Expert Opinion

Linear cyclodextrin-containing polymers and their use as delivery agents

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Cyclodextrins, cyclic oligomers of glucose, have been used in pharmaceutical formulations for decades as a result to their biocompatibilities, low toxicities and their abilities to solubilise organic small molecules via inclusion complex formation. The incorporation of cyclodextrins within polymers of numerous types, for use as drug delivery agents, has been explored. Illustrative of the flexibility in polymer chemistry and delivery application that is possible with these materials, two linear cyclodextrin-containing polymers are in preclinical and clinical development for the non-covalent delivery of nucleic acid therapeutics and covalent delivery of a small-molecule drug, respectively. This document provides an overview of the background and progress that has been made with these materials thus far, as well as suggestions for their future development and characterisation.

Keywords: camptothecin, cyclodextrins, nucleic acid delivery, polymers, siRNA, small-molecule delivery

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1. Cyclodextrins and their use in pharmaceutical formulations

Cyclodextrins (CDs) are cyclic oligosaccharides; these rings may include six (α-CD), seven (β) or eight (γ) glucose moieties. Thousands of variants of CDs have been prepared and studied, and their use in pharmaceutical formulations dates back to the 1950s [101]. There are a number of properties of CDs that make them attractive for use in pharmaceuticals, not least of which is their high water solubility. Both primary and secondary hydroxyls are contributed by each glucose moiety and endow CDs with a solubility up to 40 – 50% by weight (for hydroxypropyl-β-CD). The three-dimensional architecture of CDs is unique in that they consist of cup-like shapes with relatively polar exteriors and apolar interiors. Thus, in aqueous solution, a hydrophobic microenvironment exists within the CD interior that is suitable for water solubilisation of partial or entire organic molecules; this phenomenon is known as inclusion complex formation. Consequently, solubilisation of relatively water-insoluble small-molecule drugs has been a primary use of CDs in pharmaceutical formulations. A recent, comprehensive review of CDs and their pharmaceutical applications is available [1].

Although their study as individual molecules is much more extensive, a large body of work also exists on polymers that incorporate CDs, either pendantly or within the polymer backbone. The preparation of supramolecular CD-containing entities has revealed improvement in inclusion complex-binding strength of over five orders of magnitude; examples include randomly crosslinked CD-containing polymers (CDPs) [2] and CD-based dendrimers [3]. Grafting of CD to pre-formed polymers (including chitosan, poly[ethylene imine] [PEI] and dendrimers) has also been shown to enhance desirable polymer properties. Although this body of work with CDPs continues to grow, until the late 1990s it lacked well-defined linear polymers with CD incorporated along the polymer backbone, presumably due to the difficulty in creating a controllably difunctionalised CD monomer on a large scale.

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A description of the work in this area, both in the areas of small-molecule and nucleic acid delivery, is provided below.

2. Linear cyclodextrin-containing polymers for small-molecule delivery

The efficacy of small-molecule antitumour compounds in the clinic often suffers as a result of their poor solubility and/or high toxicity. Conjugation of these compounds to aqueous polymers has been shown to lower toxicity and improve solubility, as well as enhance tumour deposition via the enhanced permeation and retention (EPR) mechanism [4,5]. The EPR effect results from the combination of leaky tumour vasculature (which permits macromolecular extravasation) and ineffective tumour lymphatic drainage (which reduces clearance of these macromolecules). Furthermore, there is evidence that macromolecular drugs may avoid drug efflux pump-mediated multi-drug resistance [6]. Given the beneficial properties of CDs within pharmaceutical formulations as discussed in Section 1, it was logical to explore the effect of small-molecule conjugation to CDPs.

The small-molecule drug that has been most studied within conjugates to linear CDPs is 20*(S)*-camptothecin (CPT). Being a topoisomerase I inhibitor with broad antineoplastic activity, CPT itself was unsuccessful in preliminary clinical trials for precisely the reasons cited in Section 1 (insolubility, toxicity) [7,8]. To overcome these limitations, small-molecule analogues of CPT with increased solubility have been prepared: two of these analogues (irinotecan and topotecan) have already been approved for some human cancers. In order to take advantage of the aforementioned EPR effect, others sought to couple CPT to water-soluble polymers, including PEG [9] and poly(L-glutamic acid) [10].

