the PVP dispersion markedly decreased with storage, because of the formation of slow dissolving Form I crystals. On the other hand, the dissolution rate from the HP- β -CyD complex decreased only slightly due to the formation of fast dissolving form II crystals. These *in-vitro* dissolution characteristics were clearly reflected in the *in-vivo* oral absorption behavior of tolbutamide (Fig. 5) and the glucose plasma level after oral administration in dogs. The results suggested that HP- β -CyD is useful not only for converting crystalline tolbutamide to an amorphous substance, but also for maintaining the fast dissolution rate of the drug over a long period. Furthermore, the crystallization of drugs from CyD complexes, with storage, seemed to be different from those involving polymer excipients such as PVP.

Similar results were obtained for chlorpropamide-HP- β -CyD system.¹³²⁾ In the solid state, chlorpropamide was converted to amorphous complex by spray-drying with HP- β -CyD in a molar ratio of 1:1. During storage, a metastable chlorpropamide polymorph, Form C, was rapidly converted to a stable form, Form A. On the other hand, chlorpropamide was crystallized to Form C from HP- β -CyD complex, and the resulting Form C was very stable in the HP- β -CyD matrix. Under compression, Forms A and C were changed to another state, while HP- β -CyD complex kept the initial state. HP- β -CyD increased the dissolution rate of chlorpropamide and the blood levels of chlorpropamide after oral administration in dogs.

Amorphous SBE7- β -CyD is also effective to improve both the solubility and chemical stability of ONO-4819, prostaglandin E₁ analogue, among the hydrophilic CyDs.¹³³⁾

3.2. Inhibitory Effect of DM- β -CyD on P-gp Mediated Efflux of Tacrolimus Recently, P-glycoprotein (P-gp), the multidrug efflux pump, and Cytochrome P450 (CYP) 3A, the major phase I drug metabolizing enzyme in humans, are known to be present at high levels in the villus tip of enterocytes in the gastrointestinal tract, the primary site of absorption for orally administered drugs.¹³⁴⁾ A number of preclinical and clinical studies have demonstrated that the oral bioavailability of many P-gp and/or CYP3A substrate drugs can be increased by concomitant administration of P-gp inhibitors and/or CYP3A inhibitors.¹³⁵⁾ In addition, the pharmaceutical excipients such as polyethylene glycol,

pluronic P85, Tween 80 and Cremophor EL have been reported to inhibit P-gp activity in Caco-2 cell monolayers.¹³⁶⁾

We have recently found the nonlinear pharmacokinetic behavior of tacrolimus-DM- β -CyD system after oral administration to rats, in which eventual increase in the oral bioavailability of the poorly water-soluble drug was occurred.¹³⁷⁾ To gain insight into this anomalous enhancing mechanism, the effects of DM- β -CyD on the efflux of tacrolimus and rhodamine 123, typical P-gp substrates, were examined using both Caco-2 and vinblastine-resistant Caco-2 (Caco-2R) cell monolayers. Pretreatment of the apical membranes of the monolayers with DM- β -CyD decreased the efflux of tacrolimus and rhodamine 123 without an associated cytotoxicity.⁷¹⁾ DM- β -CyD decreased the P-gp level in the apical membranes of both cell monolayers, probably by allowing release of P-gp from the apical membrane into the transport buffer. DM- β -CyD, however, did not decrease the MRP2 gene expression in Caco-2 or Caco-2R cells. We have recently confirmed that P-gp localized in caveolae of Caco-2R cell monolayers, while the MRP2 resided in both caveolae and non-caveolae domains of the monolayers.⁷²⁾ DM- β -CyD decreased P-gp and MRP2 as well as cholesterol in caveolae of Caco-2R cell monolayers in a concentration-dependent manner. The inhibitory effects of DM- β -CyD on P-gp and MRP2 activity could be attributed to the release of these transporters from caveolae of Caco-2R cell monolayers, resulting from the depletion of cholesterol in caveolae. These facts suggest that the enhancing effect of DM- β -CyD on the oral bioavailability of tacrolimus is due not only to its solubilizing effect but also, at least in part, to its inhibitory effect on the P-gp-mediated efflux of tacrolimus from intestinal epithelial cells (Fig. 6).

3.3. Competitive Inclusion Complexation with Third Components Itraconazole, an orally active triazole antifungal agent, is practically insoluble in water at physiological pH and only slightly soluble under extremely acidic media. Recently, oral solution of itraconazole containing HP- β -CyD has been developed to improve the bioavailability. In this formulation, HP- β -CyD increased the bioavailability about 4 times in non-cannulated rats, compared with that of drug alone.¹³⁸⁾ However, the enhancing effect of HP- β -CyD on the oral bioavailability of itraconazole was significantly decreased by cannulation of the bile ducts of rats. This can be ascribed to the complicated factors such as dissociation of the complex due to the dilution and competitive inclusion,¹²⁶⁾ precipitation of the drug due to the pH-change, micellar solubilization of the drug, *etc.*, in GI-tract. We have found that the solubility of itraconazole decreased in a concentration-dependent manner, when bile acid was added to itraconazole/HP- β -CyD solution (pH 2.0). This may result from competitive complexation between bile acids, itraconazole and HP- β -CyD. Thus, in the case of bile duct non-cannulated condition, the drug will be readily replaced by bile acid, because the stability constant of bile acid/CyD complex is larger than that of drug/CyD complex ($K_2 > K_1$, see Fig. 7), and hence the free drug fraction available for absorption will increase. *In vivo* condition, the bile acid may prevent the precipitation of the drug in GI-tract, which is preferable for drug absorption. Therefore, bile acids may play an important role not only as a competitor, but also as a solubilizer for the improvement of oral bioavailability of itraconazole (Fig. 7).

