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# Cyclodextrins based delivery systems for macro biomolecules

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## ABSTRACT

Macro biomolecules are of vital importance in regulating the biofunctions in organisms, in which proteins (including peptides when mentioned below) and nucleic acids (NAs) are the most important. Therefore, these proteins and NAs can be applied as “drugs” to regulate the biofunctions from abnormal to normal. Either for proteins and NAs, the most challenging thing is to avoid the biodegradation or physicochemical degradation before they reach the targeted location, and then functions as complete functional structures. Hence, appropriate delivery systems are very important which can protect them from these degradations. Cyclodextrins (CDs) based delivery systems achieved mega successes due to their outstanding pharmaceutical properties and there have been several reviews on CDs based small molecule drug delivery systems recently. But for biomolecules, which are getting more and more important for modern therapies, however, there are very few reviews to systematically summarize and analyze the CDs-based macro biomolecules delivery systems, especially for proteins. In this review, there were some of notable examples were summarized for the macro biomolecules (proteins and NAs) delivery based on CDs. For proteins, this review included insulin, lysozyme, bovine serum albumin (BSA), green fluorescent protein (GFP) and IgG’s etc. deliveries in slow release, stimulating responsive release or targeting release manners. For NAs, this review summarized cationic CD-polymers and CD-cluster monomers as NAs carriers, notably, including the multi components targeting CD-based carriers and the virus-like RNA assembly method siRNA carriers.

**Key words:** cyclodextrins, drug delivery systems, proteins, nucleic acids, particles

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## 55 1. Introduction

56 Proteins and NAs are the most vital fundamental biomolecules to support lives. The peptides are  
57 the fundamental structures of functional proteins that regulate thousands of bio-reactions in  
58 lives. The nucleic acids carry genetic information for lives and regulate bio-reactions as well in  
59 some proper ways. Therefore, both proteins and NAs can be employed as drugs in vast diseases  
60 especially in metabolic disorders, respectively.

61 The proteins based drugs achieved great successes in the last 3 decades, especial after the  
62 outcome of the representative diabetes protein drug—insulin and other protein drugs such as  
63 monoclonal antibodies, recombinant proteins, protein-based vaccines, etc., applied in almost  
64 every field of diseases such as cancer, inflammatory diseases, metabolic diseases and  
65 diagnostics[1–3]...In general, compared to the conventional small-molecule drugs, the protein  
66 drugs revealed great benefits such as higher specificity, greater activity and less toxicity[4]. The  
67 specific affinity and greater activity due to the proteins could selectively effect on signaling  
68 molecules that bind to cell surface receptors specific, like ion channels or G protein-coupled  
69 receptors, then induced the intercellular effects[5]. However, the protein drugs gained their own  
70 weaknesses such as large size or molecular weight, enzymatic degradation, poor permeability,  
71 fragile structure, easy aggregation, poor stability, elicit the over immunologic response, etc. All  
72 these weaknesses posed a mega challenge—how hard dose the protein drugs permeate to cells,  
73 tissues and organs!

74 Another challenge for protein is the deficiency of formulations, most proteins deeply relied on  
75 subcutaneous or intravenous injections, unlike the chemotherapy agents, most of them couldn't  
76 be prepared for oral formulations—the most common and easy acceptable administration to  
77 patients. For proteins, even there were and are a lot of investments in non-invasive delivery  
78 strategies for proteins, however, there were just very few successful examples[6,7]. As for oral  
79 formations of proteins studies, the reality is that the oral formulations were 10% as subcutaneous  
80 does in bioavailability evaluation in general[8,9]. Although there were some approved small  
81 proteins delivered via intranasal method[10], the large proteins seem not possible delivered by  
82 intranasal formulation. No matter what kinds of formulations it would eventually take for  
83 proteins, the fundamental requirement is an appropriate delivery system for proteins, which  
84 could overcome these weaknesses mentioned and improve the bioavailability as well.

85 Gene therapy holds great potential for the treatments of vast diseases including cancers[11],  
86 metabolic disorders[12], infections of microorganism[13], vaccines deponed diseases[14] and  
87 especially for inherited diseases[15], therefore, they are considered as the terminators of  
88 diseases.

89 Typical gene therapies including plasmid DNAs, anti-sense oligonucleotides, small interfering  
90 RNAs, genetically engineered cellular therapies, etc., can be classified as DNA and RNA  
91 therapeutics. The DNA therapeutics exhibited great success in rare diseases' therapy, the first  
92 approved anti-sense oligonucleotides, *Vitravene*, for the cytomegalovirus (CMV) posed  
93 immunocompromised retinitis in the late 1990s, *Spinraza* for the spinal muscular atrophy (SMA),  
94 *Tegsedi* for the polyneuropathy of hATTR, etc.[16] Similar situation also occurred in the RNA  
95 therapeutics, such as the approved RNA drugs: *Eteplirsen* for the Duchenne muscular dystrophy;  
96 *Volanesorsen* for the familial chylomicronemia syndrome; *Inotersen* for the hereditary  
97 transthyretin amyloidosis, etc.[17]. The gene therapeutics also suggested a great potential in  
98 common diseases therapy. According to the Watts et al. summary, there are at least 431

99 RNA-targeting drug programs in different development stages and oligonucleotide companies  
 100 were drastically increased 94.2% in the last 5 years. Also in the last 5 years, the private equities  
 101 invested US\$2.8 billion only on three representative mRNA therapeutic companies (Moderna  
 102 Therapeutics, BioNtech, and CureVac)[17].

103 The key factor for the success of gene therapy is to avoid the biodegradation of gene-drugs in any  
 104 status of the transfected process to the targeted cells. While, the nucleic acid's intrinsic  
 105 properties prevent the transfection process generally, due to their large molecular size,  
 106 polyanionic phosphodiester linkage and sensitive to nuclease[18,19]. Hence, like the protein  
 107 drugs, the nucleic acids also need an appropriate delivery strategy to be transfected to targeted  
 108 cells.

109 CDs as very successful delivery molecules can play vital roles in solving these macro biomolecules  
 110 delivery issues, typical CDs are a family of basket-shape cyclic oligosaccharides, composing 6, 7  
 111 and 8 glucoses, named  $\alpha$ -,  $\beta$ - and  $\gamma$ -CDs, respectively. Hydroxyls on 6-Cs called primary hydroxyls,  
 112 on 2-Cs and 3-Cs called secondary hydroxyls, the hydroxyls on 2-Cs orienting to the inner cavity,  
 113 and hydroxyls on 3-Cs orienting to the outer cavity[20]. Hydroxyls surrounded lead to the  
 114 hydrophilic property on the outer of CDs, while the hydrophobic inner cavity attributed to the  
 115 protons H-3 and 5 pointing to the inner cavity (see fig. 1). Due to amphiphilic character, almost  
 116 nontoxicity, and cheap prices which CDs have long been employed in the food and  
 117 pharmaceutical industry. In the beginning, the CDs employed as excipients in drugs' formation,  
 118 and subsequently, as the observation of host-guest interaction (the hydrophobic groups at  
 119 appropriate size can penetrate inside the CDs hydrophobic cavity which improves the poor water  
 120 solubility), forming nanoparticles after being functionalized via self-assembly which, therefore,  
 121 raised new opportunities for drug delivery to improve the bioavailability of poorly soluble drugs  
 122 and achieved mega successes[21].

123 Though CDs applied in biomolecules delivery are not as common as in small molecules delivery,  
 124 CDs also have a long history that employed in biomolecules delivery[22,23]. CDs as delivery  
 125 carriers provided numerous of functionalization accesses which allowed desired functional  
 126 groups introduced in, and the hydrophobic cavity also could form host-guest interaction with the  
 127 hydrophobic groups of biomolecules that could improve their stability of the biofunction  
 128 structures. More importantly, that it has been shown the CDs could strengthen the effects of  
 129 proteins such as monoclonal antibodies, peptides and nucleotides[24]. As the importance of  
 130 biomolecules rising, the more vital roles CDs will play, and however there just very few  
 131 summaries of CDs applied in biomolecules delivery have been exhibited, that is, therefore,  
 132 *cyclodextrins based delivery systems for macro biomolecules* has been prepared. More details of  
 133 CDs based macro biomolecules' delivery features like the above described would be displayed in  
 134 the following context.

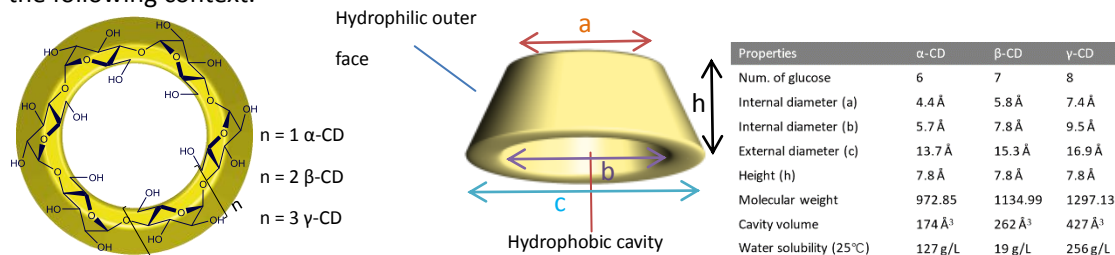


Fig. 1. Structures, amphiphilic properties and their geometric parameters of CDs

135  
 136  
 137

## 2. The delivery of proteins

As the Canadian physiologist Charles Best, Sir Frederick Banting et al. first discovered that insulin was an effectual remedy to diabetes[25], the protein drugs continue emerging, till now it becomes a giant in the whole drugs market share. However, the intrinsic properties of protein such as large size, enzymatic degradation, short circulation half-lives, fragile tertiary structures and poor membrane permeability, etc. resulting in a huge barrier for their clinical applications[2,4,26]. Hence, appropriate delivery strategies could either overcome the above disadvantages or render targeting abilities.

Generally, researchers usually investigated their studies of CDs based protein delivery systems in protein loading experiments, delivery materials biodegrade experiments, cytotoxicity experiments, protein releasing experiments, etc. If there were nanoparticles of delivery systems had formed, a lot of nanoparticle parameters also had been studied, such as size,  $\zeta$  potential, etc.

In this section the protein delivery methods would be introduced in terms of loading materials and followed the below orders: polypseudorotaxanes, supramolecular polymers, mutilate components targetable delivery system and functionalized CD monomers or dimers, displayed in table 1.

