Synthesis of Linear, β-Cyclodextrin-Based Polymers and Their Camptothecin Conjugates

Jianjun Cheng,[†] Kay T. Khin,[†] Gregory S. Jensen,[†] Aijie Liu,[†] and Mark E. Davis*[,‡]

Insert Therapeutics, Inc., 2585 Nina Street, Pasadena, California 91107, and Division of Chemical Engineering, California Institute of Technology, Pasadena, California 91125. Received May 29, 2003; Revised Manuscript Received July 22, 2003

6^A,6^D-Bis-(2-amino-2-carboxylethylthio)-6^A,6^D-dideoxy-β-cyclodextrin **1**, a diamino acid derivative of β-cyclodextrin, is synthesized and condensed with difunctionalized PEG comonomers to give linear, high molecular weight (M_w over 50 kDa) β-cyclodextrin-based polymers (**2**−**4**) with pendant functionality (carboxylate). **2**−**4** are all highly soluble in aqueous solutions (over 200 mg/mL). 20-*O*-trifluoroglycinylcamptothecin, **5a**, and 20-*O*-trifluoroglycinylglycinylglycinylcamptothecin, **5b**, are synthesized and conjugated to **2** to give polymer−camptothecin (CPT) prodrugs. The solubility of CPT is increased by more than three orders of magnitude when it is conjugated to **2**. The rates of CPT release from the conjugates **HGGG6** (high molecular weight polymer (M_w 97 kDa), glyglygly linker and 6 wt % CPT loading) and **HG6** (high MW polymer (M_w 97 kDa), gly linker and 6 wt % CPT loading) in either mouse or human plasma are dramatically accelerated over the rates of pure hydrolysis at pH = 7.4, indicating the presence of enzymatic cleavage as a rate-determining step at this pH in the release of the CPT. The pH of aqueous solution has a large effect on hydrolysis rate of CPT from **HGGG6** and **HG6**; the lower the pH, the slower the rate in the range at $4.1 \leq pH \leq 13.1$. The IC_{50}'s of polymer **2e**, CPT, and the CPT conjugates **HG6** and **HGGG6** are found to be cell-line dependent with LS174T, HT29, A2780, and PC3 cells using in vitro MTT assays. The parent polymer **2e** has very low toxicity to all cultured cells tested.

INTRODUCTION

Numerous small molecules have been investigated for use as antitumor agents. Many of these compounds have been found to have limited clinical effectiveness due in part to their high toxicity, low solubility, and/or other poor pharmaceutical parameters. It has been demonstrated that conjugation of an anticancer molecule to a water-soluble polymer can greatly enhance its aqueous solubility and reduce its cytotoxicity in vitro and in vivo (*1*−*3*). High molecular weight (MW) polymers can increase the accumulation of drug in tumor tissue through the so-called enhanced permeability and retention (EPR) effects (*4*). Therefore, development of polymeric drug delivery vehicles has attracted much attention because of these enhancing features of the polymer−drug conjugates over the parent drug molecules. Polymers that can be used for the conjugation of antitumor agents should have low-toxicity, low-immunogenicity, and high water solubility.

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of (α-1,4)-linked-α-D-glucopyranose units, and several of their derivatives are known to have low toxicity and lack immunogenicity in humans (*5*). CDs contain lipophilic cone-shaped cavities that are surrounded by hydrophilic shells that possess a large number of primary and secondary hydroxyls (Figure 1). These hydroxyl groups render many of the CD derivatives highly water-soluble. Therefore, CDs may be excellent building blocks for the synthesis of safe and water-soluble polymers for drug delivery. However, due to the multi-functionality of CD molecules (Figure 1), most CD-containing polymers that have been extensively studied are heavily cross-linked (*6*). The structures of these polymers are complex and make their characterization difficult.

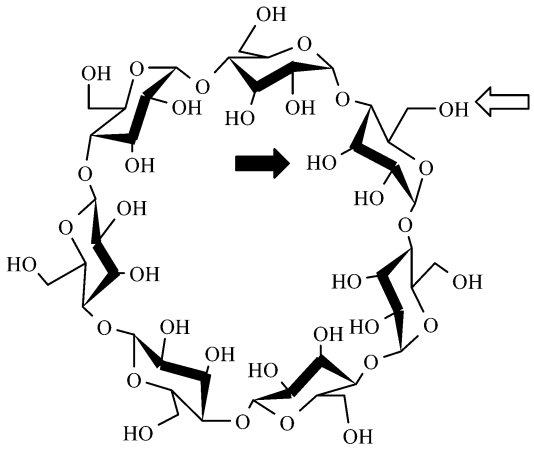


Figure 1. Hydroxyl groups located on the edged of the β-cyclodextrin ring. Primary hydroxyl groups are denoted by an open arrow. Secondary hydroxyl groups are denoted by a solid arrow.

It is possible to selectively convert only two of the many hydroxyls (e.g., 21 hydroxyls for β-CD) of CDs to other functionalities to give disubstituted CD derivatives (*7*). If properly functionalized, these CD derivatives can be used for the synthesis of linear polymers where the CD moiety is incorporated to the polymer backbone. We have described the synthesis of β-CD-based, linear polycations

* To whom correspondence should be addressed. E-mail: mdavis@cheme.caltech.edu. Tel: (626) 395−4251; Fax: (626) 568−8743.

[†] Insert Therapeutics, Inc.

[‡] California Institute of Technology.

10.1021/bc0340924 CCC: $25.00 © 2003 American Chemical Society
Published on Web 08/27/2003

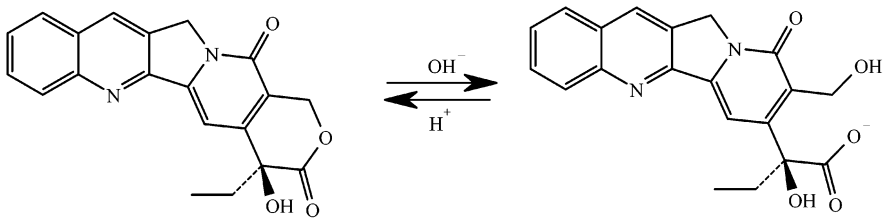
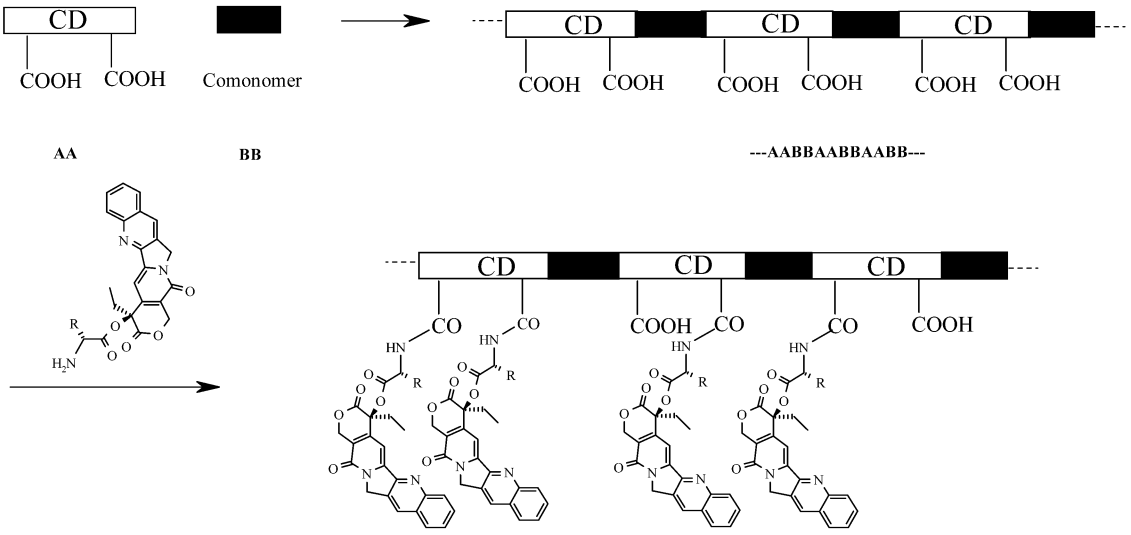


Figure 2. CPT structure and pH-dependent equilibrium between the lactone and carboxylate form. Lactone form is favored at acidic pH.

Scheme 1



(around 6 kDa) as nonviral vectors for systemic gene delivery (*8*−*12*).