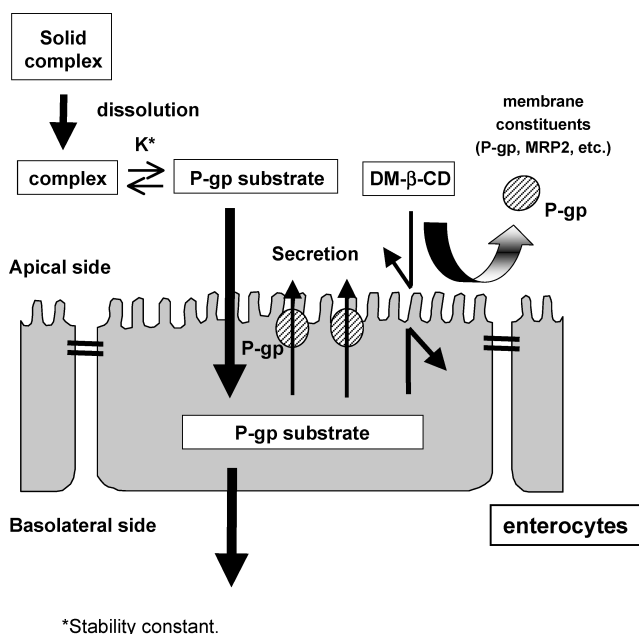


Fig. 6. Possible Enhancing Mechanism of DM-β-CyD on Oral Bioavailability of Tacrolimus (P-gp Substrate)

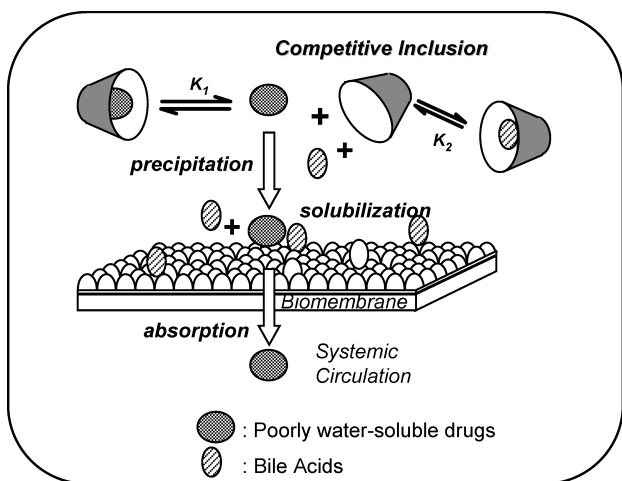


Fig. 7. Scheme Showing Drug Absorption from Drug/CyD Complex Involving Competitive Inclusion with Bile Acids in Gastrointestinal Tract
 K₁: Stability constant of drug/CyD complex,
 K₂: Stability constant of bile acid/CyD complex.

4. Release Control of Water-Soluble Drugs

There has been a growing interest in developing the rate- or time-controlled type oral preparations, because an appropriate drug release from the dosage forms is of critical importance in realizing their therapeutic efficacy. As shown in Fig. 8, the release rate of molsidomine, a water-soluble peripheral vasodilator, was markedly retarded by the complexation with per-*O*-acylated β-CyDs in the decreasing order of the solubility of the host molecules (see Table 2).³⁵⁾ Since the per-*O*-acylated derivatives with substituents longer than the hexanoyl moiety hardly released the drug, per-*O*-acetyl-(TA-), per-*O*-butanoyl-(TB-) and per-*O*-hexanoyl-(TH-) β-CyDs were used in the *in-vivo* studies following oral administration of the complexes to dogs. Among them, TB-β-CyD suppressed a peak plasma level of molsidomine and maintained a sufficient drug level for long periods, while other

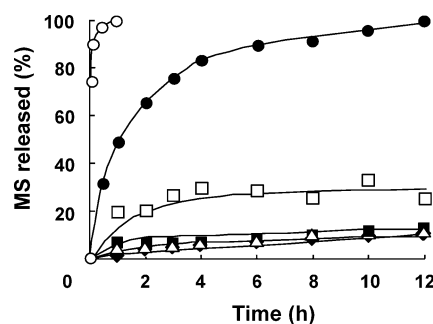


Fig. 8. Release Profiles of Molsidomine (MS) from Capsules Containing MS or Its Acylated β-CyD Complexes (Equivalent to 10 mg MS) in JP XIII Second Fluid (pH 6.8) at 37 °C, Measured by Paddle Method

○: MS alone, ●: TA-β-CyD, □: TP-β-CyD, ■: TB-β-CyD, △: TV-β-CyD, ◆: TH-β-CyD.

per-*O*-acylated β-CyDs with shorter or longer chains were ineffective in controlling the *in-vivo* release behavior of molsidomine. It is also noteworthy that TB-β-CyD improved the bioavailability of salbutamol, a short acting bronchodilator, after oral administration to dogs, probably due to the reduction of the metabolism in GI tracts. The prominent retarding effect of TB-β-CyD was ascribable to its mucoadhesive property and hydrophobicity compared with other per-*O*-acylated β-CyDs.³⁶⁾ These facts suggest that TB-β-CyD is particularly useful to modify the release rate of water-soluble drugs in the oral preparations.

In order to examine a potential use of adhesive TV-β-CyD film, we attempted to design a prolonged release system for water-soluble drugs such as molsidomine and isosorbide dinitrate (ISDN), peripheral vasodilators, with a short biological half-life, using a transdermal formulation. When the film was topically applied to the abdominal skin of rats *in vivo*, the plasma level increased up to 40–50 ng/ml within about 30 min, and then constant drug level (>10 ng/ml) was maintained for at least 3 d, suggesting a superior sustained release property of the TV-β-CyD film.⁸⁰⁾ In addition, neither the film detachment from the skin nor local skin irritation was observed under the experimental conditions, probably due to both the adhesive and bioadaptable property of TV-β-CyD. This formulation has many advantages in reducing the frequency of dosing, prolonging the drug efficacy and avoiding the local irritancy associated with long-term therapy. Moreover, TV-β-CyD film may be in preference to other synthetic polymer-films from the viewpoints of safety and environmental pollution, because of its possible degradation to non-toxic oligosaccharides and pentanoic acid.

Many attempts have been made to design the sustained release formulation of nifedipine, because this drug is poorly water-soluble and has short elimination half-life due to the first-pass effect, and also crystal growing during the storage.³⁰⁾ To maintain a prolonged efficacy of nifedipine, some extent of initial burst before slow-release is necessary to provide a more balanced oral bioavailability. On the basis of these goals, suitable formulation of double-layer tablets containing fast-release portion and slow-release portion was surveyed.¹²⁷⁾ In the fast-release portion, amorphous nifedipine powder prepared by spray-drying with HP-β-CyD and small amounts of non-ionic surfactant HCO-60 were employed to attain an initial rapid dissolution and to prevent the crystal-

growth during the storage. In the slow-release portion, hydroxypropylcelluloses (HPCs) with different viscosity grades were employed to provide an appropriate sustained-release of poorly water-soluble drug from the viscous matrices. Among the seven formulations tested, the *in-vitro* release of nifedipine from the double-layer tablets was little affected by pH of the medium and rotation speed of paddle even after accelerated storage conditions (60 °, 75% R.H.). The double-layer tablet consisting of HP-β-CyD with 3% HCO-60/(HPC-low:HPC-medium) in a weight ratio 1/(1.5:1.5) was selected as an appropriate modified-release formulation because it elicited almost comparable retarding effects with superior oral bioavailability compared with those of a commercially available slow-release nifedipine product (Adalat L-20™).¹²⁷