The first report of CPT conjugation to linear CDPs was published in 2003 [11]. β-CD was difunctionalised (using an appropriately sized capping agent, biphenyl-4,4′-sulfonic acid) at the 1- and 4-positions with L-cysteine, and then copolymerised at the amine sites with a difunctionalised PEG_{3400} (PEG–dipropanoicsuccinimide). The resulting linear polymers (which varied in molecular weight from 35 – 97 kDa, depending on the base used in the reaction) were then conjugated to CPT at the carboxylic acid sites using either a single glycine or triglycine linker; different CPT loading levels (6 or 10% by weight) were also achieved. *In vitro* release studies demonstrated that conjugation of CPT to the polymer through glycine linkers greatly extended the half-life of the active (lactone) form of CPT (as opposed to the inactive carboxylate form) compared with CPT alone.

This series of linear CPT and CDPs was then evaluated for both their maximum tolerable doses and antitumour activities in tumour-bearing (LS174T colon carcinoma) mice [12]. At their maximum tolerable doses, those conjugates with higher molecular weight (97 kDa) showed antitumour activity that was superior to both CPT at the same amount and to the optimal dose of irinotecan. Furthermore, tumour growth inhibition was better than that of a conjugate of a lower molecular weight (35 kDa) at the same CPT dose. Most importantly, as expected if these materials truly employ the EPR effect, the duration of antitumour effect seen with these conjugates was much longer than was seen with either irinotecan or CPT.

The conjugate selected for additional investigation (IT-101; Figure 1) has a molecular weight of 85 ± 23 kDa and a single glycine linker; it self-assembles into nanoparticles with a diameter > 10 nm but < 100 nm. The prolonged antitumour effect of IT-101 correlates to enhanced tumor distribution and extended plasma half-life compared with CPT alone [13]. Preclinical efficacy was further demonstrated in numerous other cancer types (including MDA-MB-231 breast cancer, Panc-1 pancreatic cancer and HT-29 colorectal cancer, all of which are resistant to treatment with irinotecan) [14] and a Phase I clinical trial of IT-101 is underway [201]. The ability to independently control polymer molecular weight, linker chemistry and drug loading makes this linear CDP drug delivery system amenable for use with other small-molecule (and protein/peptide) drugs; indeed, developmental work with several other drugs has begun.

3. Linear cyclodextrin-containing polymers for nucleic acid delivery

Research on the use of polymers (and lipids) as vehicles for the delivery of nucleic acid therapeutics has been extensive since the 1980s. Initial efforts focused on the use of naturally occurring or previously synthesised polymers (including PEI and poly[L-lysine] [PLL]) as non-viral agents for the intracellular delivery of DNA for gene therapy applications. As the field of nucleic acid therapeutics has expanded to include antisense oligodeoxynucleotides, catalytic DNAzymes and RNAzymes, small interfering RNAs (siRNAs), and others in recent years, both the number and our understanding of polymers used to deliver them have grown in parallel. Among this ever-increasing diversity of delivery materials, the presence of cationic sites within them (which permits electrostatic interaction with these polyanionic nucleic acids) is nearly universal.

In 1996, the initial efforts to create cationic, linear CDPs for nucleic acid delivery were launched in the laboratory of Dr M Davis at Caltech. Based on the aforementioned understanding (from their study as drug solubilisers and within other types of polymers for pharmaceutical applications) that CDs are relatively non-toxic and well-tolerated *in vivo*, preliminary work focused on functionalising CDs such that they would contain reactive sites suitable for reaction with a comonomer to generate a polymer. Analogous to the difunctionalisation of β-CD for delivery of small molecules discussed in Section 2, mercaptamine was used to prepare a diamino-β-CD that, when reacted with a cationic, amine-reactive comonomer (dimethylsuberimidate), yielded a water-soluble CDP [15]. The first characterisation efforts indicated that this polymer, when formulated with plasmid DNA at a positive (> 1:1) charge ratio (positive charges within