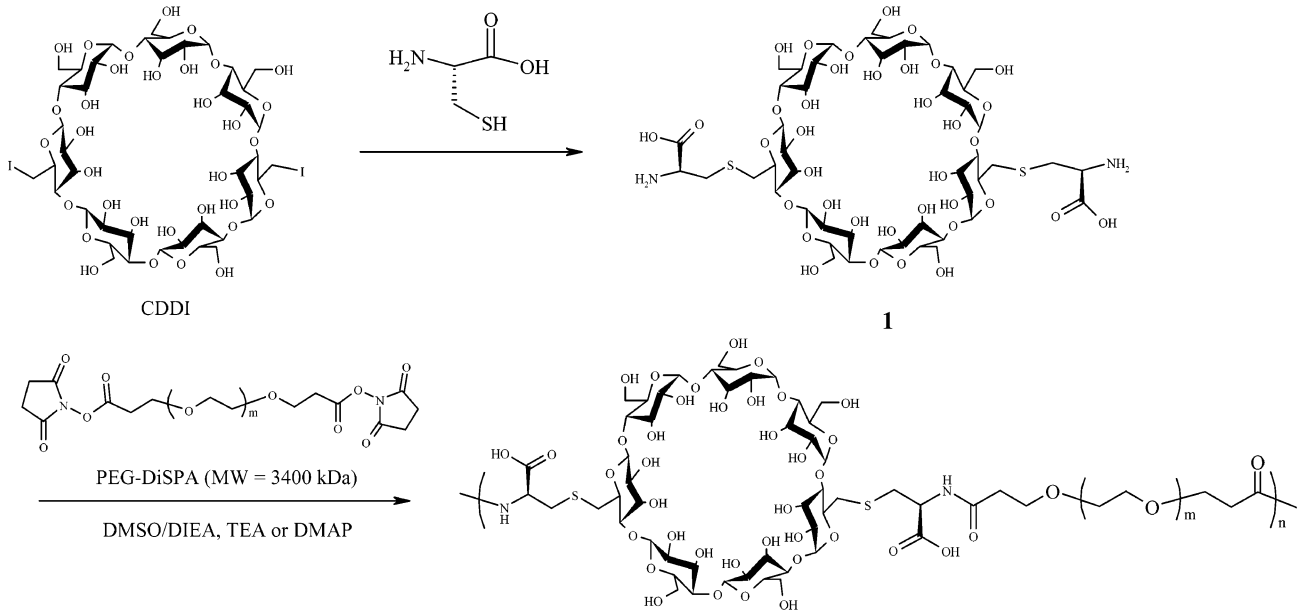
20(S)-camptothecin (CPT) was first isolated from the Chinese tree, *Camptotheca acuminata*, in the 1960s (*13*), and has shown a broad range of anticancer activity in animal models (*14*, *15*). CPT has low aqueous solubility in the lactone form and can be highly toxic in its carboxylate form (*16*). The lactone is essential for anticancer activity, while the carboxylate is inactive and favored at physiological pH (see Figure 2) (*17*, *18*). Serum albumin preferentially binds the carboxylate form of CPT and forces the distribution of CPT to further disfavor the lactone form (*16*−*18*). These features of CPT result in rapid lactone-ring opening and a loss of antitumor function. Despite these difficulties, CPT-based drugs, e.g., topotecan and irinotecan, are currently approved for use in humans (*19*, *20*).

It has been reported that substitution on 20-OH of CPT can substantially reduce the tendency for lactone ring opening (*21*). Covalent attachment of CPT via this site (20-OH) to water-soluble polymers such as poly(ethylene glycol) (PEG) (*22*−*24*), poly-*N*-(2-hydroxypropyl)methacrylamide (HPMA) (*25*, *26*) and poly-L-glutamic acid (PG) (*27*−*29*) have been reported. In this study, we show the first syntheses of linear, water-soluble, high molecular weight (M_w over 50 kDa), CD-based polymers (CDPs) that contain pendant carboxylate groups, and conjugate CPT to them for use as antitumor therapeutics. CPT functionalized at the 20-OH position was covalently coupled to the polymer to give the conjugate as illustrated in Scheme 1. The synthesis and properties of these types of conjugates are presented here.

EXPERIMENTAL PROCEDURES

General. All the anhydrous solvents, HPLC grade solvents, and other common organic solvents were purchased from commercial suppliers and used without further purification. Poly(ethylene glycol) dipropanoicsuccinimide (PEG-DiSPA, MW 3400), poly(ethylene glycol) dibutanoicsuccinimide (PEG-DiSBA, MW 3400), and poly(ethylene glycol) dibenzotrizolecarbonate (PEG-DiBTC, MW 3400) were purchased from Nektar (Huntsville, AL). Poly(ethylene glycol) di-*p*-nitrophenolcarbonate (PEG-DiNPC, MW 3400) was acquired from Sigma (St. Louis, MO). CPT was purchased from Boehringer Ingelheim (Ingelheim, Germany). Human plasma was purchased from Sigma and reconstituted with DI water. Mouse plasma was prepared by centrifuge removal of blood cells of fresh blood samples collected from BALB/C female mice (Charles River). 6^A,6^D-Diiodo-6^A,6^D-dideoxy-β-cyclodextrin (CDDI, Scheme 2) was synthesized according to previous reported procedure (*8*). Deionized water (18-MΩ-cm) was obtained by passing in-house deionized water through a Barnstead E-pure purification system. NMR spectra were recorded on a Bruker AMX 500 MHz or a Varian 300 MHz spectrometer. Mass spectral (MS) analysis was performed using either an electrospray mass spectrometer equipped with LCQ ion trap (Thermo Finnigan) and fitted with an electrospray ionization source or a MALDI-TOF mass spectrometer (Voyager DE-PRO, Applied Biosystems). MWs of the polymer samples were analyzed on a GPC system equipped with a Hitachi L-6200 Intelligent Pump, an Anspec RI detector (ERC-7512, Erma, Inc.), a Precision Detectors DLS detector (PD 2020), and double gel permeation columns (PL-aquagel-OH-40 8 μm 300 × 7.5 mm, Polymer Laboratory) calibrated using poly(ethylene gly-

Scheme 2



col) standard and eluded using PBS (1×) at a concentration of 20−50 mg/mL and at a 0.7 mL/min flow rate at ambient temperature. CD derivatives were analyzed with a C-18 reverse phase column on a HPLC system equipped with an UV detector (System Gold 168 Detector, Beckman Coulter) and an evaporative light scattering (ELS) detector (Sedex 75, Sedere, France). CPT, CPT derivatives, and polymer−CPT conjugates were analyzed on HPLC systems with a C-18 reverse phase column (HIRPB-4438, 4.6 × 150 mm, Richard Scientific) equipped with a fluorescence detector (FD-500, GTI/Spectro Vision, Groton Technology, Inc.) using a gradient of potassium phosphate buffer (pH 4.1) and acetonitile. Excitation and emission wavelengths of the fluorescence detector were set at 370 and 440 nm, respectively.

$6^A,6^D$−Bis-(2-amino-2-carboxylethylthio)-$6^A,6^D$-dideoxy-β-cyclodextrin, 1 (Scheme 2). A total of 167 mL of 0.1 M sodium carbonate buffer were degassed for 45 min in a 500-mL two-neck round-bottom flask equipped with a magnetic stir bar, a condenser, and septum. To this solution were added 1.96 g (16.2 mmol) of L-cysteine and 10.0 g (73.8 mmol) of $6^A,6^D$-diiodo-$6^A,6^D$-dideoxy-β-cyclodextrin (CDDI, Scheme 2). The resulting suspension was heated at a reflux temperature for 4.5 h until the solution turned clear (colorless). The solution was then cooled to room temperature and acidified to pH 3 using 1 N HCl. The product was precipitated by slow addition of acetone (3 times weight ratio of the solution). This afforded 9.0 g of crude material containing CD-biscysteine (90.0%), unreacted cyclodextrin, CD-monocysteine, and cystine. The resulting solid was subjected to anionic exchange column chromatography (SuperQ650M, Tosoh Bioscience) using a gradient elution of 0−0.4M ammonium bicarbonate. All fractions were analyzed by HPLC. The desired fractions were combined, and the solvent was reduced to 100 mL under vacuum. The final product was either precipitated by adding acetone or by adding methanol (3 times weight ratio of the solution). **1** was obtained in 60−80% yield. ^{1}H NMR (D_2O) δ 5.08 (m, 7H, CD-2-CH), 3.79−3.94 (m, 30H, CD-3,4-CH, CD-CH_2, Cys-CH), 3.49−3.62 (m, 14H, CD-5, 6-CH), 2.92−3.30 (m, 4H, Cys-CH_2). ^{13}C NMR (D_2O) δ 172.3, 101.9, 83.9, 81.6, 81.5, 73.3, 72.2, 72.0, 60.7, 54.0, 34.0, 30.6. ESI/MS (*m*/*z*): 1342 $[M]^+$, 1364 $[M + Na]^+$.