Combinations of CyDs with pharmaceutical excipients^{139–141} or with different kind of CyDs^{142,143} were successfully applied to obtain a prolonged release of water-solu-

ble drugs. The gel forming property of HP-β-CyD is useful to design the prolonged release of metoprolol.⁴⁶ On the other hand, SBE7-β-CyD was found to serve as both a solubility modulating and an osmotic pumping agent for the controlled-porosity osmotic pump tablets, from which the release rate of both highly and poorly water-soluble drugs can be controlled precisely.^{144–147}

The ordinary delayed release profile can be obtained by the use of enteric type CME-β-CyD.¹⁴⁸ As shown in Fig. 9, diltiazem studies were carried out in gastric acidity controlled fasting dogs with gastric pH controlled to less than two and greater than six. Diltiazem absorption was slower at high gastric acidity ($t_{max}=4.0\pm0.5$ h) than at low gastric acidity ($t_{max}=2.3\pm0.2$ h). The *in-vitro* release data measured using a pH changeable dissolution apparatus were in good agreement with the *in-vivo* data. The colon-specific delivery is also classified as a delayed-release type profile, although a fairly long lag time is required to reach the colon after oral administration. Therefore, the CyD conjugate will be useful for designing a time-controlled type oral drug delivery, which will be discussed later.

Figure 10 shows the proposed drug-release profiles from CyD complex or conjugate, and their combination in oral formulation. When the immediate-release type complex and the delayed-release type conjugate are simultaneously used, the repeated-release profile can be obtained (Fig. 10A), because the water-soluble complex will release the drug immediately in stomach or small intestine, while CyD conjugate will release the drug after arriving at large intestine. On the other hand, a combination of the slow-release preparation and the conjugate will provide a prolonged-release profile, as shown in Fig. 10B. In addition, a combination of the three different components will provide a gradient-release profile, which is useful from the viewpoint of chronopharmacology, and is also resistant to drug disposition (Fig. 10C). Typical *in-vivo* release profiles were obtained for anti-inflammatory

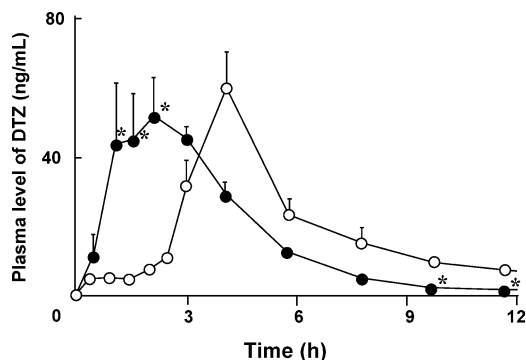


Fig. 9. Plasma Levels of Diltiazem (DTZ) after Oral Administrations of Tablets Containing CME-β-CyD Complex (Equivalent to DTZ at 30 mg/body) in Gastric Acidity Controlled Dogs

●: low gastric acidity dogs (pH>6), ○: high gastric acidity dogs (pH<2). Each value represents the mean±standard error of four dogs. * p<0.05 vs. high gastric acidity dogs.

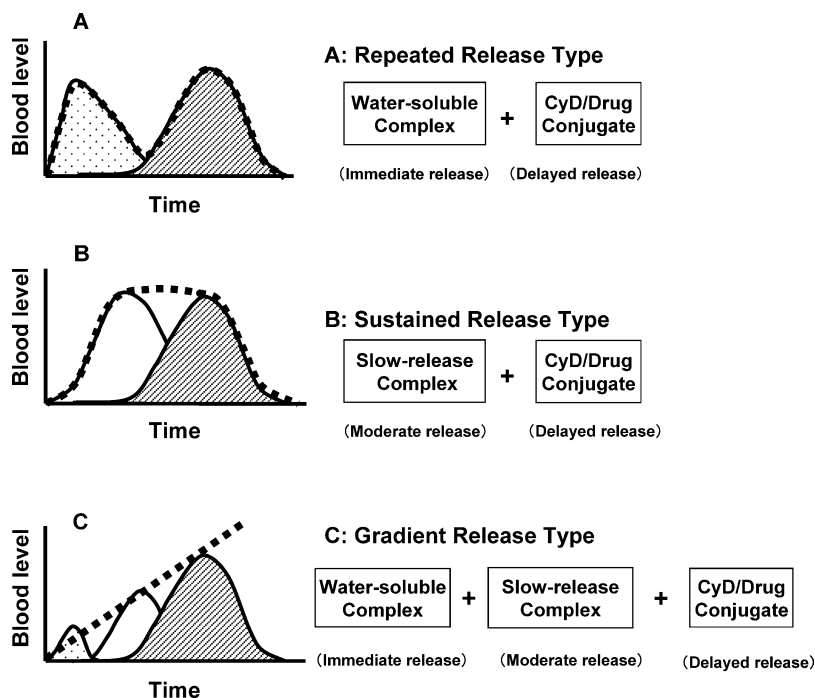


Fig. 10. Drug Release Profiles Based on Combination of CyD Complexes and CyD Conjugate

ketoprofen as a model drug.⁵⁶⁾ For example, a combination of conjugate and complex gave two peaks at about 1 and 10 h after oral administration, showing a typical repeated release profile. In sharp contrast, a combination of conjugate and the solid dispersion gave no valley in the plasma levels and maintained a constant drug level for at least 24 h, showing a prolonged-release profile. We have confirmed that these plasma drug profiles were well reflected in the anti-inflammatory effects, evaluated by using carageenan-induced acute edema models in rat paw.

5. Potential Use of CyDs in Peptide and Protein Formulation

Advances in biotechnology have allowed the economical and large-scale production of therapeutically important peptide and protein drugs to be used to combat poorly controlled diseases. The rapid progress in molecular biology, however, has not been matched by the progress in the formulation and development of delivery systems for such next generation drugs. Many attempts have addressed these problems by chemical modifications or by co-administration of adjuvants to eliminate undesirable properties of peptide and protein drugs such as chemical and enzymatic instability, poor absorption through biological membranes, rapid plasma clearance, immunogenicity, etc.^{149,150)} CyDs seem to be an attractive alternative to these approaches.^{19,151)} For example, 1) α -CyDs are preferable to solubilize cyclosporine A, a poorly water-soluble peptide; 2) hydrophilic β -CyDs inhibit the adsorption of insulin to hydrophobic surface of the containers; 3) hydrophilic CyDs improve the nasal and subcutaneous bioavailability of peptide and polypeptide drugs, due to the enhanced membrane permeation and enzymatic stabilization; 4) branched β -CyDs are particularly effective in inhibiting the aggregation of polypeptide and protein drugs such as insulin and recombinant human growth hormone (rhGH); 5) hydrophobic CyDs are useful to design a sustained-release type peptide preparation; 6) aluminum salt of CyDs is effective for sustained-release of basic fibroblast factor (bFGF). This section deals with the recent aspects of utilization of CyDs in peptide and protein drug formulations.