Table 1. The summary of proteins delivery and release methods

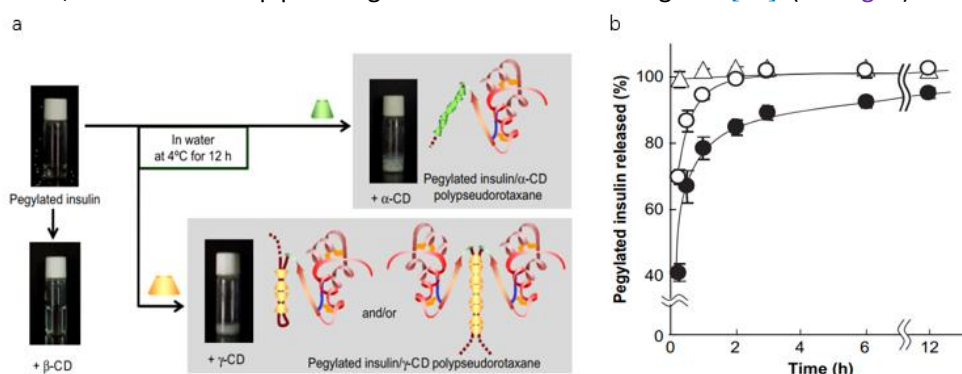
Loading materials	Proteins	Releasing methods	References	Years
CD-based polypseudorotaxanes	Insulin	Slow releasing	[27]	2008
CD-based polypseudorotaxanes	Insulin	Slow releasing	[28]	2009
CD-based polypseudorotaxanes	Lysozyme	Slow releasing	[29]	2009
CD-based polypseudorotaxanes	IgG, antibody, bromelain	Slow releasing	[30,31]	2015, 2017
CD-based polypseudorotaxanes	Insulin	Thermo responsive stimulating releasing	[32,33]	2017, 2020
Supramolecular polymers	BSA, IgG, lysozyme	Slow releasing	[34]	2008
Supramolecular polymers	Lysozyme	Slow releasing	[35]	2006
Supramolecular polymers	GFP	Photo responsive stimulating releasing	[36]	2006
Supramolecular polymers	BSA	Photo responsive stimulating releasing	[37]	2015
Targetable delivery systems	BSA, saporin, nuclease Cas9 protein	Targeted releasing	[38]	2019
Targetable delivery systems	DNAzyme	Targeted releasing	[39]	2019
Targetable delivery systems	Tyrosinase-related protein 2	Targeted releasing	[40]	2020
Functionalized CD	Insulin	Slow releasing	[41–43]	2011

monomers and dimers				
Functionalized CD monomers and dimers	BSA	Slow releasing	[44–46]	2005, 2006, 2007
Functionalized CD monomers and dimers	Lysozyme	Slow releasing	[47]	2007
Functionalized CD monomers and dimers	Bovine pancreatic trypsin	–	[48]	2004
Functionalized CD monomers and dimers	Insulin	–	[49]	2014
Functionalized CD monomers and dimers	$\alpha$ -Chymotrypsin	Slow releasing	[50]	2006
Functionalized CD monomers and dimers	Antibody	–	[51]	2014
“–” means the authors didn’t mentioned in the cited articles				

155

156 **2.1 Slow released proteins via CD-based polypseudorotaxane**

157 Slow release insulin administration is very helpful to insulin-depend diabetes patients, it can  
 158 minimize the insulin using numbers that can reduce the pain to patients marked. Uekama team  
 159 reported PEGylated insulin/CD polypseudorotaxane controlled release systems for insulin[27].  
 160 Insulin has 3 free primary amino groups, one located on A chain, another two located on B chain  
 161 of insulin which rendered multiply-PEGylated access sites to insulin. They coupled insulin with  
 162  $\alpha$ -succinimidyl-oxysuccinyl- $\omega$ -methoxy-polyoxyethylene to form PEGylated insulin[52], then,  
 163 assembled pseudorotaxanes with  $\alpha$ - and  $\gamma$ -CDs (the PEGylated insulin couldn’t form gel with  $\beta$ -CD)  
 164 to form gels. *In vitro*, insulin release studies exhibited that both  $\alpha$ - and  $\gamma$ -CDs  
 165 polypseudorotaxanes could prolong the releasing time of insulin compared to PEGylated insulin  
 166 alone. When some free  $\alpha$ - and  $\gamma$ -CDs were added to the buffer that would further decrease the  
 167 release rate of insulin than just CDs polypseudorotaxanes, because the threading and  
 168 dethreading of polypseudorotaxanes processes were dynamic equilibrating. *In vivo* release study,  
 169 the  $\gamma$ -CD polypseudorotaxane also showed a prolonged release manner than the PEGylated  
 170 insulin, which could keep plasma glucose at low level in long time[27]. (See fig. 2)



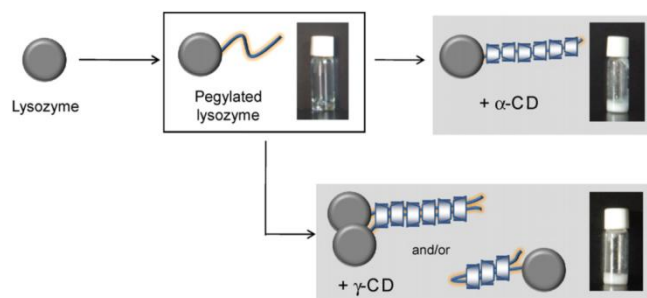
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172 Fig. 2. a) PEGylated insulin assembled polypseudorotaxanes with  $\alpha$ - and  $\gamma$ -CDs while can't form  
 173 polypseudorotaxanes with  $\beta$ -CD, b) the release profiles of PEGylated insulin alone ( $\Delta$ ) or formed  
 174 polypseudorotaxanes with  $\gamma$ -CD (o) and  $\alpha$ -CD (•)[27]

175

176 In the following study, Uekama team devised randomly- or multiply-PEGylated insulins then

177 assembled polypseudorotaxanes with  $\alpha$ - and  $\gamma$ -CDs for insulin delivery[28]. The PEGylation  
 178 procedures followed the report of Kim et al. in selectively introduced PEG to primary  
 179 amines[53,54]. That method in resulting, to give 35% of mono-substituted, 49% of di-substituted  
 180 and 9% of tri-substituted PEGylated insulins according to the HPLC spectra. The PEGylated  
 181 insulins and CDs were assembled to polypseudorotaxanes, followed the same procedure as the  
 182 above study[27]. The insulin release trials indicated that the polypseudorotaxanes were  
 183 remarkably decreased in release rate than PEGylated insulin in general. Compared the insulin  
 184 release rate of  $\alpha$ - and  $\gamma$ -CD polypseudorotaxanes, the  $\alpha$ -CD polypseudorotaxanes displayed much  
 185 slower release rate, in addition, the release rate were largely related it's concentration in  
 186 phosphate buffer, for instance, the same mounts of insulin- $\alpha$ - and - $\gamma$ -CDs polypseudorotaxanes in  
 187 1 mL, 0.85 mL and 0.45 mL of phosphate buffer displayed different release rates (the rate: 1 mL >  
 188 0.85 mL > 0.45 mL). The equilibrated threading and dethreading of polypseudorotaxanes  
 189 processes probably could explain those slow release results[28].  
 190 Inspired by the above study, Uekama et al. investigated a twice larger protein lysozyme's delivery.  
 191 A similar PEGylation strategy was employed on lysozyme's delivery, PEGylated lysozyme  
 192 assembled polypseudorotaxanes with  $\alpha$ - and  $\gamma$ -CDs. The release study suggested the release  
 193 order was PEGylated lysozyme >  $\alpha$ -CD polypseudorotaxane >  $\gamma$ -CD polypseudorotaxane[29]. From  
 194 insulin to lysozyme, it indicated that the CDs-PEG polypseudorotaxane systems could be used for  
 195 different proteins' delivery that provided a slow release model to different proteins or other  
 196 molecules delivery. (See fig. 3)



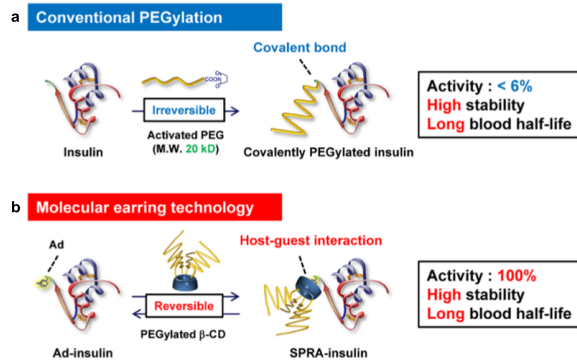
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198 Fig. 3. PEGylated lysozyme assembled polypseudorotaxanes with  $\alpha$ - and  $\gamma$ -CDs[29]

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200 However, in terms of some study, that the PEGylation of insulin or lysozyme could reduce their  
 201 bioactivities, due to the steric hindrance from large PEG chains or PEGylation originated structure  
 202 transformation of proteins, for insulin the activity reduced to less than 6%, and for lysozyme, the  
 203 number was 70%[24]. (See fig. 4a) Therefore, the Arima team's resolution for this problem was  
 204 applied an ignorable small molecule—adamantane (Ad) to conjugate to insulin, and then the  
 205 PEGylation occurred on CDs, which subsequently, formed host-guest complexes with Ad-insulin  
 206 that could retain the activity of insulin after release, this had been called "self-assembly  
 207 PEGylation retaining technology" (SPRA) by authors[55]. (See fig. 4b)





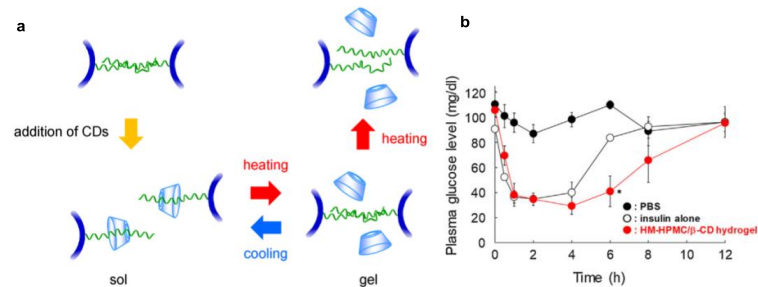
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Fig. 4. Activity of PEG-insulin and Ad-insulin assembled with PEGylated-CDs[24]

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211 Uekama and Hirayama team also developed a thermo-responsive injectable sustained release  
 212 drug delivery system based on CD/hydrophobically modified hydroxypropylmethylcellulose  
 213 (HM-HPMC) formed hydrogel[32,33]. The working mechanism was due to the HM-HPMC/CD  
 214 hydrogel with a low viscosity at low temperatures, therefore, the hydrogels were easy to inject,  
 215 while, at a high temperature like 37°C, the gels were in high viscous status, that resulted a  
 216 sustained molecule release manner. The hydrogels assembled via host-guest interactions by free  
 217 CDs and HM-HPMC to form polyrotaxanes (see fig. 5a). The sustained-release effect of the  
 218 HM-HPMC/CD hydrogels was investigated as following, the model drug insulin was loaded then  
 219 injected to the rats, the free insulin was also injected to another group as comparability and the  
 220 PBS was injected to the 3<sup>rd</sup> rat group as blank control. As the conclusion, the plasma glucose level  
 221 tested results showed the HM-HPMC/CD/insulin injection group kept at 40 mg/dL for 6 h while  
 222 for free insulin injection group was 4 h and increased to 80 mg/dL very fast in 2 h[33] (see fig. 5b).  
 223 In terms of the release study results and the working mechanism, the thermo-responsive strategy  
 224 displayed great potential in sustained-release drug delivery not just including protein drugs.