Table 1. Polymerization of 1 with Difunctionalized PEG

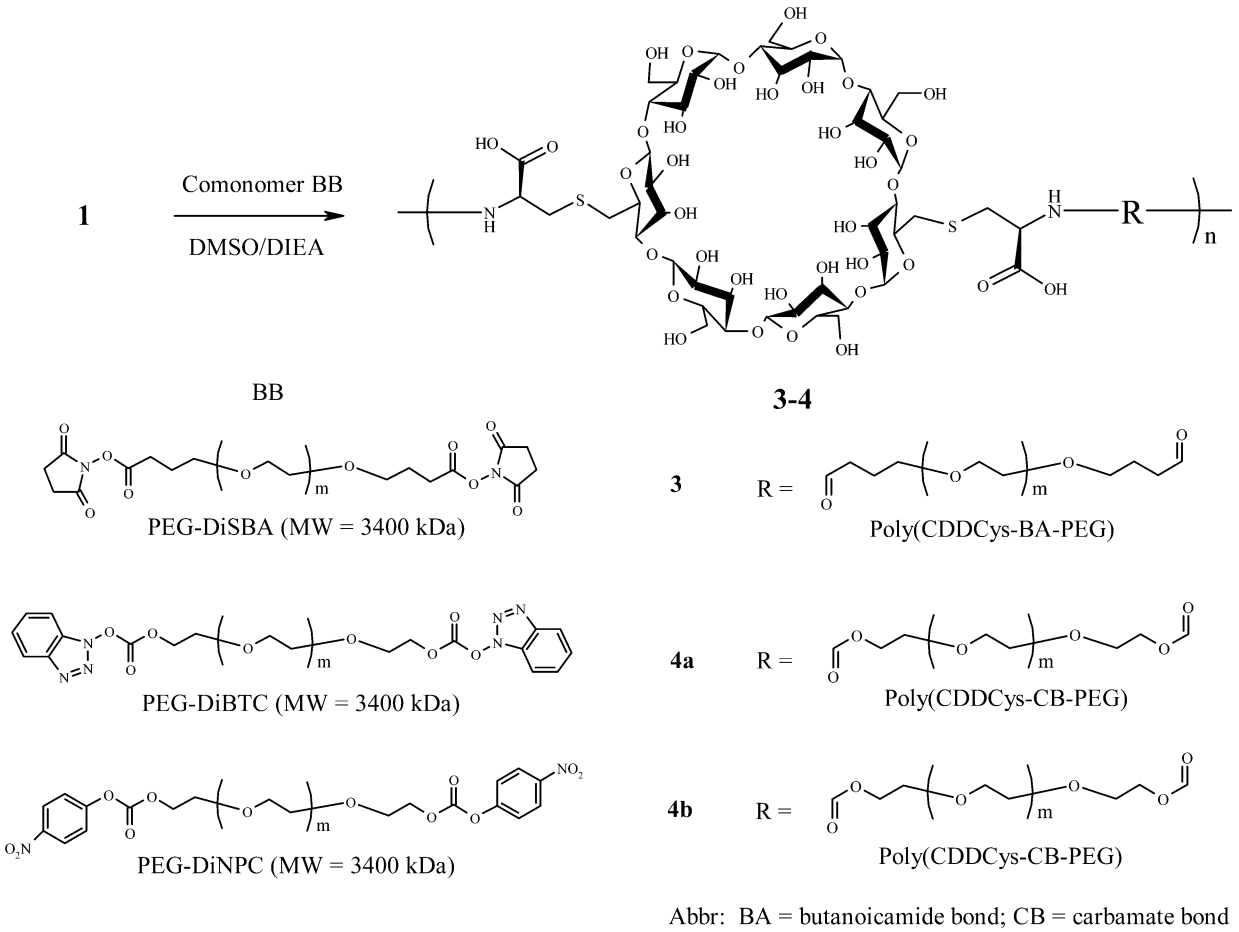
CDP	PEG comonomer	base	polymerization time (h)	M_w (kDa)	M_n (kDa)	M_w/M_n	yield (%)
2a[a]	PEG-DiSPA	DIEA	120	57.4	41.7	1.38	90
2b[a]	PEG-DiSPA	DMAP	120	54.2	38.1	1.42	91
2c[a]	PEG-DiSPA	TEA	120	57.4	42.6	1.35	91
2d[b]	PEG-DiSPA	DIEA	120	93.6	58.0	1.48	96
2e[b]	PEG-DiSPA	DIEA	144	97.3	58.0	1.67	94
2f[b]	PEG-DiSPA	DIEA	2	35.3	25.6	1.38	95
3	PEG-DiSBA	DIEA	120	114.7	77.9	1.47	96
4a	PEG-DiBTC	DIEA	120	67.6	39.4	1.47	95
4b	PEG-DiNPC	DIEA	120	86.5	57.2	1.51	96

[a] **1** was washed with acetone before polymerization. [b] **1** was washed with methanol before polymerization.

Synthesis of Poly(CDDCys-PA-PEG), 2a (Scheme 2). 1 (after precipitation with acetone, 63 mg, 0.047 mmol) and PEG-DiSPA (MW 3400, 160 mg, 0.047 mmol) were dried under vacuum for 8 h. Anhydrous DMSO (1.26 mL) was added to the mixture under argon. After 10 min of stirring, anhydrous diisopropylethylamine (DIEA, 19 μL, 2.3 equiv) was added under argon. The reaction mixture was stirred under argon for 120 h. The polymer containing solution was dialyzed using a 10 000 MWCO membrane (Spectra/Por 7) against water for 48 h and lyophilized to yield 196 mg of **2a** (92%, Table 1). M_w = 57.4 kDa, M_n = 41.7 kDa, M_w/M_n = 1.38. ^{1}H NMR (D_2O) δ 5.08 (m, CD-2-H), 4.27 (m, Cys-CH), 2.72−3.76 (m, CD-3,4,5,6-CH, CD-CH_2, PEG-CH_2), 2.44 (m, Cys-CH_2).

Synthesis of other poly(CDDCys-PA-PEG) (**2b**−**f**), poly(CDDCys-BA-PEG) (**3**), and poly(CDDCys-CB−PEG) (**4a**) (Schemes 2 and 3) were achieved under polymerization condition similar to that of **2a.** Details for the polymer-

Scheme 3



ization conditions, monomer selection, polymer molecular weight, polydispersity, and yields are listed in Table 1. **3**: ^{1}H NMR (D_2O) δ 5.10 (m, CD-2-H), 4.25−4.37 (m, Cys-CH), 2.72−3.86 (m, CD-3,4,5,6-CH, CD-CH_2, PEG-CH_2), 2.21 (m, Cys-CH_2). **4a**−**b**: ^{1}H NMR (D_2O) δ 5.05 (m, CD-2-H), 4.56 (m, Cys-CH), 2.70−3.93 (m, CD-3,4,5,6-CH, CD-CH_2, PEG-CH_2), 2.38 (m, −OC*H*$_2$CH$_2$CH$_2$C(O)-NH-), 2.34 (m, Cys-CH_2), 1.90 (m, −OCH$_2$C*H*$_2$CH$_2$C(O)-NH-).

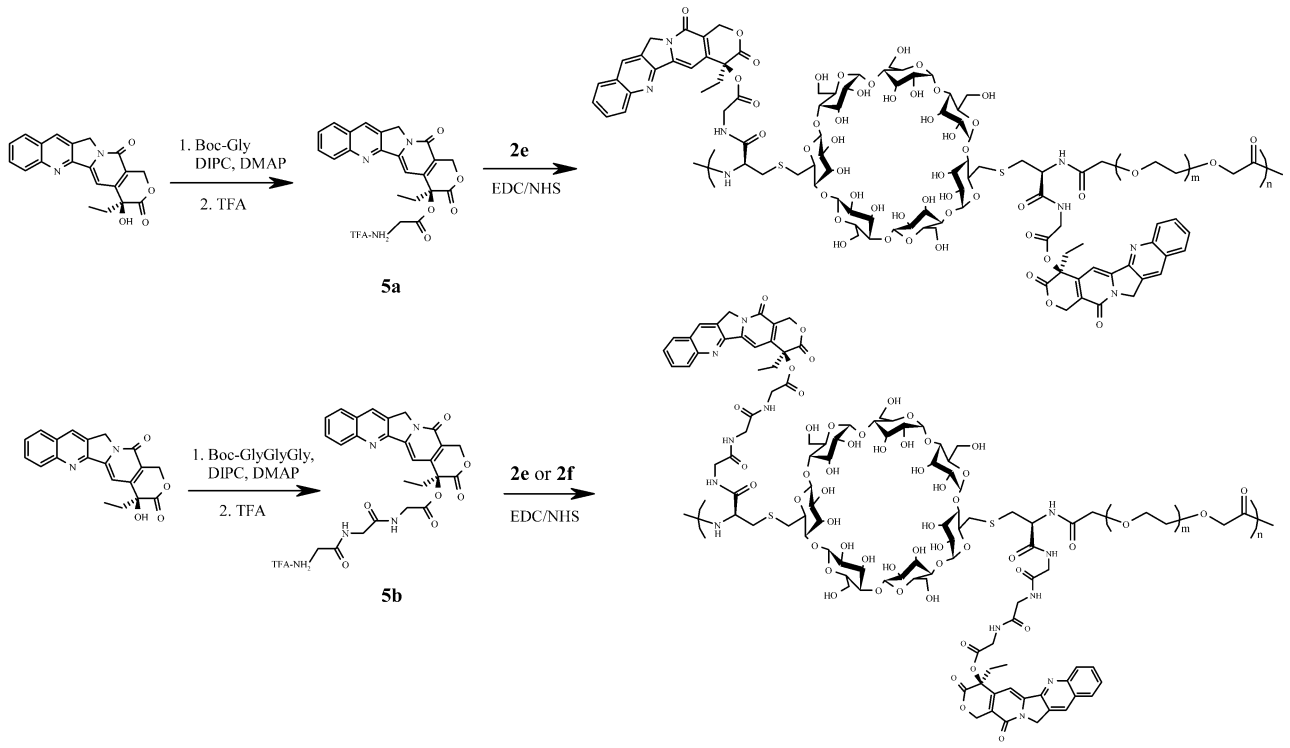
Molecular Weight Control of CD Polymers. 1 (after precipitation with methanol) (56.2 mg, 0.0419 mmol) and PEG-DiSPA (147 mg, 0.0419 mmol) were dried under vacuum for 4−8 h. To the mixture was added dry DMSO (1.1 mL) under argon. After 10 min stirring of the sample, DIEA (16 μL, 2.2 equiv) was added under argon. A portion of polymerization solution (150 μL) was removed and precipitated with ether at selected times (2, 18, 43, 70, 168, and 288 h). MWs of the precipitated polymers were determined as described above.

