Original Article

Novel poly-β-cyclodextrin derivatives as advanced carriers for 5-fluorouracil for tumor: the impact of charge on antitumor efficiency

Hailang Li[1], Shanshan Zhu[1], Li Xu[1], Yupei Chen[2], Xin Li[3], Wenlin Wu[4]

[1]Department of Pharmacy, Xiamen Medical College of Fujian Province, Xiamen, China; [2]Engineering Research Center of Natural Cosmeceuticals College of Fujian Province, Xiamen Medical College, Xiamen, China; [3]Shanghai Serum-China Bio-Tech. Co., Ltd., Shanghai, China; [4]College of Oceanology and Food Science, Quanzhou Normal University, Quanzhou, China

Contributions: (I) Conception and design: H Li, W Wu; (II) Administrative support: H Li, S Zhu; (III) Provision of study materials or patients: L Xu; (IV) Collection and assembly of data: H Li, S Zhu; (V) Data analysis and interpretation: H Li, X Li, W Wu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Wenlin Wu. College of Oceanology and Food Science, Quanzhou Normal University, Quanzhou 362000, China.
Email: wenlinw@163.com.

Background: With the emergence of more and more cyclodextrin derivatives, cyclodextrin becomes an effective adjuvant for improving the prescription of drugs. Its application in pharmacy, especially in the sustained and controlled release, targeting, transdermal and mucosal drug delivery systems, is also being expanded and deepened. In this study, novel cyclodextrin derivatives were developed to investigate the impact of the charge on antitumor efficiency by introducing different groups (carboxymethyl or quaternary ammonium group) to poly-β-cyclodextrin (β-CD).

Methods: These novel β-CD derivatives were prepared by the nucleophilic substitution reaction and characterized by IR and ^{1}H NMR. Fluorouracil (5-FU) was adopted as a model drug to form inclusion compounds. The content of 5-FU in inclusion compounds was evaluated using fluorine element analysis. Also, the cytotoxicity of poly-β-CD derivatives was studied. Finally, the effect of negative and positive charges on the antitumor activity of poly-β-CD derivatives-5-FU inclusion compounds on HepG2 cancer cells was evaluated. Human liver cancer HepG2 cells (CYP3A4G/7R clone 87, RRID: CVCL_1×10) were purchased from Cell Bank, Shanghai Institutes for Biological Sciences (China).

Results: The results of IR and 1H NMR indicated consistently that both carboxymethyl poly-β-CD (poly-CM-β-CD) and glycidyl trimethyl ammonium chloride (GTMAC) poly-β-CD (poly-GTAC-β-CD) were successfully prepared. Fluorouracil was successfully loaded into poly-β-CD derivatives. The results of fluorine analysis indicated that the content of 5-FU in 1 g poly-β-CD, poly-GTAC-β-CD and poly-CM-β-CD was 1,214, 921 and 1,187 μg, respectively. No cytotoxicity of poly-β-CD derivatives on HepG2 cells was observed. The killing effect of poly-β-CD-5-FU on HepG2 cells was similar to that of poly-GTAC-β-CD-5-FU. Poly-CM-β-CD-5-FU had the worst killing effect on HepG2 cells.

Conclusions: Charge had impact on antitumor efficiency. These novel poly-β-CD derivatives have potential applications in tumor sustained-release targeted therapy.

Keywords: Poly-β-cyclodextrin (β-CD); different groups; advanced carriers; 5-fluorouracil (5-FU); tumor

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Introduction

Cancer is regarded as one of the main problems threatening the health of humanity. The percentage of the reported patient numbers was expected to be increased by 75% by the next two decades (1). Liver cancer is the most obstinate malignant tumors. This enlightened researchers on developing low cost, efficient, and safe therapies for cancer or taking advantage of new technologies to improve the potential of the currently used drugs (2). 5-fluorouracil (5-FU), commonly used anticancer drug, has an efficient effect on active cancers (3). However, 5-FU drugs can not only kill the cancer cells but also affect the normal cells, causing several negative impacts in the course of treatment (4). Furthermore, an overdose of 5-FU can result in impair the hematological, neural, cardiac, gastrointestinal tract as well as the dermatological reactions (1). Thus, it was a requisite for developing a novel carrier to control the drug release in the specific lesion, and minimize its side effect as far as possible (5). To achieve this goal, numerous materials, such as bentonite, biodegradable polymers, chitosan, alginate, and cellulose, etc., were inspected and evaluated (6). Polymeric nano-carriers which were biocompatible, nontoxic, and stable in the biological systems were promising drug carriers with high encapsulation and controlled release properties (7).