5.1. Absorption Enhancement by Hydrophilic CyDs

Cyclosporin A, an immunosuppressive drug, is a poorly water-soluble cyclic undecapeptide, exhibiting a low oral bioavailability and a wide range of variability in absorption.^{152–154)} Hydrophilic CyDs were found to increase the solubility of cyclosporin A in water with a positive deviation from linearity, forming higher order complexes. The solubilizing ability of natural CyDs increased in the order of γ -CyD < β -CyD < α -CyD. Of the hydrophilic CyDs, DM-CyDs showed the greatest solubilizing ability. The dissolution rate of the drug was markedly augmented by complexation with DM-CyDs. In closed loop *in-situ* experiments using rat small intestine, DM- β -CyD considerably augmented the cumulative amounts of the drug in the blood, with decreasing the amount ratio of M1, one of the major metabolites of the drug. The inhibiting ability of DM- β -CyD in the bioconversion of cyclosporin A in the small intestinal microsome of rat was greater than that of DM- α -CyD. An *in-vivo* study revealed that DM-CyDs enhanced the transfer of cyclosporin A to blood not lymph, with low variability in the absorption after oral administration of the drug suspension to rats. The

variability of bioavailability of DM-CyDs complexes was lower than that of SandimmuneTM, although the extent of bioavailability of the drug or its DM- α -CyD complex was appreciably decreased by the cannulation of the bile duct of rats, probably due to the competitive inclusion with bile acids, but the extent of the lowering in the bioavailability in the presence of DM- β -CyD was much less serious than that of drug alone.

Internasal delivery of peptide and protein drugs is severely restricted by presystemic elimination due to enzymatic degradation or mucociliary clearance and by the limited extent of mucosal membrane permeability. α -CyD has been shown to remove some fatty acids from nasal mucosa and to enhance the nasal absorption of leuprolide acetate in rats and dogs.¹⁵⁵⁾ The utility of chemically modified CyDs as absorption enhancers for peptide drugs in rats has been demonstrated.¹⁵⁶⁾ For example, DM- β -CyD was shown to be a potent enhancer of insulin absorption in rats, and a minimal effective concentration of DM- β -CyD for absorption enhancement exerted only a mild effect on the *in-vitro* ciliary movement.¹⁵⁷⁾ The scope of interaction of insulin with CyDs is limited, because CyDs can only partially include the hydrophobic amino acid residues in peptides with small stability constants.¹⁵⁸⁾ Under *in-vivo* conditions, these complexes will readily dissociate into separate components, and hence the displacement by membrane lipids may further destabilize the complexes. The direct interaction of peptides with CyDs is, therefore, of minor importance in the enhancement of nasal absorption. Of the hydrophilic CyDs tested, DM- β -CyD had the most prominent inhibitory effect on the enzymatic degradation of both buserelin acetate, a leuteinizing hormone-releasing hormone (LHRH) agonist, and insulin in rat nasal tissue homogenates.¹⁵⁹⁾ Because of the limited interaction between peptides and CyDs, they may reduce the proteolytic activities of enzyme-substrate complexes. This view is supported by the following observations. Leucine aminopeptidase in the nasal mucosa is known to cleave the B-chain of insulin from the N-terminal end. DM- β -CyD and HP- β -CyD reduce the activity of leucine aminopeptidase in a concentration-dependent manner. The inhibition of proteolysis by these CyDs may participate in the absorption enhancement of peptides.¹⁶⁰⁾ Another potential barrier to the nasal absorption of peptide and protein drugs is the limitation in the size of hydrophilic pores through which they are thought to pass. The methylated CyDs significantly extracted membrane lipids, depending on the size and hydrophobicity of the CyD cavity in which lipids were included.⁷²⁾ Therefore, lipid solubilization mediated by CyDs may result in transcellular processes, and these changes could be transmitted to the paracellular region, which is the most likely route for the transport of polypeptides.

Scanning electron microscopic observations revealed that DM- β -CyD induced no remarkable changes in surface morphology of the nasal mucosa at a minimal concentration necessary to achieve substantial absorption enhancement. These facts suggest that DM- β -CyD could improve the nasal bioavailability of buserelin and is well tolerated by the nasal mucosa. HP- β -CyD is useful as a biocompatible solubilizer for lipophilic absorption enhancers involved in the nasal preparations of peptides such as insulin¹⁶²⁾ and buserelin acetate.¹⁶³⁾ When insulin was administered nasally to rats, si-

multaneous use of an oily penetration enhancer, 1-[2-(de-cylthio)-ethyl]azacyclopentane-2-one), HPE-101,¹⁶²⁾ or oleic acid¹⁶³⁾ solubilized in HP- β -CyD showed a marked increase in serum immunoreactive insulin levels and marked hypoglycemia (Fig. 11). In the case of buserelin, the combination of HPE-101 and HP- β -CyD was more effective than that of oleic acid with HP- β -CyD. In particular, the combination of 1% w/v HPE-101 with 15% w/v HP- β -CyD provided an approximately 6-fold increase in the extent of nasal bioavailability of buserelin, reaching about 60% of that based on intravenous administration. The potentiation of the enhancing effect of HPE-101 by HP- β -CyD can be explained by the facilitated transfer of HPE-101 into the nasal mucosa. In addition, the enzymatic degradation of buserelin in rat nasal mucosa homogenates was suppressed by the addition of oleic acid, HPE-101 and/or HP- β -CyD. In particular, the HPE-101/HP- β -CyD system showed the prominent inhibitory effect on the enzymatic degradation of buserelin. Studies on the release of membrane proteins and scanning electron-microscopic observations of rat nasal mucosa indicate that the combination of 1% w/v HPE-101 with 15% HP- β -CyD has the most preferable balance between efficacy and safety. Furthermore, the hyper-permeable state of the nasal mucosa induced with this combination rapidly returned to normal physiological level within 1 h after the nasal application. This approach will provide a rational basis for design and development of aqueous nasal formulations for peptide and protein drugs to optimize their therapeutic efficacy.