20-Hydroxyl-Modified Camptothecin. Synthesis of 20-*O*-trifluoroglycinylcamptothecin (**5a**) and 20-*O*-trifluoroglycinylglycinylglycinylcamptothecin (**5b**) were synthesized according to previous reported procedures (*23*) with slight modification.

*Synthesis of **5a** (Scheme 4).* *t*-Boc-glycine (0.9 g, 4.7 mmol) was dissolved in 350 mL of anhydrous methylene chloride at room temperature, and to this solution were added DIPC (0.75 mL, 4.7 mmol), DMAP (382 mg, 3.13 mmol), and CPT (0.55 g, 1.57 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and remain stirring for 16 h. The organic solution was extracted with cold pH 2 aqueous solution, dried with $MgSO_4$, and evaporated under reduced pressure to yield a light yellow solid, that was recrystallized from methanol to give camptothecin-20-ester of *t*-Boc-glycine 0.45 g (57%): ^{1}H NMR(DMSO-d_6) δ 7.5−8.8 (m), 7.3 (s), 5.5 (s), 5.3 (s), 4 (m), 2.1 (m), 1.6 (s), 1.3 (d), 0.9 (t). Camptothecin-20-ester of *t*-Boc-glycine (0.608 g, 1.2 mmol) was dissolved in a mixture of methylene chloride (10 mL) and TFA (10 mL) and stirred at room temperature for 1 h. Solvent was removed and the residue was recrystallized from methylene chloride and ether to give 0.55 g of **5a** (87%). ^{1}H NMR (DMSO-d_6) δ 7.7−8.5 (m); 7.2 (s), 5.6 (s), 5.4 (s), 4.4 (m), 2.2 (m), 1.6 (d), 1.0 (t), ^{13}C NMR (DMSO-d_6) δ 168.6, 166.6, 156.5, 152.2, 147.9, 146.2, 144.3, 131.9, 130.6, 129.7, 128.8, 128.6, 128.0, 127.8, 119.0, 95.0, 77.6, 66.6, 50.5, 47.9, 30.2, 15.9, 7.9. ESI/MS (*m*/*z*) expected 405; Found 406 (M+H).

*Synthesis of **5b** (Scheme 4).* *t*-Boc-GlyGlyGly (1.359 g, 4.7 mmol) was dissolved in 350 mL of anhydrous methylene chloride at room temperature and to this solution were added DIPC (0.75 mL, 4.7 mmol), DMAP (382 mg, 3.13 mmol), and CPT (0.55 g, 1.57 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and remain stirring for 16 h. The organic solution was extracted with cold pH 2 aqueous solution, dried with $MgSO_4$, and evaporated under reduced pressure to yield a light yellow solid, which was recrystallized from methanol to give camptothecin-20-ester of *t*-Boc-GlyGlyGly 0.47 g (65%): ^{1}H NMR(DMSO-d_6) δ 8.40 (s), 8.25 (d), 7.91 (d), 7.78 (m), 7.65 (t), 7.26 (s), 7.05 (br, s), 5.65 (d), 5.40 (d), 5.25 (s), 5.10 (br, s), 3.75−4.42 (m), 2.15−2.35 (m), 1.45 (s), 0.95 (t) Camptothecin-20-ester of *t*-Boc-GlyGlyGly (1.5 g, 2.08 mmol) was dissolved in a mixture

Scheme 4



of methylene chloride (20 mL) and TFA (20 mL) and stirred at room temperature for 1 h. Solvent was removed under vacuum, and the residue was redissolved in methylene chloride. The solution was poured into ether to give an instant yellow precipitate. The precipitate was filtered and washed with cold ether to give 1.31 g of **5b** (86%). ^{1}H NMR (DMSO-d_6) δ 8.79 (s), 7.75−8.61 (m), 7.10 (s), 5.55 (s), 3.90−4.37 (m), 3.86 (s), 3.54 (s), 2.11−2.23 (m), 0.95 (t). ESI/MS (*m*/*z*) expected 519; Found 520 (M+H).

Synthesis of Poly(CDDCys-PA-PEG)-CPT Conjugates. *Synthesis of Poly(CDDCys-PA-PEG)-GlyGlyGly-CPT (**HGGG6**).* **2e** (1.37 g, 0.30 mmol of repeat unit) was dissolved in dry DMSO (136 mL). The mixture was stirred for 10 min. **5b** (419 mg, 0.712 mmol, 2.36 equiv), DIEA (0.092 mL, 0.712 mmol, 2.36 equiv), EDC (172 mg, 0.903 mmol, 3 equiv), and NHS (76 mg, 0.662 mmol, 2.2 equiv) were added to the polymer solution and stirred for ca. 15 h. The polymer was precipitated with ethyl ether (1 L). The ether was poured out and the precipitate was washed with CH_3CN (3 × 100 mL). The precipitate was dissolved in water (600 mL). Some insoluble solid was filtered through 0.2 μm filters. The solution was dialyzed using 25 000 MWCO membrane (Spectra/Por 7) for 10 h at 10−15 °C in DI water. Dialysis water was changed every 60 min. The polymer−drug conjugate solution was sterilized by passing it through 0.2 μM filters. The solution was lyophilized to yield a yellow solid (1.42 g, 85%).

*Synthesis of Poly(CDDCys-PA-PEG)-GlyGlyGly-CPT (**LGGG10**).* Conjugation of **5b** to **2f** was performed in a manner similar to that used to produce **HGGG6** except that this conjugate was dialyzed with 10 000 MWCO membrane (Spectra/Por 7) instead of with 25 000 MWCO membrane. The yield of **LGGG10** was 83%.

*Synthesis of Poly(CDDCys-PA-PEG)-Gly-CPT (**HG6**).* Conjugation of **5a** to **2e** was performed in a manner similar to that used to produce **HGGG6**. The yield of **HG6** was 83%.

*Synthesis of Poly(CDDCys-PA-PEG)-GlyGlyGly-CPT (**HGGG10**).* **2e** (1.5 g, 0.33 mmol of repeat unit) was dissolved in dry DMSO (150 mL). The mixture was stirred for 10 min. **5b** (941 mg, 1.49 mmol, 4.5 equiv), DIEA (0.258 mL, 1.49 mmol, 4.5 equiv), EDC (283 mg, 1.49 mmol, 4.5 equiv), and NHS (113 mg, 0.99 mmol, 3 equiv) was added to the polymer solution and stirred for ca. 24 h. Another portion of EDC (142 mg, 0.75 mmol, 2.3 equiv) and NHS (56 mg, 0.5 mmol, 1.5 equiv) were added to the conjugation solution. The polymer was stirred for an additional 22 h. The workup procedure was same as that for the synthesis of **HGGG6**. The yield of **HG6** was 77%.

Determination of wt % CPT on the Conjugates. Stock solutions of **HGGG6**, **LGGG10**, **HG6**, and **HGGG10** were prepared at a concentration of 10 mg/mL in DMSO. An aliquot of corresponding stock solution was diluted to 100 μg/mL using 1 N NaOH. CPT was completely hydrolyzed in this basic solution and transformed to its carboxylate form within 2 h at room temperature. An aliquot of this solution was diluted to 10 μg/mL using 8.5% H_3PO_4, and the CPT carboxylate form was transformed to its lactone form. 30 μL of this solution was injected into the HPLC. The peak area from the CPT lactone form was integrated and compared to a standard curve.

Comparison of Lactone Stability of CPT and 5 in Phosphate Buffered Saline (PBS). CPT or **5** (**5a** and **5b**) was dissolved in DMSO at 1 mg/mL and then diluted to 1 μg/mL with PBS (1×, pH 7.4). 30 μL of solution were injected into the HPLC at room temperature at selected time intervals. The peak area from the CPT lactone form of CPT or **5** (**5a** and **5b**) were integrated.

Release of CPT from HGGG6 and HG6. *Release of CPT in PBS.* **HGGG6** and **HG6** were prepared at 1 mg/mL in PBS (1×, pH 7.4). A 100 μL aliquot of the solution was transferred to a 1.5 mL Eppendorf tube and incubated at 37 °C. The incubated samples were quenched at selected time intervals and stored at −80 °C until the

analysis. Each solution was diluted with 8.5% H_3PO_4 to a 5 mL total volume in a volumetric flask. 30 μL of such solution was injected into the HPLC. The peak area from the CPT lactone form was integrated and compared to a standard curve.

Analysis for the release of CPT from **HGGG6** and **HG6** in PBS containing acetyl cholinesterase (an esterase, 100 units/mL), in KH_2PO_4 buffer (pH 6.1, 0.1 M) and in the KH_2PO_4 buffer (pH 6.1, 0.1 M) containing cathepsin B (a cysteine proteinase, 200 μM, preactivated on ice for 30 min in this buffer containing 2 mM DTT and 1 mM EDTA) were performed in a manner similar to that described above for PBS alone.