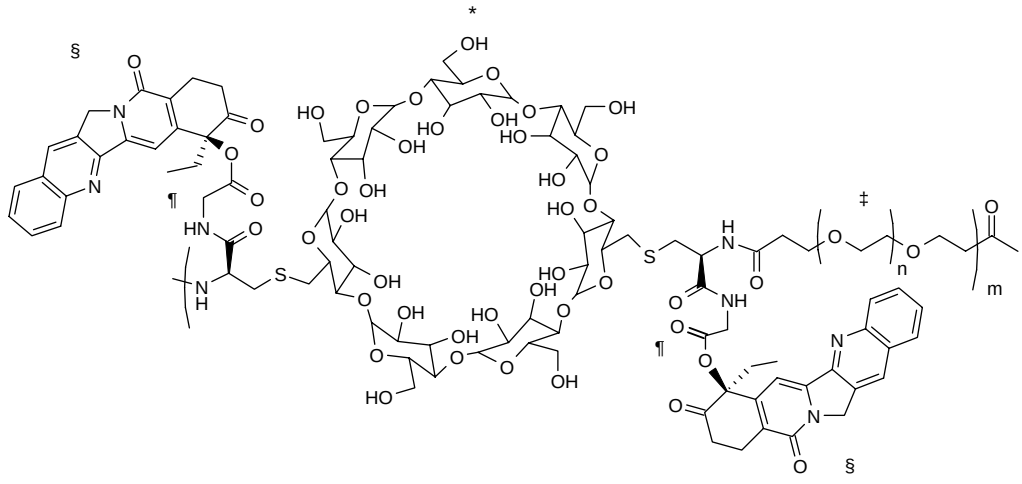

Figure 1. The structure and features of a linear, cyclodextrin-containing polymer (IT-101) that is used for camptothecin delivery.
*Indicates a β-cyclodextrin moiety that provides water solubility and biocompatibility. ‡Indicates a PEG (PEG_{3400}) moiety within the polymer backbone. §Indicates camptothecin molecules that function as anticancer agents through inhibition of the topoisomerase I enzyme. ¶Indicates single glycine linkages through which camptothecin is conjugated to the polymer.
PEG: Poly(ethylene glycol).

CDP:negative charges within DNA), condensed DNA into particles having a diameter of ~ 100 nm, which were capable of achieving significant gene expression (comparable to or exceeding that of PEI, PLL and other commercially available lipid reagents) in cultured cells.

This initial success instigated years of thorough investigation into how each of the many parameters of the polymerisation affected the properties and function of the resulting CDPs. The effect of charge spacing (specifically, the number of methylene units between the cationic sites within the comonomer) was examined first [16]. Although varying the number of methylene units (4 – 10) was not observed to have a significant impact on CDP molecular weight or on the size of CDP/DNA complexes, substantial alterations in gene expression and cytotoxicity were observed; an intermediate spacer length (6 methylenes) was deemed to be optimal. Further work was performed to examine carbohydrate size and its distance from charge centres [17], the charge-centre type [18], as well as the CD type and functionalisation [19]; the optimal polycation contained β-CD and amidine charge centres separated by six methylenes.

Although this polymer optimisation work was underway, the need to better understand and, ultimately, modify the CDP/DNA complexes was realised. Three distinct, significant improvements to these complexes were achieved. First, the issue of stabilisation of complexes for *in vivo* use was identified and addressed via modification with PEG. Second, targeting ligands were introduced to these complexes to enhance biodistribution of complexes to the desired target cells *in vivo*. Finally, modification to the polymer termini was made that enhanced endosomal escape and, thereby, enhanced the functional delivery of the nucleic acid payload.

Dynamic light scattering measurements of complex size indicated that complexes were ~ 100 – 150 nm in diameter and were stable in water. In addition, CDP/DNA complexes had a strongly positive zeta potential (surface charge) and, if physiological levels of salt were added, displayed rapid salt-induced aggregation. These last two properties can be tolerated and may actually be beneficial in *in vitro* applications, but they would have negative consequences *in vivo*. Drawing on previous work with PEI [20] and PLL [21], attempts were made to graft a neutral polymer (PEG) to CDP to reduce the surface charge and increase the stability of CDP/DNA complexes; these efforts were unsuccessful, most likey due to the shorter nature and fewer reactive amines of CDP compared with the other polycations. Pegylation was successful when an alternate strategy was performed: PEG was conjugated to the small molecule adamantane (AD), which can form inclusion complexes inside the cavity of β-CD [22,23]. Addition of AD–PEG_{5000} or AD–PEG derivatives to CDP/DNA complexes allowed for 'tunable' reduction in zeta potential and prevented salt-induced aggregation. Furthermore, mixing of AD–PEG and CDP prior to their addition to DNA allowed for even smaller (< 100 nm) complexes to be prepared.