225

226

Fig. 5. a) Thermo-responsive HM-HPMC/CD gel working mechanism[32], b) HM-HPMC/CD delivered insulin exhibited marked plasma glucose control manner[33]

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## 2.2 Supramolecular interaction-based bio-degradable and stimulating responsive release

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### protein carriers

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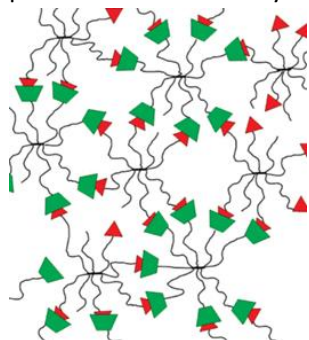
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Hennink team reported a bio-degradable—PEG-β-CD/PEG-cholesterol—hydrogel applied for  
 proteins delivery. They designed and prepared 8-arm PEG with different molecular weights (10,  
 20 and 40 kDa) grafted β-CD or cholesterol, then, formed gel via self-assembling (see fig. 6)[34].  
 They employed 3 different proteins lysozyme, BSA and IgG to evaluate the release profiles of the  
 mentioned hydrogels. The 3 proteins were loaded on 22.5% (w/w) PEG<sub>8</sub>20K-β-CD/PEG<sub>8</sub>20K-chol  
 (PEG<sub>8</sub> represents 8-arm PEG, 20K represents its mole weight grade, chol represents cholesterol)  
 gel, respectively, then under the release conditions[56] to determine their release rates. The

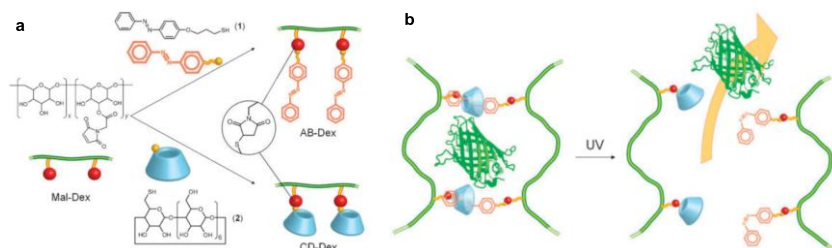
238 results exhibited a striking sustained release manner that lasted more than 9 days. The authors  
 239 attributed this astonished slow release effect to the slower surface erosion, in addition, when the  
 240 proteins released the hydrogels were degraded which was perfect for the clinical application[57].



241  
 242 Fig.6. PEG-β-CD/PEG-cholesterol hydrogel[34]

243

244 Stimulating responsive controlled release proteins delivery is always sought by modern  
 245 pharmaceuticals, the noncovalent interactions like supramolecular interaction provide a perfect  
 246 option for the stimulating responsive manner for its reversible interaction. Kros team reported a  
 247 cyclodextrin based photo-switchable crosslinked hydrogel applied on protein delivery[58]. The  
 248 components of this hydrogel consisted a “guest chain”–maleimide functionalized dextran  
 249 (Mal-Dex), on which an azobenzene grafted via the thiol-maleimide “click” reaction[59], and a  
 250 “host chain”–β-CD grafted on another maleimide functionalized dextran (CD-Dex), also via the  
 251 thiol-maleimide “click” reaction (see fig. 7a). To evaluate the stimulating release property of this  
 252 gel, GFP as model protein was loaded. Under UV irradiation ( $\lambda = 365$  nm), the azobenzene  
 253 transformed to cis isomer, dissociated inclusion interaction, caused the dissolution of the  
 254 hydrogel[36], then added GFP, next removed the UV irradiation, the GFP was physically  
 255 entrapped due to the azobenzene transformed to trans, formed inclusion interaction again. The  
 256 release study exhibited when the hydrogel under UV irradiation ( $\lambda = 365$  nm), the GFP release  
 257 improved markedly in a stimulating responsive release manner (see fig. 7b).

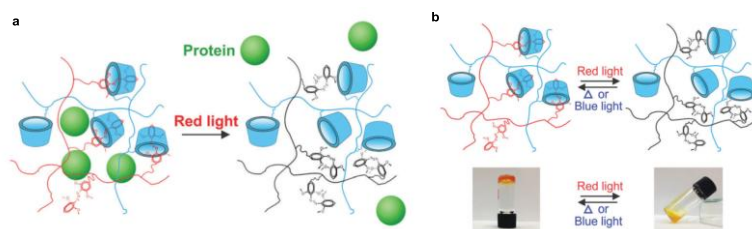


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259 Fig. 7 a) components of supramolecular interaction based light responsive protein carriers, b) the protein released  
 260 after UV irradiation[58]

261

262 Based on similar supramolecular interaction strategy, Wu team[37] applied  
 263 tetra-*ortho*-methoxy-substituted azobenzene (mAzo) and β-CD as host-guest interaction  
 264 components both grafted on backbone poly(acrylic acid). When the system irradiated by red light  
 265 (600-900 nm), the mAzo transformed to trans isomer, allowed the system to form host-guest  
 266 complex (gel form) at the same time encapsulated protein (BSA), however, when the system  
 267 irradiated with blue light, the mAzo transformed to *cis* isomer, the host-guest complex broken to  
 268 give solution form, allowed the system to release proteins. (See fig. 8)



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Fig. 8. a) Proteins were loaded under gel form (host-guest interaction), and released under solution form after red light irradiation, b) the gel and solution form could transform under different lights irradiation[37]

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### 2.3 Targetable protein vectors based on CDs

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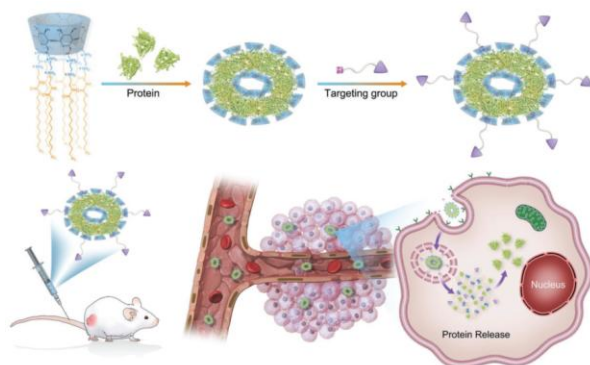
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CD-based targeting proteins delivery also attempted by scientists, Feng team developed a targeted intracellular protein delivery system based on functionalized  $\beta$ -CD called CDEH by the authors[38]. The CDEH is made of the moieties of selective conjugated hydrophobic chain via ionic linker to CD on the primary face. They chose BSA as model protein to evaluate the CDEH's protein loading capacity, the results revealed that the mass ratio of CDEH and BSA reached 2:1, the BSA could be entrapped completely. Under the same ratio of CDEH and saporin, the saporin which has been used for cancer therapy in clinical trials, could also almost be entrapped completely. MDA-MB-231 cells were selected for *vitro* cellular uptake study, MDA-MB-231 cell membranes overexpressed nucleolin receptors which could bind to AS1411 aptamer (AP)[60]. Then the AP as recognition molecule to MDA-MB-231 was introduced through the conjugation to adamantane, which allowed the AP physically grafted on CDEH via the host-guest interaction. (See fig. 9) The authors compared the free saporin, CDEH/saporin and targeted CDEH-AP/saporin in their cellular uptake efficiency suggested that the free saporin didn't cause any cell death due to its poor cell membrane permeability, CDEH/ saporin exhibited significant higher cell growth inhibition, and CDEH-AP/saporin showed the best inhibition.



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Fig. 9. Saporin loaded on targetable CDEH to form nanocomplexes and their uptake mechanism on mice[38]

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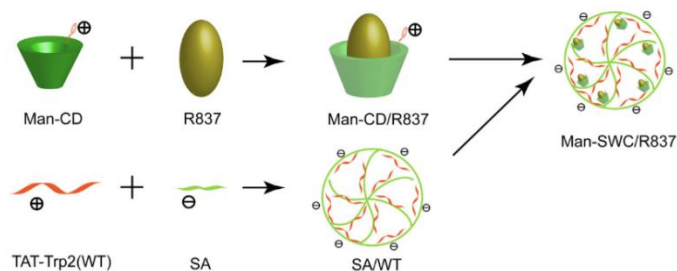
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The authors also applied this system on the delivery of nuclease Cas9 protein and sgRNA (small guide RNA), which both are employed in the prevalence genome editing tool today, called CRISPR/Cas9 genome editing system[61,62]. In this system, the sgRNA can specifically recognize the complementary DNA and guide the Cas9 protein to cleave the target DNA sequences precisely and then edit the genome successfully[63]. Nevertheless, how to deliver the Cas9 protein and sgRNA remains a real challenge nowadays, therefore, the authors employed the modified CDEH nanoparticles for the Cas9 protein loading. Polo-like kinase 1 (PLK1), usually over expressed on many tumor cells and which had been inhibited that could induce tumor cell

300 apoptosis. Therefore, PLK1 targeted sgRNAs were co-loaded with Cas9 protein on CDEH then  
 301 treated the Hela cells resulted in 47.1% cleavage of the target gene and rendered the  
 302 proliferation inhibition significant.[38] In terms of the above results, the CD-based CDEH  
 303 delivery system holds great potential on multi-functions, it could be applied as protein or  
 304 protein/RNA delivery which provided a fresh new option for genome editing.

305 Chemotherapy is still one of the most important cancer treatments nowadays, while the  
 306 resistance of anticancer agents is largely reducing the chemotherapy efficiency somehow.  
 307 P-glycoprotein 170 encoded via multi-drug resistance 1 gene (MDR1) over expressed plays a key  
 308 role in the drug resistance[64]. The Nematollahi-Mahani team[39] reported a downregulating the  
 309 MDR1 strategy to reduce the P-glycoprotein 170 expressing, subsequently, decreased the  
 310 drug-resistance effect. The mRNA-cleaving DNzyme (DNZ) can regulate the mRNA expressing  
 311 was implicated by numbers of evidences[65], therefore, the authors chose a mRNA-cleaving DNZ  
 312 which could target the mRNA of MDR1 in doxorubicin (Dox) resistance breast cancer, further  
 313 downregulated the P-glycoprotein expressing resulted in reducing the drug resistance effect. The  
 314 authors applied the reported chitosan cross-linked  $\beta$ -CD and pentasodium tripolyphosphate (TPP)  
 315 as the delivery vector for DNZ[66]. The WST1 study results revealed that Dox associated with  
 316 DNZ-MDR1 could induce cell death in the drug resistance cells significantly compared to 5  $\mu$ g/mL  
 317 DNZ-unspecific treated MCF-7 cells. The MDR1 mRNA and P-glycoprotein 170 expression reduced  
 318 results also cross-validated the above results[39].