Release of CPT in Human Plasma. An aliquot of **HGGG6** and **HG6** stock solution were diluted to give final concentration of 0.5 mg/mL in PBS (1×, pH 7.4). This solution was added to a lyophilized powder of human plasma to reconstitute 100% human plasma by the recommended amount. The solution was divided into equal volume (250 μL) to 1.5 mL Eppendorf tubes, incubated at 37 °C, and stopped at selected time point. Samples were stored at −80 °C until the analysis. Samples were separated from plasma by solid-phase extraction columns. The solid-phase extraction cartridge (Oasis HLB 1 cm^3 cartridge from Waters) was preconditioned with 1 mL of acetonitrile and then with 1 mL of 8.5% H_3PO_4 before loading. Samples were acidified with equal volume of 8.5% H_3PO_4 prior to loading. After the acidified solution was loaded on the cartridge, the bed was washed with 3 × 1 mL of water. Released CPT and polymer conjugate were eluted with 3 × 1 mL of a solution mixture of acetonitrile and potassium phosphate buffer (pH 4.1) (60/40 v/v). The eluted solution was diluted to 5 mL total volume in a 5 mL volumetric flask. 30 μL of such solution was injected into the HPLC. The peak area from the CPT lactone form was integrated and compared to a standard curve.

Release of CPT from **HGGG6** and **HG6** in PBS containing 4% human plasma (PBS/reconstituted human plasma solution = 96/4 (v/v)), in mouse plasma and in reconstituted human albumin (PBS solution) were performed in a manner similar to that described above for pure human plasma.

Release of CPT in Solution at Variable Different pH. **HGGG6** and **HG6** were prepared at 1 mg/mL in buffer solution with pH values ranging from acidic (pH = 1.2) to basic (pH = 13.1) and incubated at 37 °C for 24 h. An aliquot of each solution was diluted with 8.5% H_3PO_4 to about 100 μg/mL. 30 μL of such solution was injected into HPLC. The peak area from the CPT lactone form was integrated and compared to a standard curve.

IC_{50} via MTT Assay. The human ovarian carcinoma A2780 cell line was obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). The human colorectal adenocarcinoma HT29, human prostate carcinoma PC-3, and human colonic adeoncarcinoma LS174T cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded in 96-well plates at 5000 cells/well and grown in medium containing 10% fetal bovine serum at 37 °C for 24 h in a humidified 5% CO_2 atmosphere. The medium was replaced with fresh medium containing CPT, **2e**, **HGGG6** or **HG6** in concentrations ranging from 1 nM to 10 μM of CPT and **2e** (CPT equivalent for **HGGG6** and **HG6**). At each concentration, three wells per plate were treated. The effect of the compounds on cell growth was measured by the MTT assay after 72 h. The medium was removed, the cells were rinsed with PBS, MTT solution was added at a concentration of 0.5 mg/mL, and the plates were incubated for 4 h at 37 °C. The medium was removed and the formazan crystals were solubilized in DMSO. Absorbance was measured at 560 nm using a SPECTRAFluor Plus plate reader (Tecan, Durham, NC). The percentage of cell survival was calculated relative to untreated cells, and IC_{50}'s were determined from plots of dose versus cell survival.

RESULTS

Synthesis of Difunctionalized Cyclodextrin Monomer, 1. 6^A, 6^D-bis-(2-amino-2-carbonylethylthio)-6^A,6^D-dideoxy-β-cyclodextrin, **1**, was synthesized from 6^A,6^D-diiodo-6^A,6^D-dideoxy-β-cyclodextrin (CDDI, Scheme 2) in a basic buffer solution (pH 11.6, sodium bicarbonate buffer) at 100 °C (Scheme 2). High pH buffer was essential to increase the nucleophilicity of the thiol group on the L-cysteine. This reaction was complete within 5 h. **1** was isolated from other impurities in yields between 60 and 80% using a final anion exchange. After the purification, **1** usually still contained small amounts of free cystine, a side product from the oxidation of cysteine. To achieve higher purity product, **1** was dissolved in water and precipitated with either acetone or methanol. Cystine cannot be completely removed by the acetone-precipitation method as detectable amounts of cystine (0.5−1 wt %) still remains in **1** (Figure 3). Precipitation of **1** with methanol completely removes cystine (Figure 3), most likely due to the higher solubility of cystine in methanol as compared to acetone.

Polymerization of 1 with PEG Comonomers. Polymerization of **1** with PEG-DiSPA was carried out in anhydrous DMSO solution under argon (Scheme 2). Addition of a nonnucleophilic organic base (such as DIEA) was essential for this polymerization as no viscosity changes of the polymerization solutions were observed after 48 h if no base was added. When 2.3 equiv of DIEA was added, the viscosity of polymerization solution increased dramatically after 4−6 h of reaction. DIEA deprotonates the amino groups of **1** to render them more nucleophilic for coupling with PEG-DiSPA. There were essentially no differences in the polymerizations if other bases, such as TEA or DMAP, were used (**2b**−**c**, Table 1). Polymerization using **1** recovered by the two different precipitation methods (acetone and methanol) produced polymers with different MWs. **1** that was purified by the methanol precipitation method (contains no free cystine) gave higher MW polymer (**2d**−**e**) as compared to the less pure **1** that was obtained from the acetone precipitation method (**2a**). Polymerization of **1** with PEG-DiSPA typically produced polymer in yields greater than 90%.

As shown in Figure 4a, the polymer MW increased to around 80 kDa over the course of 48 h of reaction, then gradually increased to around 100 kDa after the polymerization was carried out for another 4−5 days. Prolonged polymerization (> 1 week) generally produced aggregation that rendered the MW measurements inaccurate (Figure 4b).

1 was polymerized with other activated monomers such as PEG-DiSBA, PEG-DiBTC, and PEG-DiNPC (Scheme 3). Reaction of **1** with PEG-DiSBA gave a polymer **3** with similar linkages as **2** (amide bond, but one more -CH_2 group than **2** at the linker) with M_w over 100 kDa, while reaction of **1** with PEG-DiBTC and PEG-DiNPC generated polymers (**4a** and **4b**, respectively) with connecting carbamate moiety and M_w's over 50 kDa (Table 1).

Polymers **2**−**4** are highly soluble in aqueous solution. They can be easily dissolved in water or phosphate buffered saline (PBS) solution at concentrations of at

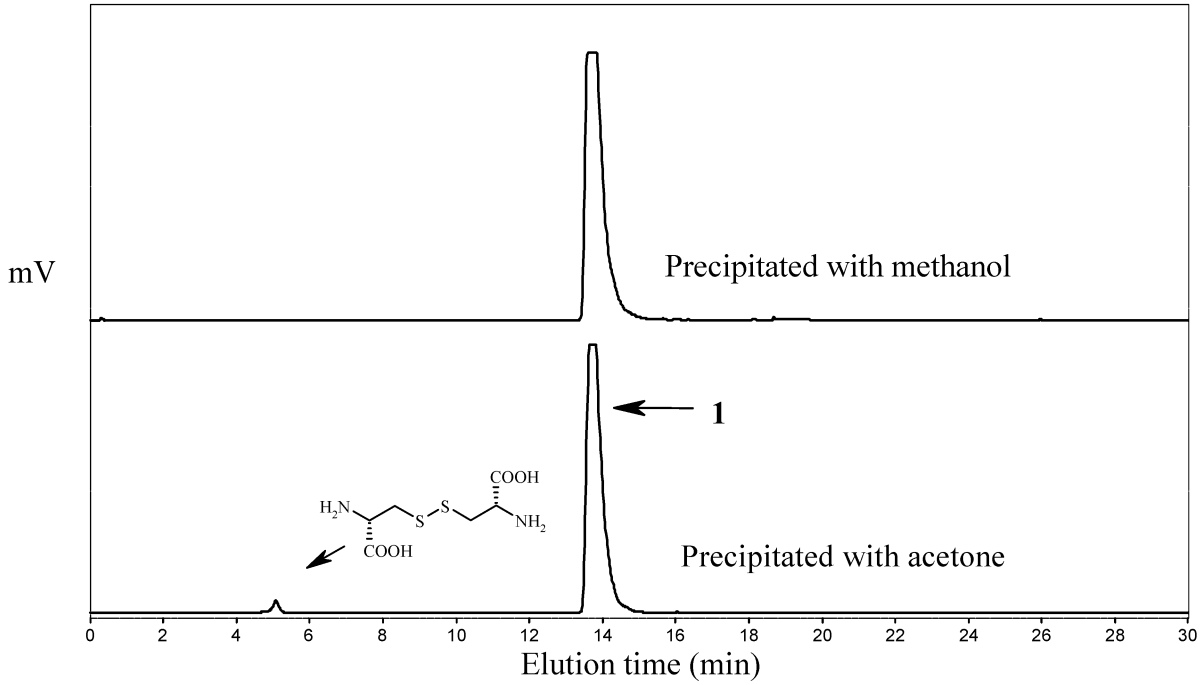


Figure 3. Purification of **1** by precipitation with acetone or methanol.