Branched CyDs,^{23,24,164–166)} β -CyD sulphate^{167–169)} and sulfoalkyl ethers of CyDs¹²²⁾ may be a new class of parenteral drug carriers because they are highly hydrophilic and less hemolytic than parent and other hydrophilic CyDs. When insulin solutions containing SBE4- β -CyD were injected into the dosal subcutaneous tissues of rats, the plasma immunoreactive insulin (IRI) level rapidly increased and maintained higher IRI levels for at least 8 h (Fig. 10).⁶⁷⁾ The bioavailability of insulin/SBE4- β -CyD system was about twice that of insulin alone and approached 96%. The enhancing effects of SBE4- β -CyD may be in part due to the inhibitory effect of SBE4- β -CyD on the enzymatic degradation and/or the adsorption of insulin onto the subcutaneous tissue at the injection site, although this does not apparently facilitate capillary permeability. Moreover, the prolonged release profile observed for SB4- β -CyD system may be due to the decrease in solubility of insulin at the injection site, where p*K*_i value of insulin may shift to neutral pH upon binding to SB4- β -CyD. These results suggest that the hydrophilic CyDs are useful for improving the pharmaceutical properties of insulin injection.

5.2. Sustained-Release of Peptide and Protein Drugs

Buserelin acetate reduces the plasma levels of endogenous sex hormones to a castrate level through down-regulation, when administered continuously or daily. These paradoxical and pharmacological effects are utilized in the treatment of endocrine-dependent diseases such as endometriosis, precocious and uterine leiomyoma. We have reported that the oily injection of buserelin with a sustained-release feature can be achieved by ethylated β -CyDs.¹⁷¹⁾ Then, the possible use of bioadaptable per-*O*-acetylated CyDs (TA-CyDs) as sustained release carriers was studied, since the release of buserelin from the peanut oil suspension into the aqueous phase was

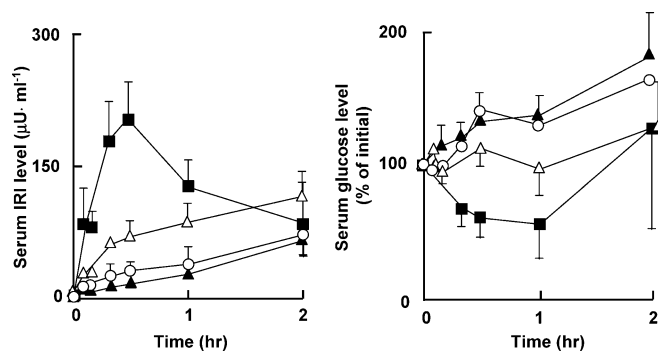


Fig. 11. Plasma Levels of Immunoreactive Insulin (IRI) and Glucose after Nasal Administration of Insulin (21 U/body) with HPE-101 (1% w/v) to Rats

○: insulin alone, ▲: with HP- β -CyD, △: with HPE-101, ■: with HP- β -CyD and HPE-101.

significantly retarded by the addition of TA-CyDs.⁴⁰⁾ A single subcutaneous injection of the oily suspension of buserelin containing TA- β -CyD or TA- γ -CyD in rats enhanced the retardation of plasma buserelin levels, giving 25 and 30 times longer mean residence times, respectively, than that with buserelin alone. Simultaneously, the suppression of plasma testosterone levels to induce castration, the pharmacological effect of buserelin, continued for 1 to 2 weeks and significant weight reduction in genital organs was observed due to the antigonadal effect. Both TA-CyDs were degraded enzymatically in the rat skin homogenates. For example, the residual amounts of TA- β - and TA- γ -CyDs were 72.4 and 59.9% after 8 h incubation, respectively. Thus, both TA-CyDs have potential for use as bioabsorbable sustained-release carriers for water-soluble peptides following subcutaneous injection of oily suspension.

TA-CyDs are also useful to prolong both plasma and lymph cyclosporin A levels.¹⁵¹⁾ When cyclosporin A was orally administered to rats as the complexes with hydrophobic acylated β -CyD derivatives, both plasma and lymph levels of the drug were prolonged up to at least 36 h, although the bioavailability decreased particularly in the case of the per-*O*-butanoyl- (TB-) and per-*O*-octanoyl- (TO-) β -CyD complexes. The administration of the cyclosporin A/olive oil solution in combination with HP-CyDs, especially HP- γ -CyD, further increased the plasma and lymph levels of the drug. Therefore, the relatively hydrophobic TA-CyDs will be useful for design of the CyD-based sustained-release formulation of poorly water-soluble peptide drugs.

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. A water-insoluble aluminum salt of sulphated β -CyD (Al- β -CyD-sul) was prepared, and its possible utility as a stabilizer and sustained-release carrier for bFGF was evaluated.¹⁷²⁾ An adsorbate of bFGF with Al- β -CyD-sul was prepared by incubating the protein with a suspension of Al- β -CyD-sul 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- β -CyD-

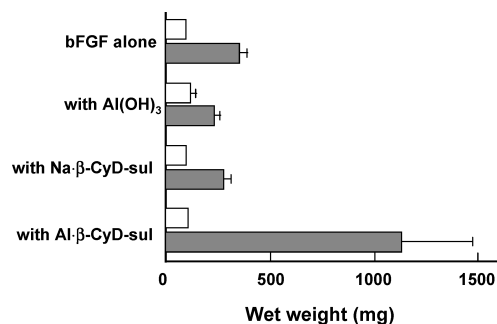


Fig. 12. Effects of bFGF Preparations (10 mg/disc as bFGF) on Granulation Tissue Formation in Rats

□: without bFGF, ■: with bFGF.

sul significantly protected bFGF from the proteolytic degradation by pepsin and α -chymotrypsin compared with their sodium salts and other oligosaccharides. The *in-vitro* release of bFGF from the adsorbate was sustained in proportional to the increase in the Al- β -CyD-sul/protein ratio in the adsorbate. Of the bFGF preparations tested, the adsorbate of bFGF with Al- β -CyD-sul, when given subcutaneously to rats, showed the most prominent increase in the formation of granulation tissues (Fig. 12), probably due to the stabilization and sustained delivery of the mitogen. These results suggest that the adsorbate of bFGF with Al- β -CyD-sul has a potent therapeutic efficacy for wound healing, and can be applicable to oral protein formulation for the treatment of intestinal mucosa erosions. In fact, the oral administration of Al- β -CyD-sul loaded with bFGF had prominent healing effects on the acetic acid induced gastric ulcers and cysteamine-induced duodenal ulcers, probably in a similar manner to sucral-fate.¹⁷³⁾

5.3. Inhibitory Effects of CyDs on Aggregate Formation of Polypeptides The propensity of polypeptide and protein drugs to form reversible and irreversible aggregates in solution is of great concern as it may lead to the loss of biological potency, immunogenic reactions, and unacceptable physical appearance in long-term therapeutic system. To overcome these drawbacks, several approaches have been proposed, including the use of amphiphatic excipients, chemical modification and site-directed mutation.¹⁷⁴⁾