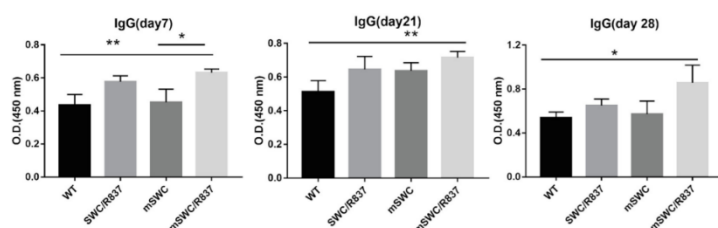
319 The antitumor protein vaccines hold great potential in tumor therapy which successfully  
 320 attracted a lot of attention from scientists. Before the vaccine proteins working as vaccines, one  
 321 mechanism is that the protein should be uptake by dendritic cells (DCs). Enhanced the cellular  
 322 uptake efficiency of DCs, triggers a strong immune efficacy but avoided toxicity over the  
 323 non-specific effects at the same time. In order to improve the cellular uptake efficiency, Hu team  
 324 developed a CD-based protein co-delivery system for both melanoma antigen protein  
 325 Tyrosinase-related protein 2 (Trp2) and Toll-like-receptor-7 (TLR-7) agonists imiquimod (R837)[40].  
 326 Trp2, a confirmed melanoma tumor-associated antigen[67] and R837, a robust vaccine adjuvant  
 327 could promote immune efficacy[68] were chosen to combine as a vaccine in Hu team's devise. CD  
 328 provided a hydrophobic cavity for the R837 and the mannose grafted on CD could specifically  
 329 interact with the DCs, on which surface abundantly expressed mannose receptors. TAT, a  
 330 cell-penetrating peptide (CPP), was employed to improve the solubility of Trp2 via a conjugation  
 331 between TAT and Trp2 to give a product named WT[69] that would increase the cellular uptake  
 332 ability to WT by DCs. Subsequently, the co-delivery nanocomplex system was assembled  
 333 Man-CD/R837, sodium alginate (SA) and WT via electrostatic interaction. (See fig. 10)



334 TAT-Trp2(WT) SA SAWT  
 335 Fig. 10. Preparation of mSWC/R837[40]

336  
 337 The authors prepared two kinds of nanocomplexes, Man-CD/R837, CD/R837 and SA/WT, called

338 mSWC/R837 and SWC/R837, respectively. The cytokines secretion determination study results  
 339 displayed that the nanocomplex delivered vaccine induced highest level of cytokines secretion  
 340 compared to WT alone or SWC/R837, especially IFN- $\gamma$  secretion in BMDCs. And also the  
 341 mSWC/R837 also induced a higher level of IgG in serum than WT alone, mSWC alone and  
 342 SWC/R837 in day 7, 21 and 28 which indicated that the mSWC/R837 induced greater  
 343 lymphocyte activation effector cytokine secretion and led to a synergistic cellular immune  
 344 response effect that endowed this co-delivery system for vaccines great potential in clinical  
 345 applications[40]. (See fig. 11) Additionally, an earlier study in 2016 was investigated by Ishii  
 346 team[70], which also proved that the hydroxypropyl- $\beta$ -CD employed as adjuvant of vaccine could  
 347 also stimulate a serum IgG response via intranasal administration.



348

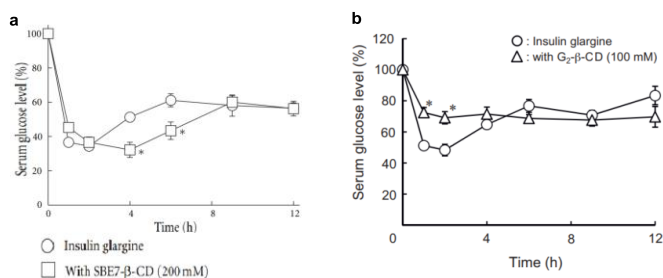
349 Fig. 11. WT, SWC/R837, mSWC and mSWC/R837 induced IgG secretion level in 7, 21 and 28 days[40]

350

#### 351 2.4 CD-functionalized monomers or dimers applied as protein delivery vectors

352 Except the CDs-PEG polypseudorotaxane insulin delivery systems, Uekama team also developed  
 353 sulfobutyl ether- $\beta$ -CD (SBE- $\beta$ -CD) and maltosyl- $\beta$ -CD ( $G_2$ - $\beta$ -CD) as insulin delivery systems. The  
 354 authors discovered that the SBE7- $\beta$ -CD and  $G_2$ - $\beta$ -CD could influence the aggregation of insulin,  
 355 even not in the same mechanism[71] but all of them could enhance the bioavailability and  
 356 persistence, and applied in insulin administration could prolong the release process marked,  
 357 compared to insulin glargine (see fig. 12a and 12b). The authors proposed this outcome to the  
 358 inhabitation of SBE7- $\beta$ -CD on the enzymatic degradation of insulin[41,43]. Notably, the  
 359 SBE7- $\beta$ -CD and  $G_2$ - $\beta$ -CD/insulin administrations exhibited peak-less sustained hypoglycemic effect  
 360 compared to insulin glargine[42,72].

361



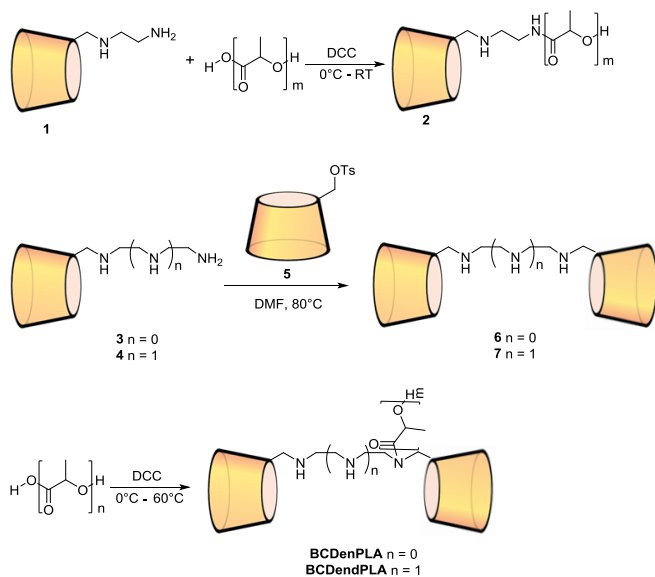
362

363 Fig. 12. Both SBE7- $\beta$ -CD and  $G_2$ - $\beta$ -CD loaded insulin displayed comparative sustained hypoglycemic effect  
 364 compared to insulin glargine and peak-less effect as well[42,72]

365

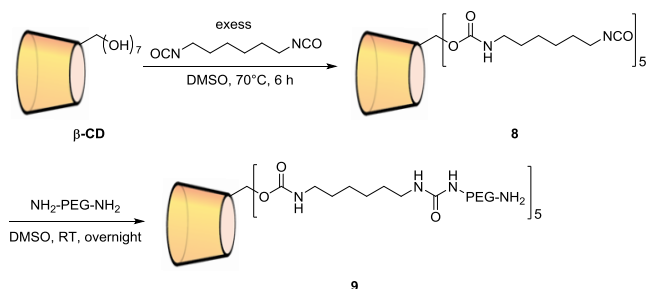
366 Ma team[44] reported a copolymer, consisted of the poly(DL-lactide) (PLA) and  $\beta$ -CD via the  
 367 linkage of ethylenediamine, CDenPLA (2) which could fabricate nanoparticles with protein,  
 368 subsequently, to fulfill the purpose of protein delivery. They compared the loading capacity of  
 369 bovine serum albumin (BSA) with encapsulation efficiency (EE%) between CDenPLA and its  
 370 conjugated PLA alone, found out that the EE% of CDenPLA polymers is much higher than PLA

371 polymers. In addition, different nanoparticle fabrication methods[73,74] showed different  
 372 loading capacities. And the BSA release profiles of CDenPLA were faster compared to its  
 373 conjugated PLA alone but both releasing processes could sustain to a month or more[44,46].  
 374 Then they figured out that the bisaminated  $\beta$ -CDs were easier to form a complex with BSA than  
 375 the monoaminated  $\beta$ -CD[75], hence,  $\beta$ -CD dimer was grafted on PLA to form so called BCDenPLA  
 376 and BCDendPLA delivery systems (see scheme 1). The BCDenPLA and BCDendPLA system in  
 377 model protein BSA's delivery exhibited good loading capacity while related to its particle size, for  
 378 instance, 300-400 nm in diameter particles' EE reached 70-83%, in contrast, sized 150-250 nm  
 379 particles' EE was 40-50%. In addition, the BCDenPLA and BCDendPLA systems revealed a BSA  
 380 slow release behavior that could last 30 days as well[45].



381  
 382 Scheme 1. Synthesis of CDenPLA (2), BCDenPLA and BCDendPLA

383  
 384 CDs and PEG both hold remarkable biocompatibility and hydrophilicity characteristics in drug  
 385 delivery, hence, the Caliceti team devised a series of CD/PEG hydrogels consisted different  
 386 CD/PEG molar ratios, to deliver proteins like lysozyme[47]. They applied  $\beta$ -CD to link with  
 387  $\text{NH}_2$ -PEG- $\text{NH}_2$  via hexamethylene diisocyanate, obtained CD-PEG conjugates[76,77]. (See scheme  
 388 2)

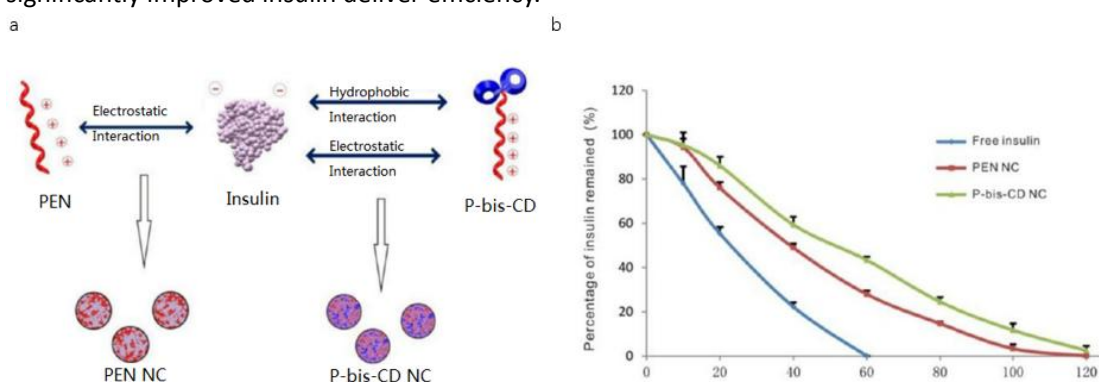


389  
 390 Scheme 2. Synthesis of CD-PEG conjugates

391  
 392 The lysozyme loading study suggested that when the CD/PEG ratio went higher, the loading  
 393 capacity decreased while the release rates were similar in different CD/PEG components. To  
 394 explain that the author attributed the CD/PEG hydrogel formed tight networks as a major factor  
 395 which prevented the lysozyme penetrated in hydrogels[47].



396 To improve the cell-penetration ability, Huang team inspired by the cell-penetrating peptides  
 397 (CPPs) as penetratin (PEN) used for biomolecules delivery[78], especially insulin delivery[79],  
 398 therefore, developed a PEN conjugate to the bis- $\beta$ -CD delivery system for insulin[49]. Generally,  
 399 the CPPs were considered as nontoxic in their working concentration[80] so as CDs. The CPPs  
 400 could interact with the insulin charged groups via electrostatic effects, while the CDs could  
 401 include some insulin hydrophobic groups formed complex[81]. Therefore, the PEN-bis-CD  
 402 (P-bis-CD) could form a complex with insulin both through electrostatic and hydrophobic  
 403 interactions following led nanocomplexes assembly.  
 404 The authors then assembled two nanocomplexes employed insulin with PEN directly and insulin  
 405 with P-bis-CD (see fig. 13). After the complex stability, enzymatic stability and cellular uptake  
 406 studies, the P-bis-CD displayed better results in all parameters than PEN, and much better than  
 407 free insulin[49]. This design successfully combined both PEN and CD's advantages and  
 408 significantly improved insulin deliver efficiency.