Another advance was made when these pegylated CDP/DNA complexes were further modified to include targeting ligands. Systemic administration of these complexes was expected to achieve preferential uptake in the desired target cells/tissues only if a suitable targeting moiety was appropriately incorporated into the formulation. The first targeting ligand to be investigated was galactose for selective uptake by hepatocytes, which express abundant levels of the surface asialoglycoprotein receptor, for which galactose is a ligand. Indeed, incorporation of galactose, either itself or

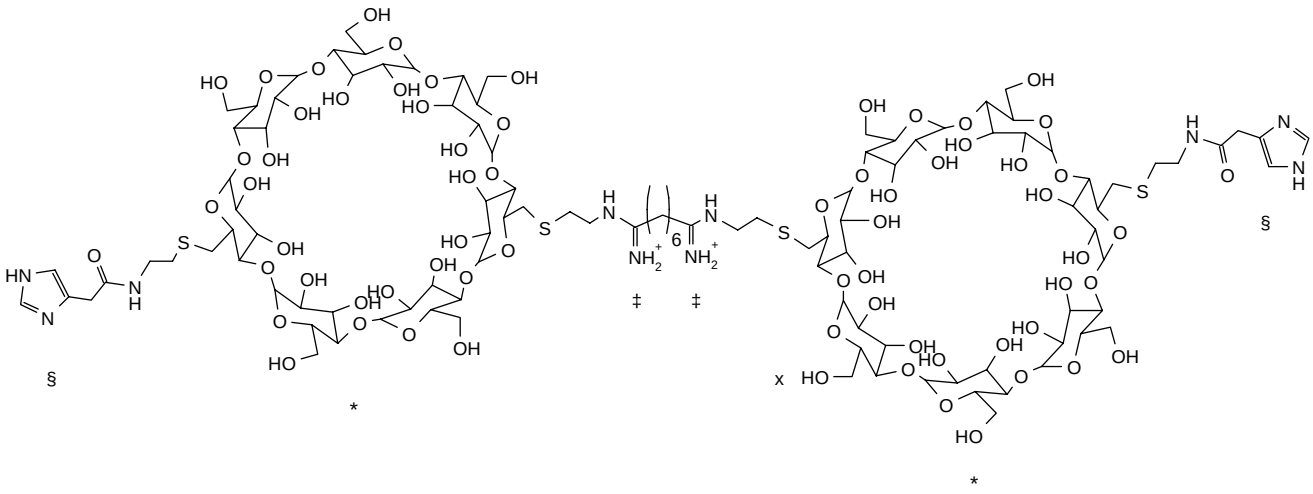

Figure 2. The structure and features of a linear, cyclodextrin-containing polymer with imidazole groups used for nucleic acid delivery.
*Indicates the β-cyclodextrin moieties that reduce toxicity and serve as sites for non-covalent modification via inclusion complex formation with adamantane–poly(ethylene glycol) conjugates. ‡Indicates the cationic amidine groups that are necessary for electronic interaction with, and condensation of, anionic nucleic acids. §Indicates terminal imidazole moieties that buffer intravesicular acidification of complexes and enhance intracellular release of nucleic acids.

within lactose, had been previously shown to enhance hepatocyte uptake of polymer/DNA complexes [24,25]. Galactose (or glucose as a negative control) was incorporated into complexes at the distal end of an AD–PEG conjugate (i.e., AD–PEG–galactose or AD–PEG–glucose) [22]. Galactosylated CDP/DNA complexes consistently show higher uptake by cultured hepatoma cells than glucosylated complexes; furthermore, a competitor for asialoglycoprotein receptor (asialofetuin) reduced uptake of galactosylated, but not glucosylated, complexes. In a similar fashion, subsequent efforts successfully incorporated a protein ligand, human transferrin (Tf), into CDP/DNA complexes (via an AD–PEG–Tf conjugate) [26] and demonstrated uptake of these complexes by tumour cells in mice that overexpress the Tf receptor [27]. In the cases of galactose and Tf, use of AD–PEG–ligand conjugates allowed for non-covalent introduction of targeting ligands such that the DNA-binding nature of CDP was not disturbed. In addition, these two examples illustrate that both stable targeted DNA-containing formulations can be prepared entirely by self-assembly, as well as indicating the diverse nature of ligand (from a small sugar to an ~ 80-kDa protein) that could be incorporated within CDP/DNA complexes.