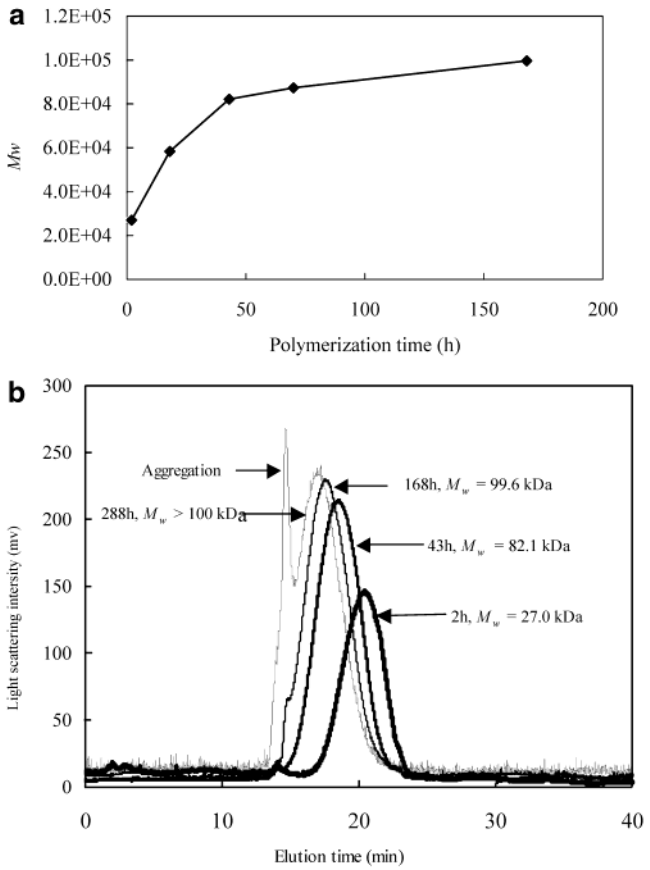


Figure 4. (a) Molecular weights of poly(CDDCys-PA-PEG) as a function of polymerization times. (b) GPC curves of poly-(CDDCys-PA-PEG) as a function of polymerization times.

least 200 mg/mL. Solubility of these polymers in aqueous solution at concentrations higher than 200 mg/mL was not attempted due to the high viscosity. These polymers were also soluble in DMF, DMSO, and methanol, slightly soluble in CH_3CN and $CHCl_3$, but insoluble in THF and ethyl ether.

Table 2. Properties of Polymer–CPT Conjugates

polymer	CPT-linker	CPT (wt %)	conjugate[a]	yield (%)
2e	**5b**	6.1	**HGGG6**	85
2f	**5b**	10.2	**LGGG10**	83
2e	**5a**	6.8	**HG6**	87
2e	**5b**	9.6	**HGGG10**	77

[a] Abbreviations: **H** = high M_w polymer (97 kDa), **L** = low M_w polymer (35 kDa), **GGG** = triglycine linker, **G** = glycine linker, **6** = drug loading around 6 wt %, **10** = drug loading around 10 wt %.

Synthesis of Poly(CDDCys-PA-PEG)–CPT Conjugates. CPT was reacted with *N-tert*-butyloxycarbonyl (Boc) glycine or triglycine to form an ester bond between the 20-hydroxyl group of CPT and the carboxylate of the amino acid in the presence of diisopropylcarbodimide and DMAP. **5a** and **5b** were obtained after removing the BOC groups of the corresponding ester intermediate with trifluoroaceticacid (TFA) (Scheme 4). **5a** and **5b** were isolated with yields over 50%.

5a and **5b** were conjugated to **2e** or **2f** (Scheme 4 and Table 2) using conventional coupling methods. Due to the instability of the ester linker of **5a** and **5b** in aqueous solution, the conjugation was conducted in anhydrous DMSO under argon. An organic base was required to deprotonate the TFA salts of **5a** and **5b** to facilitate the coupling. For polymer conjugation with **5b**, the weight percent (wt %) drug loading was around 6–10% (a prolonged reaction time did not lead to higher loadings). The theoretical maximum drug loading is around 13% using PEG with MW of 3400 Da; maximum values can be increased by decreasing the MW of the PEG segments. Solubilities of all conjugates in water or PBS were more than 200 mg/mL (equivalent to a 12–20 mg of CPT/mL for 6–10 wt % drug loading, respectively).

CPT Lactone Ring Stability by 20-Substitution. The rate of lactone ring opening for **5a**, **5b,** and CPT were studied in PBS buffer (pH 7.4). Both **5a** and **5b** were very stable against ring-opening, and no carboxylate forms of **5a** and **5b** were detected throughout the study (7 h). On

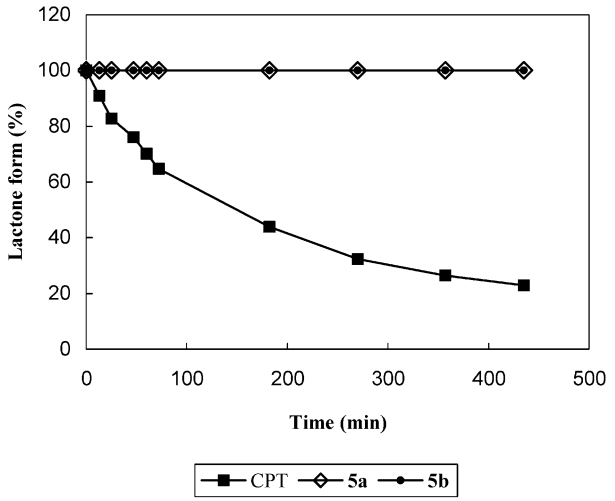


Figure 5. Lactone stability of CPT, **5a** and **5b** in PBS (pH 7.4).

Table 3. Half-life ($t_{1/2}$, in h) of the Release of CPT from HG6 and HGGG6[a]

conjugate	PBS[b]	4% HP[c]	HP[d]	MP[e]	Alb[f]	Ac Cho[g]	pH 6.1 buffer[h]	Cath B (pH 6.1)[i]
HG6	59	25	1.7	2.6	62	33	>144	>144
HGGG6	32	22	1.6	2.2	73	43	>144	>144

[a] $t_{1/2}$ is defined as time (h) for the release of half of the total conjugated CPT. Abbreviations: HP means human plasma, MP means mouse plasma. [b] pH 7.4 PBS 1× buffer. [c] Reconstituted human plasma mixed with PBS (v/v = 4/96). [d] Reconstituted human plasma. [e] Fresh mouse plasma. [f] In reconstituted human albumin PBS buffer. [g] In the presence of acetyl cholinesterase PBS solution (100 units/mL). [h] pH 6.1 phosphate buffer (0.1M). [i] pH 6.1 phosphate buffer in the presence of cathepsin B.

the other hand, more than 60% of the CPT lactone form was transformed to its carboxylate form in the same period of time (Figure 5).

Release of CPT from Conjugates. The release of CPT from two polymer-CPT conjugates (**HG6** and **HGGG6**) with identical MW (M_w 97 kDa) and very similar drug loading (ca. 6 wt %) was investigated. The release kinetics for CPT from **HG6** and **HGGG6** was measured at 37 °C in different aqueous solutions (Table 3). In PBS (1×, pH 7.4), the half-lives ($t_{1/2}$) for releasing CPT from **HG6** and **HGGG6** were 59 and 32 h, respectively. The half-lives decreased to 25 and 22 h, respectively, in the presence of 4% human plasma, and to 1.7 and 1.6 h, respectively, in 100% human plasma and 2.6 and 2.2 h, respectively, in 100% mouse plasma. CPT release rates for both **HG6** and **HGGG6** in the presence of albumin or acetyl cholinesterase were on the same order of magnitude as in PBS. In a buffer solution at a pH lower than PBS (pH 6.1) with or without the enzyme cathepsin B (active at pH 6.1 (*30*)), less than 50% of total conjugated CPT was released from both **HG6** and **HGGG6** for times up to 144 h (Table 3).

The pH of aqueous solution has great effect on the CPT release rates from both **HG6** and **HGGG6**. The amounts of CPT released from **HG6** and **HGGG6** at 37 °C after 24 h in buffer solutions with pHs ranging from 1.1 to 13.1 are illustrated in Figure 6. The glycinyl−CPT ester bonds of both **HG6** and **HGGG6** were very stable in acidic pH (1.1−6.4) as less than 7% of CPT were released in 24 h. The CPT hydrolysis rate was drastically increased with an increase of pH to pH 7 or above. CPT was completely hydrolyzed at pH 13 in 24 h. The polymer backbone of **2e** was stable against degradation at pH 5 or above, and

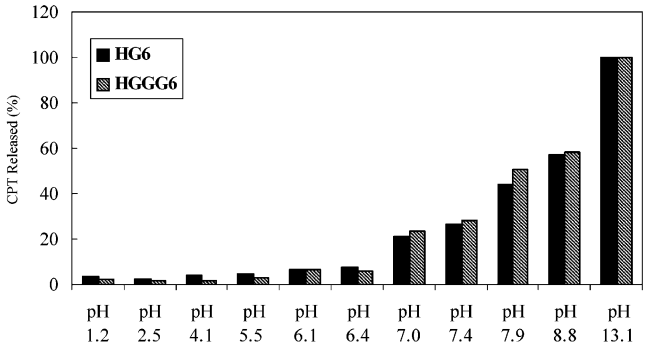


Figure 6. Release of CPT from **HG6** and **HGGG6** at various pH (37 °C, 24 h).

Table 4. IC_{50} of CPT, Unconjugated Polymer 2E and CPT Conjugates HG6 and HGGG6 in Various Cell Lines

cell line	**2e** (μM)	CPT (μM)	**HG6** (μM)	**HGGG6** (μM)
LS174T	>300	0.005	0.050	0.010
HT29	300	0.020	0.050	0.030
A2780	100	0.007	0.025	0.020
PC3	>300	0.075	0.25	0.15

slowly degrades to low MW species over a period of several months at pH 4 or below (data not shown).