409  
 410 Fig. 13. a) Preparation of PEN/insulin nanocomplexes and P-bis-CD/insulin nanocomplexes, b) the stability of  
 411 different loaded insulins over trypsin[49]  
 412

### 413 3. The delivery of DNA and RNA

414 Since the biophysicist Crick and biochemist Watson developed the revolutionary model of the  
 415 double-strand DNA in the 1950s, these magic molecules have been attracted so much attention  
 416 from not only the scientists but also the public.[82] As people continuously studying in genetic  
 417 science, they found out that almost every disease could be traced to genes except some physical  
 418 injured. Therefore, gene therapy is a promising strategy, that may allow doctors to treat diseases  
 419 by inserting a certain gene into patients' cells instead of utilizing drugs or surgeries, which can be  
 420 the final solution for vast diseases. But how to deliver the nucleic acids (NAs) safely and  
 421 efficiently into patients' cells is still a mega challenge to us by now. Basically, there are two types  
 422 of major transferred techniques, the viral and non-viral vectors. As for the viral vector, it  
 423 possessed carrying capacity, high cost, immunogenic response, toxicity and oncogenicity  
 424 issues.[83–85] Therefore, the liposome based non-viral vector attracted a lot of attention and  
 425 became an alternative for it can avoid most viral vector's disadvantages.[86] However, the  
 426 non-viral based carrier has its own limitation such as toxicity (such as high density charge of  
 427 poly-ethyleneimines (PEI))[87] as well, and even low efficiency and poor selectivity compared to  
 428 the viral one.[88] To get across the above shortcomings of the non-viral vector, CDs came out as  
 429 an attractive alternative tool for the scientists to operate, for its appropriate properties such as  
 430 good water solubility, easy functionalization accessible and non-toxicity.

431 Generally, CDs-based NAs delivery systems, researchers usually investigated their studies on Ns

432 loading experiments, delivery materials biodegrade experiments, cytotoxicity experiments, NAs  
 433 transfection and releasing experiments, etc. if there were nanoparticles formed, a lot of  
 434 nanoparticles parameters also been studied, such as size,  $\zeta$  potential, etc.  
 435 In this section the NAs delivery methods would be introduced in terms of loading materials and  
 436 will be described as the below orders: polypseudorotaxanes, supramolecular polymers, mutilate  
 437 components targetable delivery system and functionalized CD monomers or dimers, displayed in  
 438 [table 2](#).

439 Table 2. The summary of NAs delivery methods

Loading materials	NAs	References	Years
Cationic polymers	pDNA	[89–92]	1999, 2001, 2003, 2004
Cationic polymers	siRNA	[93]	2013
Cationic polymers	siRNA	[94]	2011
Cationic cluster monomers	pDNA	[95]	2008
Cationic cluster monomers	pDNA	[96–100]	2009, 2011, 2013
Cationic cluster monomers	pDNA	[101,102]	2004, 2011
Cationic polyrotaxanes	pDNA	[103,104]	2007, 2009
Cationic polyrotaxanes	pDNA	[105]	2012
Cationic polyrotaxanes	pDNA	[106]	2008
Cationic polyrotaxanes	pDNA	[107]	2006
Cationic polyrotaxanes	pDNA, siRNA	[108,109]	2012, 2017
Cationic polyrotaxanes	siRNA	[110]	2013
Cationic polyrotaxanes	siRNA	[111]	2012
Targetable cationic polymers	pDNA	[112]	2011
Targetable cationic polymers	pDNA	[113–116]	2004, 2007, 2009, 2011
Targetable cationic polymers	pDNA, siRNA	[117,118]	2016
Targetable cationic polymers	siRNA	[119]	2017
Monomers assembled supramolecular materials	siRNA	[120]	2018

440

### 441 3.1 DNAs' delivery

442

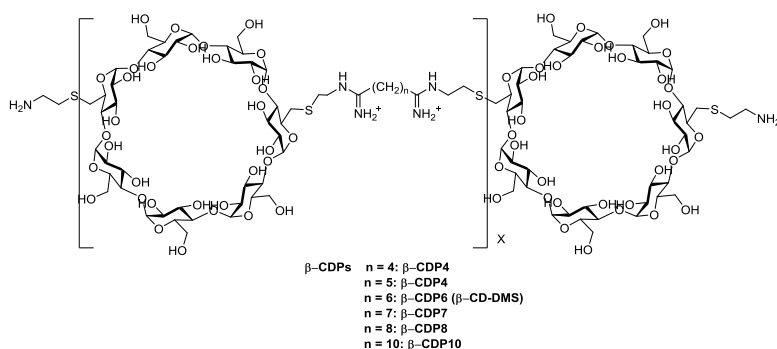
#### 442 3.1.1 Cationic polymers applied as DNA delivery vectors

443

443 Davis and co-works devised and prepared a series of linear cationic (because the linear of NAs  
 444 shown negative charges, which can coordinate to the cations)  $\beta$ -cyclodextrins (CDs)-based  
 445 effective and low toxic gene delivery vectors. They introduced the ethanethioamine as the charge  
 446 centers on the primary rim of  $\beta$ -CD to form monomer,  
 447  $6^A, -6^D$ -dideoxy- $6^A, -6^D$ -di(2-aminoethanethio)- $\beta$ -CD. Subsequently, they cross-linked the monomer



448 over the DMS to obtain the  $\beta$ -CD contained polymers (molecular weight 8800) in 24% of overall  
 449 yield. The polymer and plasmid DNA (pDNA) binding experiments resulted the charge ratio was  
 450 1:1.5 which exhibited that the polymer was able to bind the pDNA completely. The *in vitro*  
 451 transfection and toxicity of DNA complexes assay show that 1) the Luciferase protein activity in  
 452 BHK-21 cells transfected in serum-free conditions reached a stable maximum at 30+/- with  
 453  $\sim 1 \times 10^9$  RLU/mg of protein; 2) the toxicity was minimized with the presence of 10% serum during  
 454 transfection. The results approved the polymers containing  $\beta$ -CD as gene delivery cargo with  
 455 satisfied the effective and low toxic criteria[89]. To better understand the length of the spacer,  
 456 the authors varied the length of the spacers then they found that the toxicity was increasing as  
 457 the length increases in going from  $\beta$ -CDP4 to  $\beta$ -CD8.[90](See fig. 14)

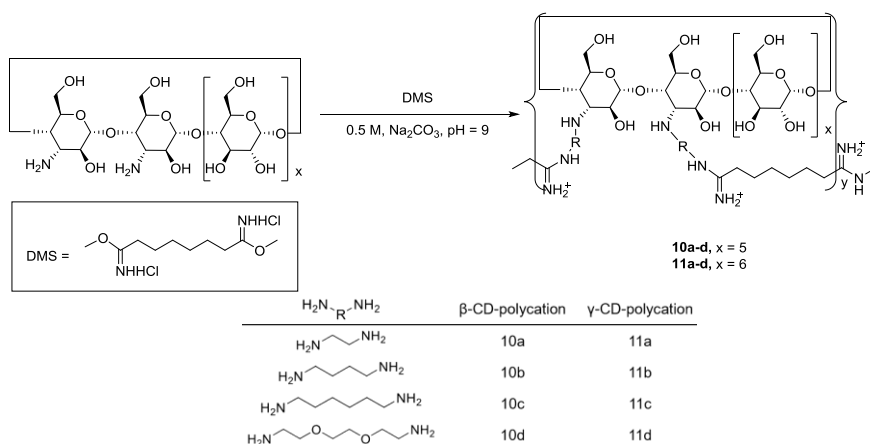


458

459 Fig. 14. Different spacer lengths of CD polymers

460

461 Inspired by their previous work, Davis and co-workers introduced aminoalkylamine and  
 462 aminoalkoxyamine as pedals on the secondary rim of  $\beta$ - or  $\gamma$ -CD then conjugated with the DMS as  
 463 the charge center to form 3<sup>A</sup>,3<sup>B</sup>-di(aminoalkylamino)- $\beta$ -cyclodextrin and  
 464 3<sup>A</sup>,3<sup>B</sup>-di(aminoalkoxyamino)- $\gamma$ -cyclodextrins (**10a-d** and **11a-d**). (See scheme 3) The toxicity assay  
 465 demonstrated that the polycations **10a-c** and **11a-c** in cell viability with a pronounced decreased  
 466 as the spacers prolonged while, **10d** and **11d** were non-toxicity at all concentrations employed by  
 467 authors. In addition, when increased the charge ratio of polycation and pDNA, the cell viability  
 468 decreased for both **b** and **c** analogues, but for the  $\beta$ -CD was worse compared to  $\gamma$ -CD. As for the  
 469 transfection efficiency, the polycations, **10a-c** and **11a-c**, prolonged the spacers, produced a  
 470 better transfection efficiency.[91] In general, the introduced CDs could increase the cell viability  
 471 and the NAs transfection efficiency apparently.

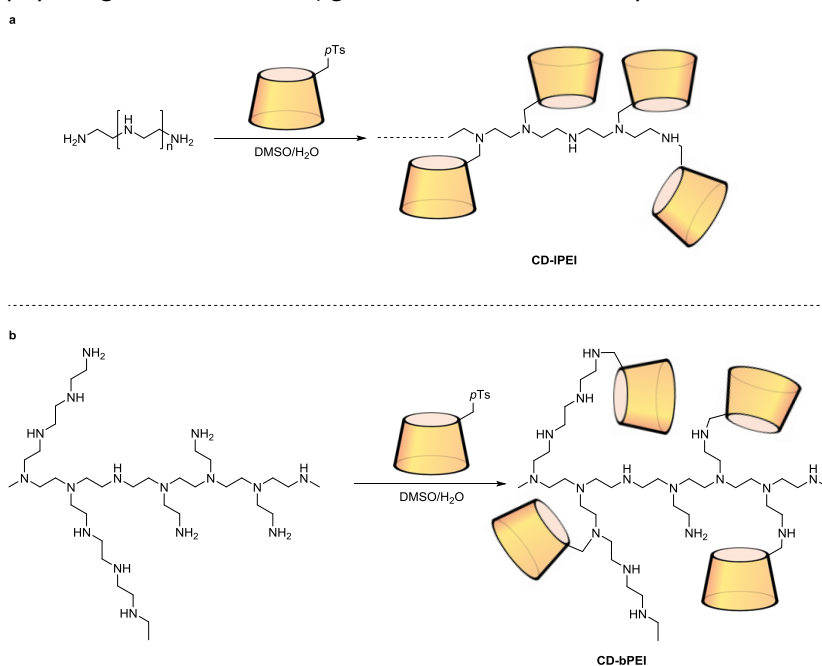


472

473 Scheme 3. Synthesis of linear DMS linked CD polymers

474

475 Poly(ethylenimines) (PEI) can provide efficient gene transferring *in vitro*[121], nevertheless, the  
 476 toxicity and difficulty in formulation of PEI is limiting its application in gene delivery. The LD<sub>50</sub> of  
 477 linear PEI (IPEI) were approximately 4 mg/kg (Balb/C mice)[122], while the CD-based polymers  
 478 were 200 mg/kg (Balb/C mice)[90]. To combine both benefits of PEI and CD polymers and  
 479 minimize the toxicity of PEI, Davis[92] team designed and synthesized the β-CD grafted PEI  
 480 (CD-PEI) polymers applied non-viral gene delivery. They employed both the linear and branched  
 481 PEI (IPEI and bPEI) conjugated with β-CD to prepare the CD-PEI polymers (see scheme 4a and 4b),  
 482 then, added adamantane-poly ethylene glycol (AD-PEG) to the polymers to increase the stability  
 483 and formulation ability of CD-PEI polymers. In the transfection and toxicity assays in PC3 cells,  
 484 displayed in 3 general outcomes, 1) reduced the toxicity acutely, 2) stabilized the vectors in  
 485 physiologic salt solutions, 3) good transfection efficiency, as the authors anticipated.