Although the stabilisation and introduction of targeting ligands, two improvements that were essential for *in vivo* applications of CDP/DNA complexes, were accomplished, it was evident even in early cell culture work that there was room for improvement in expression of the delivered transgene. Although there are many cellular processes between CDP/DNA-complex uptake and transgene expression that are potential rate-limiting steps, microscopic analysis of complex-treated cells shed some light on the most critical of these. When made with fluorescently labelled DNA, intracellular complexes that were analysed by confocal microscopy primarily exhibited a punctate staining pattern that was consistent with their sequestration within the endocytic pathway. Indeed, subsequent transmission electron microscopy revealed enlarged, electron-dense intracellular vesicles that contained virtually all of the complexes that had been endocytosed [28]. To overcome this apparent endosomal entrapment, published work with PEI and other polycations was examined, in which nitrogens that were protonatable within the pH range of endosomal acidification (pH ~ 5 – 7) were thought to achieve enhanced endosomal release via pH buffering – the so-called proton-sponge hypothesis [29,30]. By modifying the termini (primary amines) of CDP to contain imidazole groups (CDPim), which protonate within this pH range, intravesicular pH buffering and significant enhancement of gene expression were seen with minimal effect on DNA binding, cellular uptake and cytotoxicity [31-33]. The structure of CDPim is shown in Figure 2.

In vivo applications of these linear CDPs and all three of these modifications (pegylation, targeting and imidazole incorporation) include the previously cited delivery of DNAzymes [27] and that of a different nucleic acid therapeutic, siRNA. When targeted to the fusion point of a chromosomal translocation that results in a cancer-causing fusion gene, siRNA was incorporated within Tf-targeted polyplexes within a disseminated murine model of Ewing's sarcoma [34]. Twice-weekly administration of complexes over 4 weeks resulted in significantly reduced tumour size compared with mice that were similarly injected with carrier solution, uncomplexed siRNA, formulated control siRNA, or formulated but untargeted siRNA. Real-time PCR analysis confirmed that formulations containing siRNA against the fusion gene, but not a control siRNA, could achieve knockdown in

target gene transcript levels. Furthermore, histological examination of mice after receiving this treatment regimen failed to show any evidence of damage to major organs. Single administration of these different formulations to fully immunocompetent mice did not reveal any treatment-related changes in cytokine, liver enzyme or blood cell levels. Therefore, this work suggests that this delivery system is safe and efficacious for *in vivo* administration of therapeutic nucleic acids. Indeed, efforts to commercialise this technology for cancer applications are currently underway.

4. Expert opinion

The advances in linear CDPs as delivery agents for therapeutics, in regard to preparation, understanding and intellectual property, have been significant. Although there are already numerous issued patents concerning both the composition of the matter and the use of individual CDs in pharmaceutical applications, the polymeric nature of the materials discussed in this review distinguishes them from such patents. Indeed, multiple new patents have already been issued for this class of materials [102-104], and their novelty has required them to be tested extensively to demonstrate safety prior to administration to humans. This testing has indicated these materials to be well tolerated and, indeed, the first-ever human clinical trial involving these materials is ongoing.

Characterisation of anionic CD–PEG copolymers and their CPT-loaded variants has been extremely thorough: drug loading levels and release kinetics, IC_{50} values, *in vivo* pharmacokinetics and biodistributions, and the effect of different dosing regimens on the treatment of > 6 different tumour types have all been examined and published. With this information in hand, the two most important next steps in the development of this class of materials (other than the accrual of clinical data) are the investigation of the effects of incorporating i) targeting moieties; and/or ii) different drugs and/or drug types. Although biodistribution results with CPT-loaded polymer (in tumour-bearing nude mice) indicate that the tumour contains more of this polymer than any other organ examined (i.e., the heart, liver, spleen and lungs), it may be possible to enhance preferential tumour uptake even further by incorporating a tumour-specific ligand, perhaps most easily via the AD–PEG–ligand conjugates discussed in this paper. The versatility of this delivery platform should also be explored further with therapeutics of other types, including peptides and/or proteins.

Variations in linear, cationic CDPs and their effect on nucleic acid delivery have been thoroughly investigated and characterised, but much more extensive work on understanding the nature of the complete formulations (containing polymer, nucleic acid, AD–PEG and AD–PEG–ligand) is needed. Suggested investigations include: i) determination of complex stoichiometry (especially with respect to the nucleic acid and targeting ligand components); ii) assessment of binding strength of AD–PEG and AD–PEG–ligand to CD moieties within the polymers; and iii) measurement of the effect of targeting ligand density on *in vivo* efficacy. Additional development, including different targeting ligands (different proteins or sugars but also aptamers and/or antibodies) and nucleic acid sequences (targeting separate tumour-associated genes), should also be performed. Given the positive results that have been seen thus far, and the body of work on CD–polymer–CPT conjugates ahead of it, it appears reasonable to expect clinical evaluation of a nucleic acid therapeutic containing a linear CD polymer to become a reality in the near future.

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