β-cyclodextrin (β-CD), a known natural polymer with size in nanoscale, is composed of seven α-D units of glucose which linked to echo with α-1,4-glucose bonds (8). The central cavities of β-CD are lipophilic and its outer surface is hydrophilic. These properties make β-CD interact with lipophilic guest molecules, forming a host-guest inclusion compound by self-assembly (9). It was reported that β-CD can be used as excipient materials, improving the stability and oral bioavailability of the drug (10). The notable feature of cyclodextrins is their ability to form solid inclusion host-guest complexes with a wide range of solid, liquid, and gaseous compounds by a molecular complexation (11). The driving force for the formation of an inclusion compound was mainly from the release of water molecules in the cavity of cyclodextrin. It was difficult to fully form a hydrogen bond between water molecules in the hydrophobic cavity of cyclodextrin. Nevertheless, the potential of water molecules to form hydrogen bonds was not fully released. Therefore, water molecules in the hydrophobic cavity of cyclodextrin had considerable enthalpy. When these high-energy water molecules were replaced by suitable guest molecules with smaller polarity and released from the hydrophobic cavity of cyclodextrin, the energy of these systems would be reduced, facilitating the formation of inclusion compounds. During the formation of inclusion compounds, the removal of high energy water molecules from the hydrophobic cavity of cyclodextrins was the main driving force. Additionally, the driving force also included van der Waals force (12), hydrogen bond interaction (13), hydrophobic interaction (14), non-classical hydrophobic interaction, the reduction of ring tension in the formation of inclusion compounds and the decrease of surface tension of solvent (15). It was also documented that integrating or grafting the hydrophilic monomer with β-CD can increase its complexation efficiency with the drugs and decrease the cost of β-CD polymer (16). Additionally, it was reported that polymerizing β-CD with other polymers can increase its solubility and bioavailability (17). This advantage can result in the pro-apoptosis of melanoma cells by using the complexation of harman, a natural β-carboline alkaloid, with β-CD (18). On the other hand, the curcumin, a natural polyphenol, with β-CD and γ-CD showed superior in antioxidant, antibacterial, and anticancer activities (19). However, the biggest problem of cyclodextrin drug carrier is its low drug loading efficiency (20).

It was demonstrated that the gap of tumor vascular endothelial cells is 380–780 nm (21). This abnormal permeability of tumor vessels makes the permeability of macromolecular and nanoparticles increase and lymphatic clearance reduce. However, there is no difference in the distribution of small molecule drugs between tumor tissues and normal tissues. The difference of permeability between tumor tissues and normal tissues makes macromolecule and nanoparticles aggregate in tumor tissue, which is called enhanced permeability and retention (EPR). EPR is the basis of tumor-targeting for nanoparticles (22). The cell membrane is composed of the phospholipid bilayer and the surfaces of the cell are negatively charged. Therefore, it was expected that the introduction of carboxymethyl or quaternary ammonium group to β-CD resulted in a novel β-CD derivatives, which can increase the solubility of 5-FU by facilitating its loading and enhance the potential of 5-FU for inhibiting the tumor cells by boosting the combination of β-CD inclusion compounds to tumor cell.

In this study, the novel β-CD derivatives by grafting different groups (carboxymethyl or quaternary ammonium group) to poly-β-CD were developed as delivery systems for the 5-FU drug and could be applied in the liver cancer therapy. The novel poly-β-CD was characterized by IR and ^{1}H NMR. 5-FU was adopted as a model drug to form an

inclusion compound. The content of 5-FU in poly-β-CD derivatives was evaluated using fluorine element analysis. The self-assembly and its mechanism were explored in detail. Also, the cytotoxicity of poly-β-CD derivatives was analyzed. Finally, the potential of poly-β-CD derivatives-5-FU inclusion compounds for HepG2 cancer cells was evaluated.