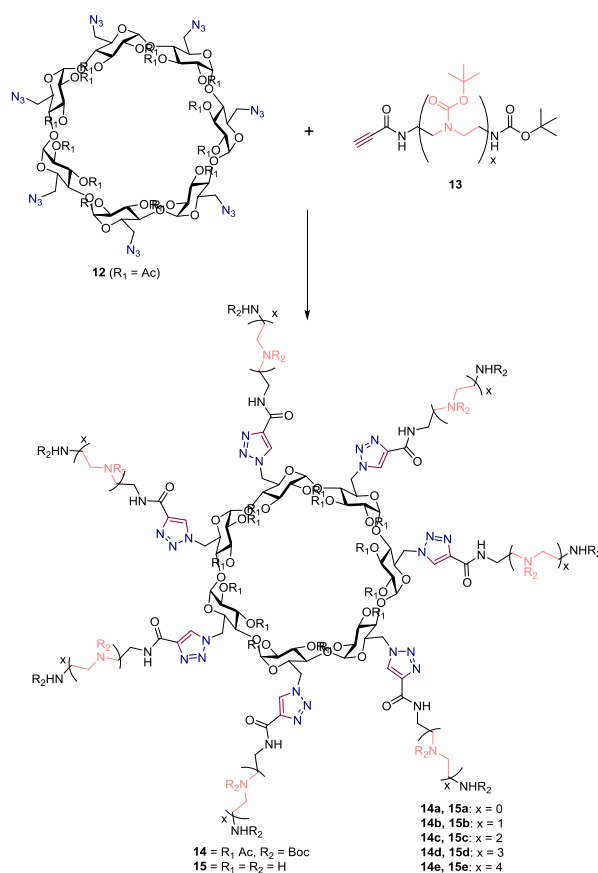
486

487 Scheme 4. Synthesis of bPEI grafted CDs (a) and IPEI grafted CDs polymers

488

### 489 3.1.2 Cationic cluster monomers applied as DNA delivery vectors

490 Reineke[95] and co-workers described a series of multivalent clusters based on β-CD. They  
 491 introduced 7 azide groups on the primary rim, then, conjugated the different alkyne dendrons via  
 492 the “click” reaction to form motifs **15a-e** (see scheme 5). The transfection and toxicity assays  
 493 showed that all the compounds **15a-e** were minimal cytotoxicity, the **15d** gave the best  
 494 transfection efficiency of all compounds nevertheless, **15b** indicated moderate efficiency, in HeLa  
 495 cell lines. To explain that the **15d** exhibited the best transfection efficiency compared to other  
 496 derivatives, the authors considered that for example the **15b** bond the pDNA weak and hence  
 497 lack of pDNA protection against enzymatic degradation. On the opposite, a much stronger  
 498 combination of **15d** and pDNA, led to a stronger protection of pDNA from enzymatic degradation,  
 499 consequently, a higher gene expression.



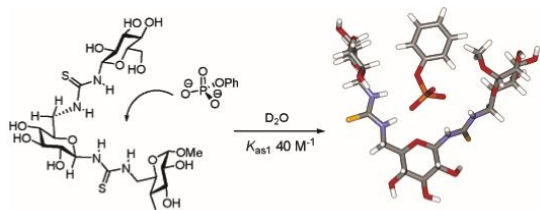
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501 Scheme 5. Synthesis of CD cluster via a "click" reaction

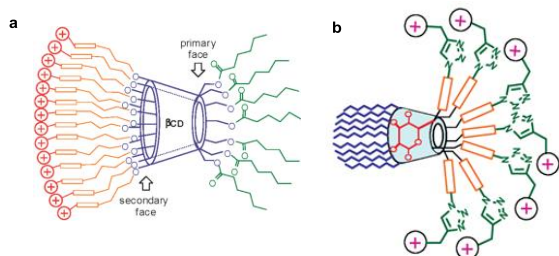
502

503 In 2006, García Fernández and co-workers[123] reported that the urea, thiourea and guanidinium  
 504 could form hydrogen bond with phosphate via their dual amino groups that made the dual amine  
 505 structure became the binder of phosphate which are the fundamental units of nucleosides. (See  
 506 fig. 15) Subsequently, therefore, García Fernández group devised monodisperse facial  
 507 amphiphiles consisting of a  $\beta$ -CD-based multivalent polycationic groups (aminoethyl amine and  
 508 aminoethyl thiourea) at the primary/secondary face and grafted hydrophobic chains at the  
 509 secondary/primary face clusters as shown in figure 16 (a and b) to deliver the NAs[99,124] They  
 510 took the similar strategy to form CD monomers **16-19**. All these monomers could form polyplex  
 511 with pDNA exhibited significantly transfection efficiency under the absence of serum  
 512 condition.[96,99,124] Nevertheless, only **17** still revealed transfection efficiency with the  
 513 presence of serum which was vital for the *vivo* trials.[100] In terms of the authors' description,  
 514 the preparation of **17** could be the handicap by its high cost, therefore an easy operable and  
 515 cheaper approach was expected. Looked back on the CD monomers **16-19**, the authors figured  
 516 out that the "click" strategy to obtain monomer **19** was a reasonable access to the CD cluster. To  
 517 improve the "click" method they employed solid-supported Cu(I) catalysts, thereby simplifying  
 518 the purification procedure. A series of triazole and thiourea products were prepared, and then  
 519 the prepared chemicals **20-22**, **23**, **24**, and **25** can fully co-assemble with pDNA to form  
 520 nanoparticles. (See fig. 17) The transfection and toxicity trials revealed that, **20** had better  
 521 transfection results than **17** under the 10% serum presence, probably because the longer  
 522 hydrophobic chains. While if the length of the hydrophobic chains were increased too much to  
 523 generate **21** and **22**, that would weaken the transfection ability. As for the triazole-thiourea CDs,

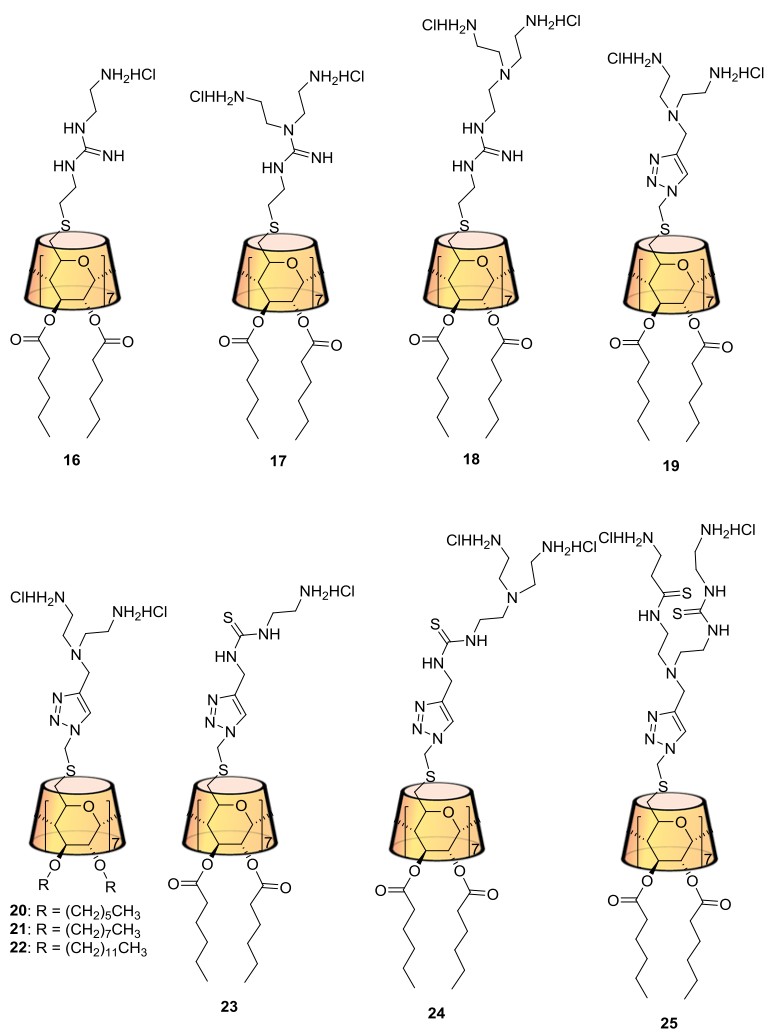
524 the derivative **25** shown better transfection performance than **16**, **17**, **18**, **19** or **23** in the absence  
 525 of serum and lower cytotoxicity but worse transfection than just **17** in the presence of 10 %  
 526 serum.[98]



527  
 528 Fig. 15. Phosphate could bind with dual thiourea[123]



529  
 530 Fig. 16. García Fernández team designed and prepared monomer with cation groups on secondary face and  
 531 hydrophobic groups on primary face of CD (a)[124] or cation groups on primary face and hydrophobic groups on  
 532 secondary face of CD (b)[99]