The **HG6** hydrolysis solution (PBS) was analyzed by HPLC and the fraction containing the released CPT molecule (same retention time as authentic CPT) was investigated by mass spectroscopy. The molecular weight of this molecule is identical to that of authentic CPT demonstrating that the component released is pure CPT.

IC_{50} Measurement by MTT Assay. The toxicities of **2e**, CPT, and the conjugates (**HG6** and **HGGG6**) were tested in various cell lines (Table 4) using the MTT assay. The IC_{50}'s of **2e** were in a range of 100 to 450 μM, indicating that this polymer was very nontoxic to all cell lines tested. The IC_{50} of CPT was around 5 nM in LS174T cells, and ca. 75 nM in PC3 cells. The IC_{50} of **2e** was thus at least 3 orders of magnitude higher than that of CPT. In all cell lines tested, the IC_{50} values decreased in the order **HG6**, **HGGG6**, and CPT.

DISCUSSION

The general strategy for the design of the linear, CD-containing polymers with pendant functionalities via the condensation of a difunctionalized CD monomer (AA) with a comonomer (BB) for the conjugation of CPT is illustrated in Scheme 1. Due to the existence of numerous hydroxyl groups on cyclodextrins, syntheses involving CD molecules typically require protection and deprotection of these hydroxyls to avoid side reactions such as cross-linking. Here, the design of a difunctionalized CD molecule allows straightforward use as a monomer for the construction of linear polymers without the need of tedious protection/deprotection chemistry during the polymerization. CD-diamino acids are used as monomers since amino groups are much more reactive for certain types of condensation reactions (e.g., coupling to succinimide) when compared to hydroxyl groups, and the carboxylates remain intact during polycondensation for use in the conjugation of CPT.

Synthesis of the CD diamino acid monomer was first attempted by reacting N_α-CBZ-lysine with CDDI. However, no product was isolated. The result is likely due to the low reactivity of the amino groups of lysine for substitution of the iodo group of CDDI that is adjacent to the bulky CD moiety. It has been reported that thiol group, as compared with amino group, has a much higher

reactivity for this substitution reaction (*8*, *9*). For example, reaction between CDDI and dicysteamine gives predominantly CD-dicysteamine with amino terminal groups. Therefore, L-cysteine, an amino acid containing a thiol group, was chosen to react amino acid moieties onto the CD (Scheme 2). **1** was successfully synthesized using this strategy and was purified with good yield (60−80%).

Due to the bulky size of the CD, a disuccinimide compound with relatively long distance between these reactive groups is used to minimize the steric hindrance between CD molecules in the construction of polymer chains. PEG-DiSPA (MW 3400) is a good comonomer for such polymerization. **1** is a multifultionalized β-CD derivative containing two amino, two carboxylates, and 19 hydroxyl groups. The amino groups of **1** ($pK_a = 10.8$ (*31*)) are protonated after purification by ion exchange and are not reactive toward PEG-DiSPA. Nonnucleophilic organic bases, such as DIEA, DMAP, or TEA, whose pK_a's are in a range of 11−12, can deprotonate the amino groups of **1** to render them very nucleophilic and facilitate coupling with the succinimides of PEG-DiSPA (the hydroxyls of the CD are not reactive to succinimide comonomers). Linear, CD-based polymers with M_w's greater than 50 kDa can be easily synthesized using this method. To the best of our knowledge, this is the first synthesis of water-soluble, linear, β-CD polymers with high MW (M_w > 50 kDa) and with pendant functional groups. No cross-linking was observed in these polymers.

As shown in Table 1, the purity of **1** plays an important role in the achievement of high MW polymers. Although there is only ca. 0.5−1 wt % free cystine in acetone-precipitated **1** (Figure 3), the mole percentage is around 3−6% due to its small molecular weight (240 g/mol) compared to **1** (1341 g/mol). Chain termination due to the presence of the impurity is likely one of the reasons lower MW polymers are obtained (Table 1).

As shown in Figure 4a for the polymerization of **1** (methanol-precipitated) with PEG-DiSPA, the M_w of poly(CDDCys-PA-PEG) (**2**) increases to around 80 kDa during the first 2 days of reaction and then slowly increases to ca. 100 kDa after another 3−5 days of reaction. Although the polymer MW should continue to increase with prolonged reaction time in this step-growth polymerization (as long as polymerization is not terminated), polymer aggregation normally occurs in this polymerization system when reaction is carried out for more than one week that can be easily identified since it gives a sharp peak in the gel permeation chromatography (GPC) with a retention time earlier than that of nonaggregated polymer peak (Figure 4b).

In addition to PEG-DiSPA, **1** was polymerized with comonomers that contain different functional groups such as benzotriazolecarbonate (PEG-DiBTC) or nitrophenol-carbonate (PEG-NPC). **1** condenses with these difunctionalized molecules to create linear, CD polymers with the desired connecting bonds (Table 1).

Direct conjugation of CPT to the CDPs **2**−**4** is problematic due to the existence of the hydroxyl groups on the CD rings. Therefore, conjugation of CPT to this type of polymer is performed by converting the 20-OH moiety of CPT into an ester via coupling to an amino acid or peptide linkers (transforms CPT hydroxyl group to a more reactive amino end group), and subsequently grafting the functionalized CPT intermediates onto the polymer backbone at the pendant carboxylate sites. It was also found that CPT lactone form (the antitumor active form) was greatly stabilized when CPT was transformed to its amino acid or oligopeptide derivatives via the ester linkage at the 20-OH site (Figure 5). Therefore, CPT should be maintained in its lactone form after conjugation to the CDP via the amino acid or oligopeptide linkers.

Insertion of linkers between CPT and polymer backbone has the advantage that CPT hydrolysis rates can be adjusted by altering the size and/or nature of the linkers. Although study of CPT conjugates with PEG showed that the introduction of amino acids with bulky side chains such as the leucine or proline can substantially lower hydrolysis rates of CPT in PBS and in plasma (*32*), excellent antitumor efficacy was found in PEG−CPT conjugates with linkers such as glycine or alanine that are known to release CPT with higher hydrolysis rates (*32*). Several recent in vivo studies of polymer−CPT conjugates have used glycine or similar linkers (*27*, *29*, *32*). Therefore, glycine and triglycine were chosen in this study to conjugate CPT to poly(CDDCys-PA-PEG).

To confirm that there was no interchain cross-linking reactions between the hydroxyls of the cyclodextrin and the pendent carboxylate groups under such coupling conditions, **2e** was treated with same coupling reagents and the molecular weight of this polymer was analyzed before and after such treatment. No increase in molecular weight was observed for this polymer after treatment. Solubilities of the polymer−CPT conjugates with the two different linkers have values greater than 200 mg/mL (equivalent to a 12−20 mg of CPT/mL) in aqueous solution. For comparison, the solubility of CPT (lactone form) is 4 μg/mL at the same condition as those for testing the polymer−CPT conjugates. Therefore, the solubilities of the CPT lactone form are increased for more than three orders of magnitude when conjugated to **2e**.

Dramatic, accelerated release of CPT from both **HG6** and **HGGG6** in the presence of plasma was observed (Table 3), and indicates that the release of CPT is enhanced by mechanism other than pure hydrolysis. Similar release rates in the presence of human or rat plasma have been reported by others using PEG-CPT conjugates with a glycine linker (*32*). Although the conjugate with the triglycine linker (**HGGG6**) releases CPT faster than the one with the glycine linker (**HG6**) in PBS (pure hydrolysis), the difference is less in plasma (enzyme-induced release dominates). Release rates of **HG6** and **HGGG6** in mouse or human plasma are essentially the same (Table 3). The release of CPT from both **HG6** and **HGGG6** was also analyzed in the presence of cathepsin B, acetyl cholinesterase and albumin, and none show any dramatic effects on the release rate of CPT.

The CPT hydrolysis from both **HG6** and **HGGG6** decrease substantially in acidic pH (Table 3 and Figure 6). A possible mechanism for uptake of polymer−drug conjugates into tumor cells is via an endocytotic pathway. Conjugates internalized in this manner can be contained in intracellular compartments such as endosomes and lysosomes that have low pH (pH 4−6.5) (*33*). Therefore, the dramatic decrease in CPT release in acidic pH may play a role in the therapeutic efficacy of these conjugates.

CDPs **2**−**4** have backbones that are repeating units of two species (PEG and CD) that are known for their low toxicities and low immunogenecities. Other linear, CD-based polymers have also been reported to have low toxicity and lack an immune response in animals (*8*). Therefore, it is not surprising to observe high IC_{50} values for **2e** in various cultured cells (Table 4).