Methods

Materials

Poly-β-cyclodextrin (poly-β-CD) (Mw, 5,000–10,000 Da) was provided by Shandong Binzhou Zhiyuan Biotechnology Co., Ltd. (Shandong, China). 5-FU, glycidol trimethyl ammonium chloride, dimethyl sulfoxide (DMSO), bromoacetic acid, cell culture media and supplements, fetal bovine serum (FBS), and alamar blues were purchased from Sigma-Aldrich (Shanghai, China). Dialysis tubing with an Mw cut-off of 1,500–2,000 Da was purchased from Spectrum Laboratories (Miami, FL, USA). Unless stated otherwise, all reagents and solvents were commercially available analytic grade reagents and were used without further purification.

Preparation of glycidyl trimethyl ammonium chloride (GTMAC) poly-β-CD (poly-GTAC-β-CD) and carboxymethyl poly-β-CD (poly-CM-CD)

To prepare poly-GTAC-β-CD, 0.01 mole GTMAC and 0.01 mole poly-β-CD were mixed in a 50 mL round-bottom flask. On the hand, 0.01 mole bromoacetic acid and 0.01 mole poly-β-CD were mixed for poly-CM-β-CD. Ten milliliters DMSO was added into the mixture and stirred. To remove the DMS and residual small molecular compound, the dialysis membrane of Spectra/Por 1,500–2,000 Da was utilized. The resultant product was collected by lyophilization.

Fourier transform IR spectra

Fourier transform IR spectra of poly-GTAC-β-CD and poly-CM-β-CD were measured over 4,000–400 cm^{-1} on a Perkin-Elmer Spectrum 2000 instrument (Perkin Elmer, Boston, MA, USA) with KBr sample pellets.

^{1}H NMR spectra

Both poly-GTAC-β-CD and poly-CM-β-CD structures were further confirmed by ^{1}H NMR. The ^{1}H NMR spectra were recorded in D_2O on a Bruker AC 200P, 200 MHz spectrometer (Bruker Corporation, Rheinstetten, Germany), using tetramethylsilane as the internal standard.

The preparation of inclusion complexes

Four-gram poly-GTAC-β-CD and poly-CM-β-CD were respectively added into 10 mL DMSO and 1 g 5-FU in 50 mL round-bottom flask. The mixture was stirred with reflux at 80 ℃ for 72 h. The DMSO and residual small molecular compound were removed by dialysis for 72 hours using the dialysis membrane of Spectra/Por 1,500–2,000 Da. The resultant product was collected by lyophilization.

Determination of the 5-FU loading content in poly-CM-β-CD and poly-GTAC-β-CD

The content of 5-FU in poly-GTAC-β-CD or poly-CM-β-CD was evaluated using fluorine element analysis. Briefly, a 100 mg sample was wrapped in ashless paper and placed in a 500 mL oxygen flask containing 5 mL absorbing liquid for combustion. Fluoride in the resultant absorbing liquid was separated using IonPac AS14-AG14 (Dionex, Sunnyvale, CA, USA) as a separating column and rinsing with a solution containing 0.001 M $NaHCO_3$ and 0.0035 M Na_2CO_3. The electric conductivity was detected.

Cell culture

Human liver cancer HepG2 cells (CYP3A4G/7R clone 87, RRID: CVCL_1×10) were purchased from Cell Bank, Shanghai Institutes for Biological Sciences (China). HepG2 was cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 1.0 mM sodium pyruvate, 0.1 mM unessential amino acid and 1.5 g/L $NaHCO_3$. All cells were cultured in a fully humidified atmosphere containing 5% CO_2 at 37 ℃.

In vitro cytotoxicity assay of poly-CM-β-CD and poly-GTAC-β-CD

HepG2 cell lines were seeded in a 24-well plate at a density of 5.0×10^4 cell/mL and incubated overnight at 37 ℃ and 5% CO_2 to attain subconfluence before treating with poly-CM-β-CD/poly-GTAC-β-CD at various concentrations. After two days post-incubation, cells in each well were exposed to 0.4 mL 2% crystal violet in 20% methanol for 30 min

at room temperature and rinsed with distilled water in preparation for image analysis.