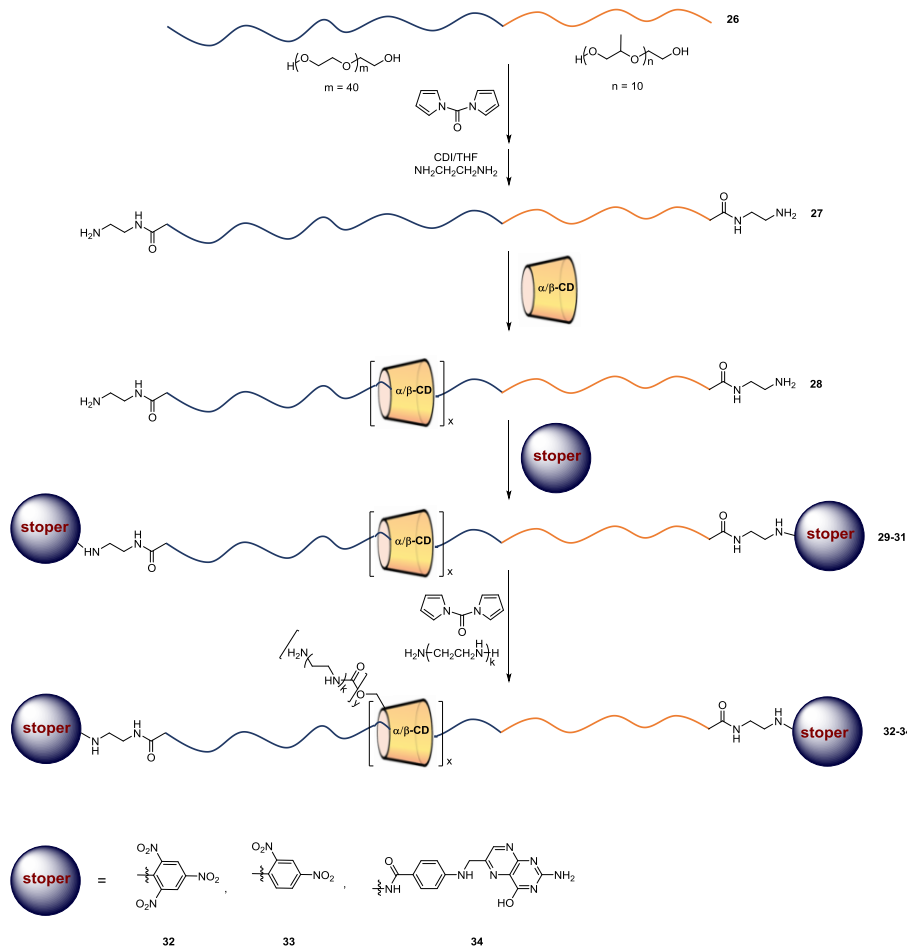


534 Fig. 17. García Fernández et al. prepared CD clusters

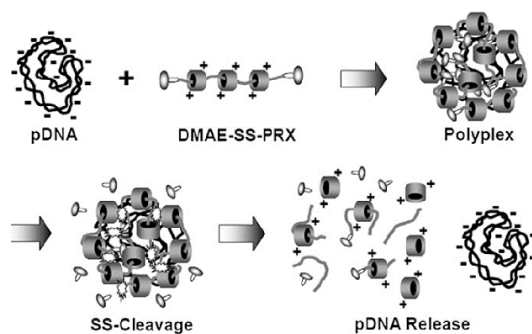
535

### 536 3.1.3 Cationic polyrotaxanes applied as DNA delivery vectors

537 Ionic CD-based on polyrotaxane (PR) system applied as nucleic acids' delivery also investigated by  
 538 numerous scientists, Li team reported a PR system as gene carrier to deliver the genes. The PR  
 539 system contained poly[(ethylene oxide)-(propylene oxide)] as axle,  $\alpha$ -CD as cationic groups  
 540 (pentaethylenhexamine salts) carry platform and two molecules of 2,4,6-Trinitrobenzene  
 541 sulfonic acid (TNBS) as terminated stoppers (**32**). (See scheme 6) Subsequently, the prepared  
 542 cationic PR system formed complexes with pDNA further to transfect cells[103]. Then they  
 543 substituted the axles with PEG and the stoppers with 2,4-dinitrobenzene (**33**), formed similar  
 544 cationic PR which possessed similar delivery properties as the above cationic PR system.[125]  
 545 Next  $\beta$ -CD as cationic groups and larger stoppers were added as the larger ring cycle size of  
 546  $\beta$ -CD[104]. With a similar strategy, Chen team applied the folate as stoppers (**34**) and at the same  
 547 time as target to folate receptors, over expressed on numerous cancer cells surface[105] which is  
 548 a very brilliant strategy that could improve the delivery efficiency with the help of targeting effect.  
 549 (See scheme 7) In order to control the release of the delivered pDNA, Harashima team  
 550 introduced disulfide linkages at both terminal sides before the stoppers that could control the  
 551 release of the pDNA via the S-S bond cleavage and improve the stability of PR gene delivery  
 552 system[126]. The S-S bond could be ruptured under such as GSH rich environment which  
 553 enriched in tumor microenvironment, which would provide a practicable tumor-targeted gene  
 554 therapy delivery method.



556 Scheme 6. Synthesis of CD-based polyrotaxanes with different stoppers



557

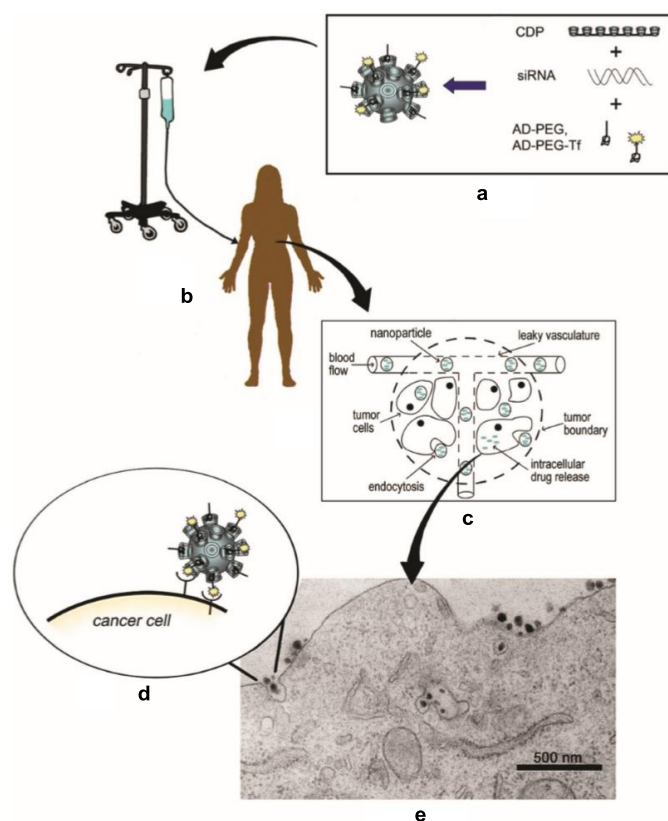
558 Scheme 7. pDNA coassembly with disulfide CD rotaxanes to form polyplex then controlled releasing pDNA via  
559 the S-S bond cleavage[106]

### 560 3.2 RNAs' delivery

561 In the human genome there over 95% of DNA sequences are non-protein-coding sequences[127],  
562 while many of them would translate massive functional non-coding RNAs (ncRNAs) which may  
563 regulate different functions physiologically. Interestingly, the ncRNAs themselves or mRNA may  
564 be also targeted by the RNA entities such as antisense RNAs (asRNAs), micro RNA (miRNAs), small  
565 interfering RNAs (siRNAs), and other small RNAs (sRNAs) that can manipulate the expression or  
566 function of target gene subsequently, control diseases[128–130]. This mechanism is generally  
567 called RNA interference (RNAi) holding vast therapeutics potential in cancer, autoimmune  
568 diseases, dominant genetic disorders, viral infections or any disease caused by the abnormal  
569 activity of genes[131]. The instances were reported by the Davis[115] team, first RNAi introduced  
570 gene silencing in human skin cancer in phase I clinical trial, demonstrated that the therapy  
571 successfully suppressed the expression of the cancer gene in 2010 and the first RNAi-related drug  
572 *patisiran* approved by the USFDA in 2018[132]. An appropriate delivery system is required  
573 intensely by the DNAs therapy which is also a vital factor to the RNAs therapy.

#### 574 3.2.1 Targetable cationic polymers applied as siRNA delivery vectors

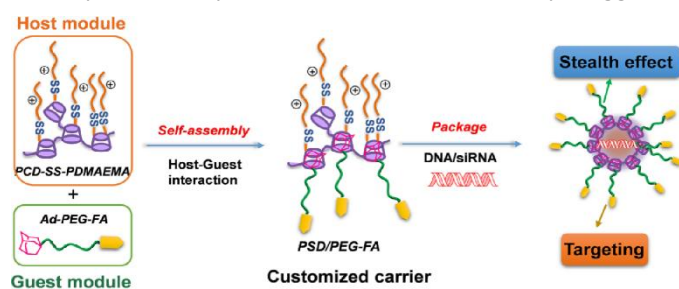
575 A classic instance for siRNA delivered by CD-based nanoparticles was reported by Davis et al. in  
576 2007, they prepared CD-based polycation (similar as previously described in DNA delivery  
577 section), polyethylene glycolized adamantine (AD-PEG) and AD-PEG-transferrin (AD-PEG-Tf) as  
578 components of the nanoparticles[113,114,133]. PEG has been applied in many different types of  
579 non-viral gene delivery vectors, demonstrated that it's helpful for increasing the serum and salt  
580 stability. On the other hand, however, the PEGylation of nanoparticles reduced the interaction  
581 between the particles and cells, consequently, reduced the transfection of delivery vectors. On  
582 the consideration of this, the Davis team introduced the Tf, which is able to bind the Tf receptors  
583 (TfR) on the surface of cancer cells[134], on the vectors to hedge the transfection reduction  
584 brought by the PEGylation[116]. This was demonstrated by the delivery of the siRNA, an inhibitor  
585 of a subunit M2 of ribonucleotide reductase (RRM2), in the phase I trial of human skin cancer  
586 mentioned above[115]. (See fig. 18) The ribonucleotide reductase (RR) plays a key role in DNA  
587 replication and repair, hence the inhibition of RRM2 can provide the inhibition of RR that induces  
588 the apoptosis of the cancer cells[135].