Self-association of the insulin molecule into oligomers and macromolecular aggregates leads to complications in the development of long-term insulin therapeutic systems and limits the 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 onto the surfaces of containers and devices, perhaps by mechanisms similar to those inducing aggregation. Thus, many attempts have been made to prevent aggregation and surface adsorption in parental formulation.¹⁷⁴⁾ We have reported the effects of hydrophilic β -CyDs on the aggregation of bovine insulin in aqueous solution and its adsorption onto hydrophobic surfaces of glass and polypropylene tubes by interacting with hydrophobic regions of the peptide in both concentration and time-dependent manners. Among the CyDs tested, HP- β -CyD and G₂- β -CyD significantly inhibited the adsorption to containers and self association of insulin at neutral pH, whereas DM- β -CyD had only a moderate effect on the ag-

gregation.^{175,176)} In addition, SBE- β -CyDs showed different effects on insulin aggregation, depending on the degree of substitution of sulphobutyl group: *i.e.*, the inhibition at relatively low substitution (SBE4- β -CyD) and acceleration at higher substitution (SBE7- β -CyD). In fact, sulfated β -CyD (β -CyD-sul) significantly accelerated the insulin aggregation. The sulfate group in β -CyD-sul and sulfonate groups in SBE7- β -CyD would remove the hydration layer from the insulin molecule in a manner similar to lyotropic anions, a situation which makes the intermolecular interaction of the peptide stronger, eventually leading to the accelerated association or aggregation of the peptide.

The hydrophilic CyDs such as G₂- β -CyD and HP- β -CyD facilitated the permeation of insulin through ultrafiltration membranes. The increased permeation of insulin was much greater than that of EDTA which is known to prevent the self-association of insulin by sequestering zinc ions from insulin oligomers. The mechanism of hydrophilic β -CyDs seems to be different from that of EDTA: *i.e.*, EDTA sequesters the binding zinc ions from insulin and dissociates the hexamer or higher-order aggregates to the dimer, whereas β -CyDs may interact with hydrophobic amino acid residues to prevent the direct contact of insulin molecules. Furthermore, G₂- β -CyD facilitated the permeation of insulin through the membrane in an acidic solution (pH 2.0), in which the peptide exists primarily as a zinc-free dimer. By the addition of HP- β -CyD or G₂- β -CyD, the surface tension of insulin solutions was increased, whereas it was decreased by EDTA. These observations suggest that hydrophilic CyDs shift the equilibrium in favor of the monomeric form.

Differential scanning calorimetric studies indicated that the self-association of insulin stabilized the native conformation of the peptide, as indicated by an increase in the mean unfolding temperature (T_m). As shown in Fig. 13, G₂- β -CyD and SBE4- β -CyD decreased the T_m value of insulin oligomers. They may shift the equilibrium in favor of the unfolded insulin by dissociating the oligomers and/or binding to hydrophobic side chains exposed on the unfolded peptide. On the other hand, β -CyD-sul, having a limited inclusion ability increased the T_m value of insulin, resulting solely from the higher degree of association of the peptide.

Electrospray ionization mass spectrometry (ESI-MS) coupled with measurement of hydrogen/deuterium (H/D) exchange rates of insulin demonstrated that the H/D exchange rate of insulin in a monomer-dimer equilibrium increased with increasing G₂- β -CyD concentrations, indicating that G₂- β -CyD shifts the equilibrium in favor of the monomer form. Furthermore, in the ESI-MS spectrum, a peak corresponding to the complex or adduct of penta-ionize insulin with G₂- β -CyD at a molar ratio of 1 : 1 was observed. ¹H-NMR studies suggested that G₂- β -CyD includes accessible hydrophobic side chains of insulin within the CyD cavity, and hence perturbs the intermolecular hydrophobic contacts between aromatic side chains across the monomer-monomer interface: *i.e.*, G₂- β -CyD significantly altered the aromatic regions of insulin, particularly B24(Phe) and B26(Tyr), which are involved in the association of insulin to form an antiparallel β -sheet around the carboxyl terminal of B-chain. By contrast, the electrostatic interaction between the positive charges of insulin and the concentrated negative charges of the sulfate and sulfonate groups of the anionic β -CyDs seems to be

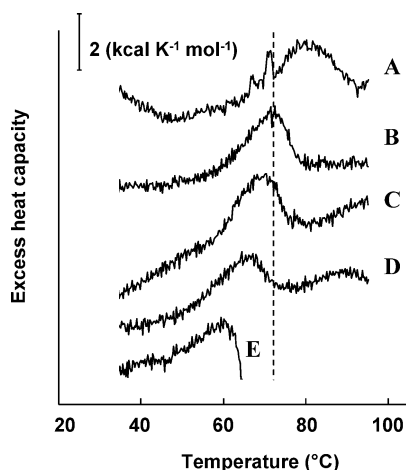


Fig. 13. Effects of CyDs (0.1 M) on DSC Thermogram of Insulin (0.1 mM) in Phosphate Buffer (pH 6.8)

A: β -CyD-sul; B: insulin alone; C: with SBE7- β -CyD; D: with SBE4- β -CyD; E: with G2- β -CyD

more of a factor than the inclusion effects. In the circular dichroism (CD) spectra of insulin, β -CyDs increased the negative CD intensity around 208 nm assigned to the α -helix structure of insulin, while it decreased that around 275 nm assigned to the antiparallel β -structure of insulin oligomers.⁶⁴ These spectral changes were in close agreement with those observed when insulin aggregates are dissociated to monomer or lower-order aggregates.

rhGH is commercially available as a lyophilized powder for treatment of hypopituitary dwarfism, but it is subject to aggregate in the industrial production. We have recently reported the effects of hydrophilic CyDs on the thermally- and chemically-induced aggregation of rhGH in aqueous solution.⁶⁹ Among the CyDs tested, G₂- β -CyD also significantly inhibited the aggregation of rhGH after refolding, where only dimer formation was observed, compared with other CyDs and linear saccharides. This can explain that the β -CyD cavity with branched sugar moieties may be preferable to prevent the aggregation of rhGH. In contrast, HP- β -CyD was effective in reduction of the aggregation induced by interfacial denaturation compared with those of branched β -CyDs due to their surface activities. On the other hand, hydrophilic CyDs showed no noticeable inhibitory effect on the oxidation and deamidation of rhGH. These results suggest that hydrophilic CyDs may interact with exposed hydrophobic side chains rather than aliphatic side chains of rhGH, resulting in inhibition of aggregation.