In summary, β-cyclodextrin-based, linear polymers were synthesized and used for the conjugation of camptothecin. These polymers are extremely soluble in aqueous solution and show low toxicity to cultured cells. When

conjugated to the CD polymers **2e** and **2f**, the solubility of CPT is increased by more than 3 orders of magnitudes. These polymer−CPT conjugates have been tested in a xenograft mouse model and the results will be reported elsewhere (*34*). Initial tumor reduction studies reveal that these conjugates have enhanced efficacy over CPT alone and one of the commercial analogues of CPT (irinotecan). Comparisons to the other polymer conjugates of CPT are difficult since dosing schedules and amounts are not the same. However, Conover et al. dosed mice containing LS174t colon tumors with PEG-CPT conjugates on days 1, 5, and 9 and repeated the dosing schedule every 21 days (*32*). In our study, we dosed mice containing LS174t colon tumors on days 1, 5, and 9 (no repeat dosing) (*34*). The median tumor sizes for the PEG-CPT treated animals were 20−29% of the median size of the tumors in the placebo treated animals when these tumors reached 1000 mg (*32*). By comparison, the cyclodextrin-containing polymer conjugates of CPT gave median tumor sizes that ranged from 10 to 12% of the median size of the tumors in the control group when they reached 1000 mg (*34*). Thus, the conjugates presented here reveal properties that are different from other polymer−CPT conjugates that have been reported previously.

ACKNOWLEDGMENT

We thank Drs. Nathalie Bellocq, Leonard Borrmann, Christopher Dartt, and Suzie Hwang Pun of Insert Therapeutics, Inc., for their helpful discussions. M. E. Davis is a consultant to and has financial interest in Insert Therapeutics, Inc.

LITERATURE CITED

(1) Duncan, R., Dimitrijevic, S., and Evagorou, E. (1996) The role of polymer conjugates in the diagnosis and treatment of cancer. *S. T. P. Pharma. Sci. 6*, 237−263.

(2) Duncan, R., Gac-Breton, S., Keane, R., Musila, R., Sat, Y. N., Satchi, R., and Searle, F. (2001) Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic. *J. Controlled Release 74*, 135−146.

(3) Duncan, R., and Kopecek, J. (1984) Soluble synthetic polymers as potential drug carriers. *Adv. Polym. Sci. 57*, 51−101.

(4) Matsumura, Y., and Maeda, H. (1986) A new concept for macromolecular therapeutics in cancer-chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. *Cancer Res. 6*, 6387−6392.

(5) Uekama, K., Hrayama, F., and Irie, T. (1998) Cyclodextrin drug carrier systems. *Chem. Rev. 98*, 2045−2076.

(6) Fenyvesi, E. (1988) Cyclodextrin polymers in the pharmaceutical-industry. *J. Inclusion Phenom. Macrocyclic Chem. 6*, 537−545.

(7) Tabushi, I., Shimokawa, K., and Fujita, K. (1977) Specific bifunctionalization on cyclodextrin. *Tetrahedron Lett. 18*, 1527−1530.

(8) Hwang, S. J., Bellocq, N. C., and Davis, M. E. (2001) Effects of structure of β-cyclodextrin-containing polymers on gene delivery. *Bioconjugate Chem. 12*, 280−290.

(9) Gonzalez, H., Hwang, S. J., and Davis, M. E. (1999) New class of polymers for the delivery of macromolecular therapeutics. *Bioconjugate Chem. 10*, 1068−1074.

(10) Reineke, T. M., and Davis, M. E. (2003) Structural effects of carbohydrate-containing polycations on gene delivery. 1. Carbohydrate size and its distance from charge centers. *Bioconjugate Chem. 14*, 247−254.

(11) Reineke, T. M., and Davis, M. E. (2003) Structural effects of carbohydrate-containing polycations on gene delivery. 2. Charge center type. *Bioconjugate Chem. 14*, 255−261.

(12) Popielarski, S. R., Mishra, S., and Davis, M. E. (2003) Structural effects of carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization. *Bioconjugate Chem. 14*, 672−678.

(13) Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhail, A. T., and Sim, G. A. (1966) Plant antitumor agents. I. Isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminata. *J. Am. Chem. Soc. 88*, 3888−3890.

(14) Muggia, F. M., Dimery, I., and Arbuck, S. G. (1996) Camptothecin and its analogs. *Ann. NY Acad. Sci. 803*, 213−223.

(15) Rothenberg, M. L. (1997) Topoisomerase I injibitors: review and update. *Ann. Oncol. 9*, 837−855.

(16) Hertzberg, R. P., Caranfa, M. J., and Hecht, S. M. (1989) On the mechanism of topoisomerase-I inhibition by camptothecin-evidence for binding to an enzyme DNA complex. *Biochemistry 28*, 4629−4638.

(17) Mi, Z., and Burke, T. G. (1994) Marked interspecies variations concerning the interactions of camptothecin with serum albumins: a frequency-domain fluorescence spectroscopic study. *Biochemistry 33*, 12540−12545.

(18) Mi, Z., and Burke, T. G. (1994) Differential interactions of camptothecin lactone and carboxylate forms with human blood components. *Biochemistry 33*, 10325−10336.

(19) ten Bokkel Huinink, W. W., Gore, M., and Carmichael, J. (1997) Topotecan versus paclitaxel for the treatment of recurrent epithelial ovarian cancer. *J. Clin. Oncol. 15*, 2183−2193.

(20) Van Cutsem, M., Cunningham, D., and ten Bokkel Huinink, W. W. (1999) Clinical activity and benefit of irinotecan (CPT-11) in patients with colotectal cancer truly resistant to 5-fluorouracil (5-FU). *Eur. J. Cancer 35*, 54−59.

(21) Zhao, H., Lee, C., Sai, P., Choe, Y., Boro, M., Pentri, A., Guan, S., and Greenwald, R. B. (2000) 20-*O*-Acylcamtothecin derivatives: evidence for lactone stabilization. *J. Org. Chem. 65*, 4601−4606.

(22) Greenwald, R. B., Pendri, A., Conover, C. D., Gilbert, C. W., Yang, R., and Xia, J. (1996) Drug delivery systems. 2. Camptothecin 20-O-poly(ethylene glycol) ester transport forms. *J. Med. Chem. 39*, 1938−1940.

(23) Greenwald, R. B., Pendri, A., Conover, C. D., Lee, C., Choe, Y. H., Gilbert, C., Martinez, A., Xia, J., Wu, D. C., and Hsue, M. (1998) Camptothecin-20-PEG ester transport forms: the effect of spacer groups on antitumor activity. *Bioorg. Med. Chem. 6*, 551−562.

(24) Rowinsky, E. K., Rizzo, J., Ochoa, L., Takimoto, C. H., Forouzesh, B., Schwartz, G., Hammond, L. A., Patnaik, A., Kwiatek, J., Goetz, A., Denis, L., McGuire, J., and Tolcher, A. W. (2003) A phase I and pharmacokinetic study of pegylated camptothecin as a 1-hour infusion every 3 weeks in patients with advanced solid malignancies. *J. Clin. Oncol. 21*, 148−157.

(25) Zamai, M., vandeVen, M., Farao, M., Gratton, E., Ghiglieri, A., Castelli, M. G., Fontana, E., d'Argy, R., Fiorino, A., Pesenti, E., Suarato, A., and Caiolfa, V. R. (2003) Camptothecin poly[N−(2-hydroxypropyl) methacrylamide] copolymers in antitopoisomerase-I tumor therapy: intratumor release and antitumor efficacy. *Mol. Cancer Ther. 2*, 29−40.

(26) Caiolfa, V. R., Zamai, M., Fiorino, A., Frigerio, E., Pellizzoni, C., d'Argy, R., Ghiglieri, A., Castelli, M. G., Farao, M., Pesenti, E., Gogli, M., Angelucci, F., and Suarato, A. (2000) Polymer-bound camptothecin: initial biodistribution and antitumour activity studies. *J. Controlled Release 65*, 105−119.

(27) Bhatt, R., Vries, P. D., Tulinsky, J., Bellamy, G., Baker, B., Singer, J. W., and Klein, P. (2003) Synthesis and in vivo antitumor activity of poly(L-glutamic acid) conjugates of 20-(S)-camptothecin. *J. Med. Chem. 46*, 190−193.

(28) Zou, Y., Wu, Q., Tansey, W., Chow, D., Hung, M., Charnsangavej, C., Wallace, S., and Li, C. (2001) Effectiveness of water soluble poly(L-glutamic acid)-camptothecin conjugate against resistant human lung cancer xenografted in nude mice. *Int. J. Oncol. 18*, 331−336.

(29) Singer, J. W., Bhatt, R., Tulinsky, J., Buhler, K. R., Heasley, E., Klein, P., and Vries, P. D. (2001) Water-soluble poly-(L-glutamic acid)-gly camptothecin conjugates enhance

camptothecin stability and efficacy in vivo. *J. Controlled Release 74*, 243−247.
(30) Mach, L., Mort, J. S., and Glossl, J. (1994) Maturation of human procathepsin B. Proenzyme activation and proteolytic processing of the precursor to the mature proteinase, in vitro, are primarily unimolecular processes. *J. Biol. Chem. 269*, 13030−13035.
(31) Stryer, L. (1998) *Biochemistry* (Fourth ed.), p 43, W. H. Freeman and Company, New York.
(32) Conover, C. D., Greenwald, R. B., Pendri, A., and Shum, K. L. (1999) Camptothecin delivery systems: the utility of amino acid spacers for the conjugation of camptothecin with poly(ethylene glycol) to create prodrugs. *Anti-Cancer Drug Des. 14*, 499−506.
(33) Duncan, R. (2003) The dawning era of polymer therapeutics. *Nature Rev. Drug Discov. 2*, 347−360.
(34) Cheng, J., Khin, K. T., and Davis, M. E. Antitumor activity of β-cyclodextrin polymer-camptothecin conjugates. *Cancer Res.*, submitted.

BC0340924