589  
 590 Fig. 18 a) siRNA contained assembled nanoparticles, then b) the aqueous solution of nanoparticles injected to  
 591 trial subjects, c) via the EPR effect the nanoparticles were “leaking” from the micro blood vessels to the tumor  
 592 tissues, d) and e) based on the Tf and Tf acceptor interaction, the nanoparticles were selectively binding to tumor  
 593 cells.[116]

594

595 Similar strategy was taken by Wang[117] teams, that was applied cationic CD-based polymer to  
 596 assemble nanoparticles with siRNA then via supramolecular chemistry to anchor folate (FA) that  
 597 could specifically target on folate receptor (FR), consistently high and uniform expression on  
 598 numerous cancer cells[136], to obtain the siRNA delivery vectors. On the primary face of  $\beta$ -CD  
 599 were introduced disulfide contained cationic groups to achieve  $\beta$ -CD-based cationic polymer[118].  
 600 Notably, the disulfide structure could be used as a trigger for the endosomal/lysosomal escape of  
 601 loaded siRNA in a reductive environment, or the siRNA would not be released. The folate was  
 602 linked to adamantane via linear PEG to form FA-PEG-Ad, in which the adamantanes could interact  
 603 with the previous prepared (PCD<sup>+</sup>)s to form host-guest complexes. (See fig. 19) The cytotoxicity,  
 604 hemolysis, stability and siRNA transfection assays suggested satisfying results.[117]



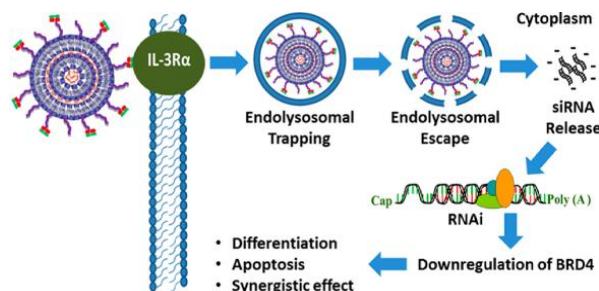
605

606 Fig. 19. 4-component of nanoparticles delivering siRNAs[117]

607



608 Acute myeloid leukemia (AML) is a deadly clonal disorder disease which with great potential  
 609 being cured by the RNA interference (RNAi) therapy[137], however the nonspecific tissue  
 610 distribution, poor cellular transfection, toxicity and short plasma half-life time et al. hedged the  
 611 RNAi therapy in clinic[138]. Therefore, the O'Driscoll team developed an antibody targeted  
 612 CD-based siRNA delivery system for the AML's treatment[119]. In this system, firstly, they applied  
 613 the CD as platform grafted cation groups then with the siRNA formed CD/siRNA complex[139];  
 614 secondly, the 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene  
 615 glycol)-2000] (DSPE-PEG-Maleimide) conjugate was prepared[140]; tertiary, prepared the  
 616 DSPE-PEG-Fab (a monoclonal antibody specific target to IL-3R $\alpha$ , a cell surface antigen for human  
 617 AML leukemia stem cells (LSCs))[141]; finally, CD/siRNA and DSPE-PEG-Fab were incubated at  
 618 60°C for 1 h with slight shaking, to give the CD/siRNA/DSPE-PEG-Fab formulation  
 619 nanoparticles[142]. The mechanism of targeted nanoparticles delivered the siRNA via Fab bind to  
 620 IL-3R $\alpha$  elicited endocytosis, subsequently, endosomal/lysosomal escaped and released siRNA.  
 621 Then, the siRNA activated RNAi effect to downregulate the corresponding gene (BRD4, an  
 622 epigenetic reader[143]) induced the leukemia apoptosis. (See fig. 20) *In vitro*, the gene silencing  
 623 in KG1 cells (an AML leukemia stem and progenitor cell line), the targeted nanoparticles exhibited  
 624 efficiently and selectively delivery of siRNA to silence BRD4 induced leukemia apoptosis, in  
 625 addition, the nanoparticles also could combine with clinically available chemotherapeutic Ara-C  
 626 and reveal the combination therapy for AML treatment[119].

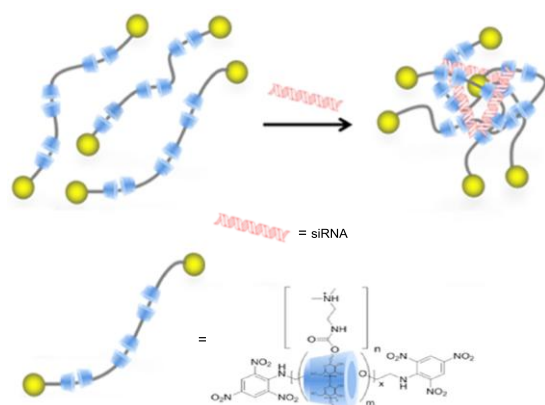


627  
 628 Fig. 20. Transfection mechanism of IL-3R $\alpha$  targeted siRNA delivery systems based on CDs[119]  
 629

### 630 3.2.2 Cationic polyrotaxanes applied as siRNAs delivery carriers

631 Thompson et al.[144] devised  $\alpha$ -CD-based cationic polyrotaxanes for the delivering the siRNA.  
 632 They prepared three polyrotaxanes derived from three different sizes of polymer axles (PEG MW  
 633 2,000, 3,400, and 10,000). The cationic polyrotaxanes were synthesized via two steps: 1<sup>st</sup>,  
 634 different sizes of axles were mixed with the free  $\alpha$ -CD to form polyrotaxanes (CD-PR), then  
 635 end-capped with 2,4,6-Trinitrobenzenesulfonic acid (TNBS); 2<sup>nd</sup>, the CD-PR reacted with  
 636 N,N'-dimethylethylenediamine (DMEDA) to obtain the cationic polyrotaxanes  
 637 (PR<sup>+</sup>)[103,104,107,125]. (See fig. 21) These PR<sup>+</sup>s were able to condense siRNA into positively  
 638 charged particles with a diameter smaller than 200 nm. The cell viability assay and cellular uptake  
 639 studies of PR<sup>+</sup> nanoparticles compared to bPEI displayed 100 to 200 times less toxic and even  
 640 better cellular uptake percentage during the N/P ratio was 20[144].





641

642

Fig. 21. Polyrotaxanes co-assembly with siRNAs form complexes for delivering of siRNAs[144]

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### 3.2.3 Cationic amphiphilic monomers applied as siRNAs delivery carriers

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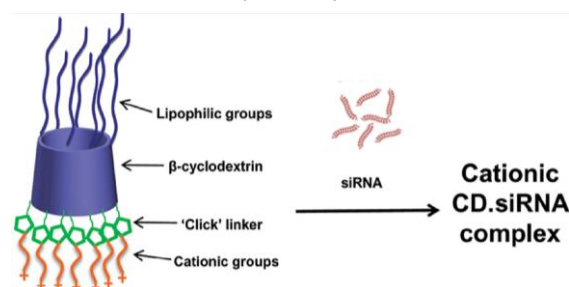
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In order to overcome the issues in delivering siRNA into neurons and the central nervous system, like neuronal uptake, vesicular escape, and blood-brain barrier et al.[145,146] to treat some nervous system diseases, O'Driscoll and Cryan teams collaborated to devise a neuronal siRNA delivery carrier based on modified  $\beta$ -CD[139]. They introduced lipophilic groups on the primary face and the cationic groups via "click chemistry" on the secondary face of  $\beta$ -CD selectively to form a functionalized CD monomer[111]. (See fig. 22) Then the candidate siRNAs were formed nanoparticles with the CD monomers, sized 200 nm approximately. Several experiments were launched to evaluate the delivery efficiency, such as serum stability, cell viability, cellular uptake and transfection efficiency via the related gene knockdown technique *in vitro*. The results revealed that, in serum condition the cargos were stable, the cells maintained 80% of viability after the transfection trials and significant gene knockdown was observed via the studies of reduction of luciferase and glyceraldehyde phosphate dehydrogenase (GAPDH) expression of up to 68% and 40%, respectively.[139]



658

659

Fig. 22. siRNA formed complexes with CD-based amphiphilic cationic monomers[111]

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### 3.2.4 A virus fabrication manner simulating RNA loaded method applied as siRNAs delivery carriers

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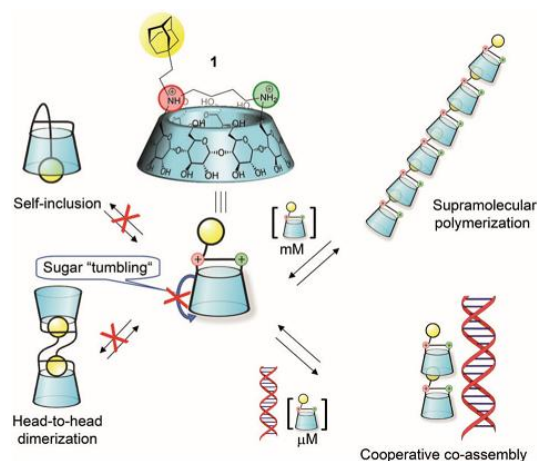
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An interesting and intelligent RNA delivery method probably is simulating the manner of virus in RNA transfection. Inspired by tobacco mosaic virus (TMV) were assembled from nucleic acids (NAs) and coat protein. Namely, the NAs were cooperative assembly with the coat protein, then to form NAs inside fiber shape virus, Sollogoub[120] team carefully mimicked the TMV assembly manner, designed a functional  $\beta$ -CD co-assembled with siRNA to form a fiber shape siRNA vector. They built a 4 carbon chain bridge on the primary rim of  $\beta$ -CD on  $6^{A,D}$  positions via benzylation and selective bisdebenzylation[147,148]. Then an adamantane was grafted on  $6^A$  position of  $\beta$ -CD

670 via reductive amination with 1-adamantaneacrtalhydride which could allow adamantane- $\beta$ -CD  
 671 (Ad-CD) co-assembled with siRNA to form the siRNA contained vectors. Notably, the introduced  
 672 bridge could prevent the adamantane grafted sugar unit tumbled that led the self-inclusion and  
 673 the head to head dimerization effect (see fig. 23). The gene silencing trials exhibited the vectors  
 674 had satisfied siRNA transfection[120]. Compared to the above examples this strategy was more  
 675 tunable and controllable in assembly to form better orderly delivery vector shapes (not just some  
 676 nanocomplexes).



677  
 678 Fig. 23. siRNA co-assembly with supramolecular polymers[120]

679

#### 680 4. Conclusions

681 Both proteins and NAs drugs play vital roles in the diseases' therapy now but more vital especially  
 682 in the future that we can imagine. However, one of the most robust barriers that could slow  
 683 down the future come to us earlier probably is the deficiency of efficient delivery systems. As for  
 684 this big issue, CDs based delivery carriers can be strong candidates that become very potential  
 685 solutions.

686 In theoretically, the proteins with hydrophobic groups can form host-guest interaction which is  
 687 able to improve the ability of nanocomplexes formation between the proteins with CDs and the  
 688 stability that largely preserve the activity of proteins and optimize the proteins' transfection that  
 689 has been proved via the above examples. However, in terms of our knowledge, cyclodextrin  
 690 based delivery systems are not very common delivery devices for proteins such as BSA[44],  
 691 IgG[51], insulin[27], etc., especially, considered the giant numbers of proteins discovered. A  
 692 proposed mechanism for explaining this phenomenon is probably that the complicate  
 693 stereo-structures that impeded the CD-protein complex formation even with the existence of the  
 694 host-guest interaction effect.

695 Compared to proteins, the NAs delivery based on CDs are much more popular and successful,  
 696 especially, after the success of Davis[115] team promoted the siRNA for the skin cancer  
 697 treatment to phase I clinical trial. In the early stage, the CDs applied as a platform to graft  
 698 numerous of cationic groups to interact with the anions of NAs to form nanocomplexes for NAs  
 699 delivery. Recently as the raising of CDs applied in supramolecular chemistry and more precise  
 700 selective functionalization methods developed, the scientists started to assembly nano structures  
 701 to mimic the "coat proteins" of the virus to carry the NAs, then transport the NAs to the desire  
 702 spots[120].

703 While numerous disadvantages would be overcome, as the continuing progresses achieved in the

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704 functionalization techniques in CDs[149], easier tunable and more appropriate CD-based  
705 materials as delivery devices for individual protein delivery will become more efficient and  
706 common.

707 As the burst of COVID19 pandemic, before the wonder drugs and vaccines (gen and protein  
708 vaccines) approved, the monoclonal antibodies are the most effective cures, hence, CDs can be  
709 functionalized to appropriate delivery systems for antibodies or gen and protein vaccines[150].

710

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