6. Site Specific Drug Delivery by CyD Conjugates

CyD complex is in equilibrium with guest and host molecules in aqueous solution, with the degree of the dissociation being dependent on the magnitude of the stability constant of the complex (see Fig. 7). This property is desirable because the complex dissociates to give free CyD and drug at the absorption site, and thus only the drug in a free form enters into the systemic circulation. However, the inclusion equilibrium is sometimes disadvantageous when drug targeting is to be attempted, because the complex dissociates before it reaches the organ or tissues to which it is to be delivered. One of the methods to prevent the dissociation is to bind a drug covalently to CyD. This type of drug release is essentially classi-

fied as delayed-release with a fairly long lag time. Therefore, the CyD prodrug approach can provide a versatile means for constructions of not only colon-specific delivery systems but also site-specific drug release system, including gene delivery.

6.1. CyD-Based Colon Specific Delivery CyDs are known to be barely capable of being hydrolyzed and only slightly absorbed in passage through the stomach and small intestine; however, they are fermented to small saccharides by colonic microflora and thus absorbed as maltose or glucose in the large intestine.⁴⁷ Such biological property of CyDs is useful as a source of site-specific delivery of drugs to colon and as a pro-moiety for reducing an adverse effect. Taking these factors into account, we have designed CyD conjugates of nonsteroidal anti-inflammatory drug, biphenylacetic acid (BPAA)^{48–50} and ketoprofen,⁵⁶ a short-chain fatty acid, *n*-butyric acid,⁵¹ and a steroidal drug, prednisolone,^{52–55} anticipating new candidates for colon-specific delivery prodrugs. The drug molecules were selectively conjugated onto the primary or secondary hydroxyl groups of CyDs through an ester- or an amide-linkage, respectively, and their physicochemical properties and drug release behavior in various solutions were investigated.

The BPAA/CyD ester conjugates, for example, are subject to the ring-opening followed by hydrolysis to the maltose and triose conjugates. The ester bond of small saccharide conjugates is subsequently hydrolyzed to form BPAA which is absorbed from the cecum and colon. On the other hand, the amide conjugates are hydrolyzed to small saccharide conjugates, but the amide bond resists the hydrolysis and thus resides as the maltose conjugate in this tract. Figure 14 shows the serum drug levels after oral administration of three ester type conjugates, comparing with drug alone, and β -CyD complex in rats. A rapid increase and decrease in the drug levels were observed for both drug alone and its β -CyD complex. On the other hand, it is obvious that the serum drug levels of the α -CyD and γ -CyD conjugates increased after a certain lag time, and reached maximum levels at about 8 h, accompanying about 5-folds increase in the AUC values. The anti-inflammatory effect of BPAA system was evaluated using the model of carrageenan-induced acute edema in rat paw. In the case of BPAA/ β -CyD complex, a rapid anti-inflammatory response was observed, compared to drug alone, because the drug was mainly absorbed from the small intestine after a fast dissolution of the complex. In sharp contrast, the BPAA/ γ -CyD conjugate needed a fairly long lag time to exhibit the drug activity, because BPAA was produced after it had reached the cecum and colon. This indicates that BPAA could be released after the ring opening of CyD followed by the ester hydrolysis, and BPAA activation will take place site-specifically in the cecum and colon.

Similarly, we have studied the alleviation of systemic side-effects of prednisolone by means of CyD conjugate.^{52–55} Prednisolone, a typical glucocorticoid, is widely used for the treatment of inflammatory bowel disease (IBD). However, when prednisolone is administered orally, almost all of the drug is absorbed from the upper GI tract and causes systemic side-effects, such as moon face and diabetes. Therefore, it is necessary to deliver the drug site-specifically to the colon in IBD therapy.¹⁷⁷ Prednisolone succinate was introduced into one of the secondary hydroxyl groups of CyDs through ester

linkage in a 1 : 1 molar ratio, using a direct coupling agent, carbonyldiimidazole (CDI). All the conjugates prepared were extremely soluble in water, compared to drug alone. The NMR study was conducted to gain insight into the enhanced mechanism of solubility of the conjugates. By the analysis of two-dimensional ROESY spectra, self-inclusion mode was estimated, which probably inhibits the intermolecular stacking association of the conjugates to form less soluble crystals. The anti-inflammatory effect and adverse effect of prednisolone/ α -CyD conjugate were evaluated using IBD model rats prepared by administration of trinitrobenzenesulfonic acid (TNBS). Following oral administration, the anti-inflammatory effect of the conjugate was comparable to that of drug alone. However, thymus/body weight ratio, a measure of typical systemic side-effect in steroid therapy was significantly reduced. The lower side effect of the conjugate was attributable to passage of the conjugate through the stomach and small intestine without significant degradation or absorption, followed by the degradation of the conjugate site-specifically in the large intestine. The oral administration of prednisolone alone gave higher plasma levels of the drug, giving the significant systemic side effect. Therefore, the CyD conjugate of prednisolone could be useful for colon-specific delivery, owing to the alleviation of systemic side effect, while maintaining the anti-inflammatory effect (Fig. 15).

6.2. Application of CyD in Gene Therapy Gene therapy requires carriers that can efficiently and safely transfer

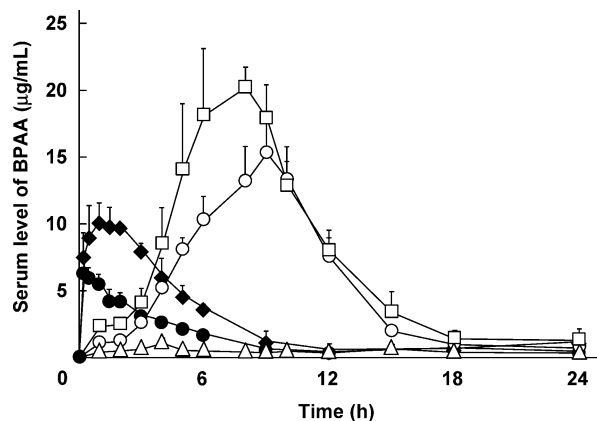


Fig. 14. Serum Levels of BPAA after Oral Administration of α - (○), β - (△), γ -CyD Ester Conjugates (□), BPAA (●), or BPAA/ β -CyD Complex (◆) (Equivalent to 10 mg/kg BPAA) to Rats

Each point represents the means \pm S.E. of 3 experiments.

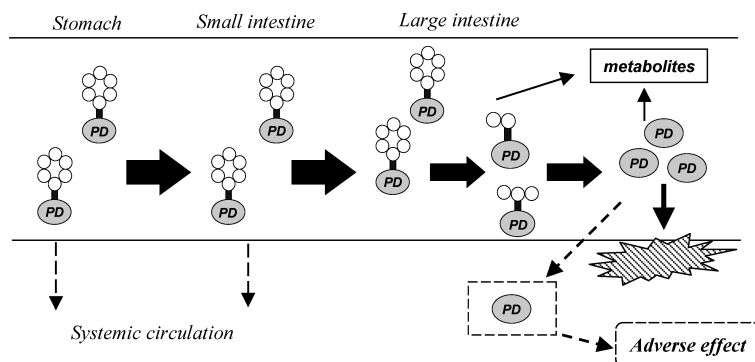


Fig. 15. Proposed Mechanism of Anti-inflammatory Effect and Decreased Side Effect after Oral Administration of Prednisolone (PD) Succinate/ β -CyD Conjugate to TNBS-Induced Colitis Rats

the gene into the nucleus of the desired cells. There are two categories of gene therapy vectors, *i.e.*, viral vectors and non-viral vectors. The nonviral vectors have many advantages over viral vectors, such as ease of manufacture, safety, low immunogenicity, and molecular attachment of targeting ligand.¹⁷⁸⁾ However, the problem is that the efficiency of nonviral vector-mediated gene transfer to cell is markedly low, compared to the viral vectors. To improve the transfection efficacy of nonviral vector, we synthesized the starburst polyamidoamine dendrimer (generations 2—4) conjugates with α -, β -, and γ -CyDs (CDE conjugates), expecting the synergistic effect of dendrimer and CyDs⁵⁷⁾ The plasmid DNA which contains the firefly luciferase gene under the control of the SV40 promoter was used. Figure 16 shows the chemical structures of CDE conjugates prepared. From the *in-vitro* and *in-vivo* evaluation,^{58,59)} it could be concluded that the conjugate having generation 3 dendrimer and average degree of substitution of 2.4 of α -CDE is the best compound among the 9 conjugates tested. This compound showed the greater gene transfer activity with lower cytotoxicity, compared with commercially available non-virus vectors such as LipofectinTM and TransFastTM. Figure 17 shows the proposed scheme on the enhancement of gene expression by α -CDE conjugate. The superior gene transfer activity could be ascribed to the synergistic effects of the proton-sponge effect of dendrimer and the tentative membrane-disrupting effect of α -CyD on endosomal membranes. The inclusion ability of the conjugate with phospholipids was confirmed by a separate experiment, using liposomal membrane. Therefore, α -CDE conjugate is a potent candidate of non-viral vector and may be useful for design of the other non-viral vectors. To achieve the cell specific gene transfer, we have recently designed the mannosylated α -CyD with the dendrimer as the targetable ligand to cells which express the mannose-receptor such as macrophages, dendritic cells and epithelial cells.

7. Perspective

A number of CyD derivatives, CyD polymers and CyD conjugates have been designed and evaluated for practical uses in various fields. These CyDs are applicable to pharmaceutical formulations in the forms of complex and conjugate. In the case of the complex, hydrophilic CyDs are widely used for enhancing the dissolution and absorption of poorly water-soluble drugs. In addition, HP- β -CyD is effective for control of solid state properties of drug molecules such as polymorphic transition and crystallization rates during stor-

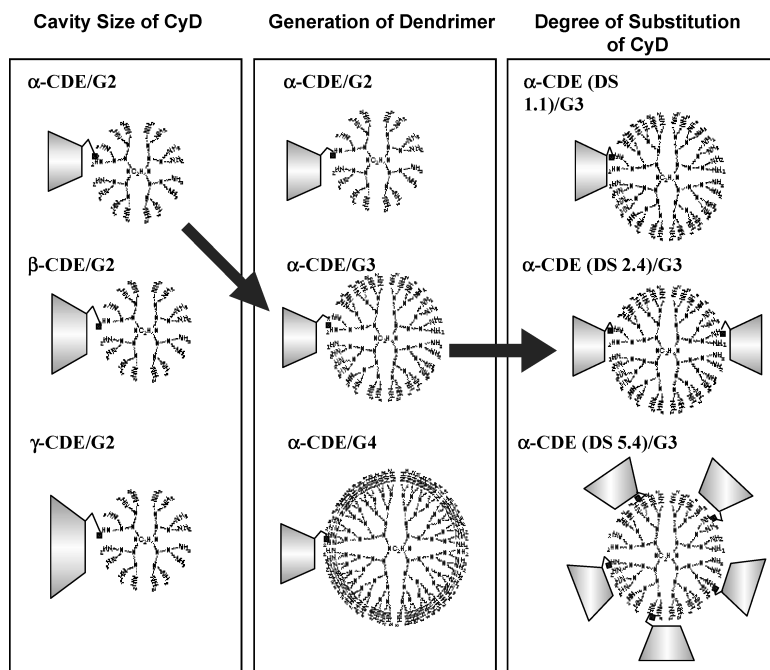


Fig. 16. Molecular Design of CyD/Polyamidoamine Dendrimer Conjugates as Non-viral Gene Transfer Vector

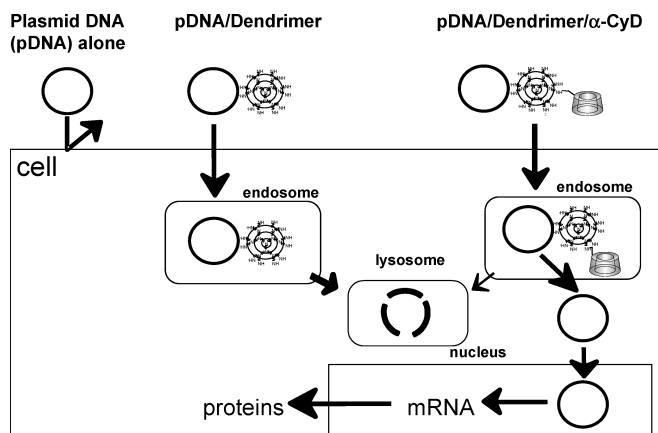


Fig. 17. Proposed Scheme of Gene Expression by β -CyD/Polyamidoamine Dendrimer Conjugate

age. By contrast, the hydrophobic CyDs are useful as sustained-release carriers of water-soluble drugs. Amphiphatic CyDs are able to improve the transdermal and transmucosal absorptions of drugs, and also chemical stability of peptide and protein drugs. Since CyDs are able to extend the function of pharmaceutical additives, the combination of molecular encapsulation with other carrier materials will become effective and valuable tools in the improvement of drug formulation. A combined use of different CyDs and pharmaceutical additives will provide more balanced oral bioavailability with prolonged therapeutic effects. On the other hand, the drug/CyD conjugate may be useful for colon specific delivery, time controlled release, gene delivery, and prolonged drug release, with the assistance of some carrier materials such as liposome. In particular, cationic polymer/CyD conjugate can be a novel candidate for non-viral vectors to enhance the gene transfer. Owing to the increasingly globalized nature of the CyD-related science and technology, development of the CyD-based pharmaceutical formulation is also

rapidly progressing. The future should see a number of commercial products using various CyD derivatives.

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