Evaluation of the potential of poly-CM-β-CD-5-FU/poly-GTAC-β-CD-5-FU inclusion compounds for HepG2 cancer cells

The potential of poly-CM-β-CD-5-FU/poly-GTAC-β-CD-5-FU inclusion compounds was evaluated using HepG2 cells and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 μL cell culture medium and incubated overnight to obtain 75–80% confluency. The culture medium was then replaced with fresh, serum free medium, and a serial sample of poly-CM-β-CD-5-FU/poly-GTAC-β-CD-5-FU inclusion compound was added to the cells. Cells were incubated with poly-CM-β-CD-5-FU/poly-GTAC-β-CD-5-FU inclusion compound at a 5-FU concentration of 5 μg/mL for the originally seeded cells at 37 ℃. Then, cells were incubated for 24 h. A total of 10 μL MTT solution (25 μg/mL) was added to the 100 μL of culture medium in each well before incubation at 37 ℃ for 4 h. The MTT-containing medium was replaced with 100 μL solubilization solution DMSO. Finally, the absorbance was measured at 595 nm using an ELISA plate reader (Thermo Fisher, Waltham, MA, USA) with a reference filter of 650 nm. The viability of non-treated control cells was arbitrarily defined as 100%. The experiment was repeated four times for each sample treatment. Cell viability (%) was calculated according to the following Eq. [1]:

$$\frac{OD_{595}(sample)-OD_{650}(sample)}{OD_{595}(control)-OD_{650}(control)}\times100 \quad [1]$$

where OD_{595}(sample) and OD_{650}(sample) represent measurements from the wells treated with poly-CM-β-CD-5-FU/poly-GTAC-β-CD-5-FU inclusion compounds and OD_{595}(control) and OD_{650}(control) represent measurements from the wells treated with only DMEM containing 10% fetal calf serum.

Statistical analysis

All experiments were repeated four times and measurements were collected in quadruplicate. Data are expressed as the mean ± standard deviation based on four measurements. Statistical analysis was performed using Student's *t*-test. $P<0.005$ was considered to indicate a statistically significant difference.

Results

^{1}H spectrum of poly-GTAC-β-CD and poly-CM-β-CD

The successful synthesis of poly-GTAC-β-CD and poly-CM-β-CD was confirmed by ^{1}H NMR spectra. Typical ^{1}H spectra of poly-GTAC-β-CD and poly-CM-β-CD were showed in *Figures 1* and *2*, respectively. Chemical shifts and corresponding protons were analyzed in *Table 1*.

IR spectroscopy of poly-GTAC-β-CD and poly-CM-β-CD

To further confirm the formation of poly-GTAC-β-CD and poly-CM-β-CD, IR spectroscopy of poly-GTAC-β-CD and poly-CM-β-CD was performed. The FT-IR spectrum of poly-β-CD, poly-GTAC-β-CD, and poly-CM-β-CD, with or without reaction, was illustrated in *Figure 3*. According to the *Table 2*, it was indicated that the FT-IR spectrum of poly-GTAC-β-CD and poly-CM-β-CD can reappear the characteristic absorption peaks of 2,3-epoxypropyltrimeth ylammonium chloride and Br-CH_2COOH. These results were consistent with the expected chemical structures poly-GTAC-β-CD and poly-CM-β-CD.

Determination of 5-FU content in poly-GTAC-β-CD/poly-CM-β-CD

To determine the percentage of 5-FU loaded in poly-GTAC-β-CD/poy-CM-β-CD, the fluorine element analysis was conducted following freeze-drying of the conjugate. The result indicated that the content of 5-FU in 1 g poly-β-CD, poly-GTAC-β-CD, and poly-CM-β-CD was 1,214, 921 and 1,187 μg, respectively.

In vitro cytotoxicity of poly-CM-β-CD and poly-GTAC-β-CD

For the concerns of efficient drug delivery, biocompatibility and cytotoxicity of poly-GTAC-β-CD and poly-CM-β-CD, HepG2 cell lines were selected for the *in vitro* cytotoxicity analysis and incubated with poly-GTAC-β-CD or poly-CM-β-CD for 72 h. Crystal violet stain was used to assay cell viabilities in the presence of poly-GTAC-β-CD or poly-CM-β-CD, and phosphate buffered saline was utilized as the control. As illustrated in *Figure 4*, *Figure 4A1*,A2,*B1* and *B2*, for poly-CM-β-CD and poly-GTAC-β-CD,

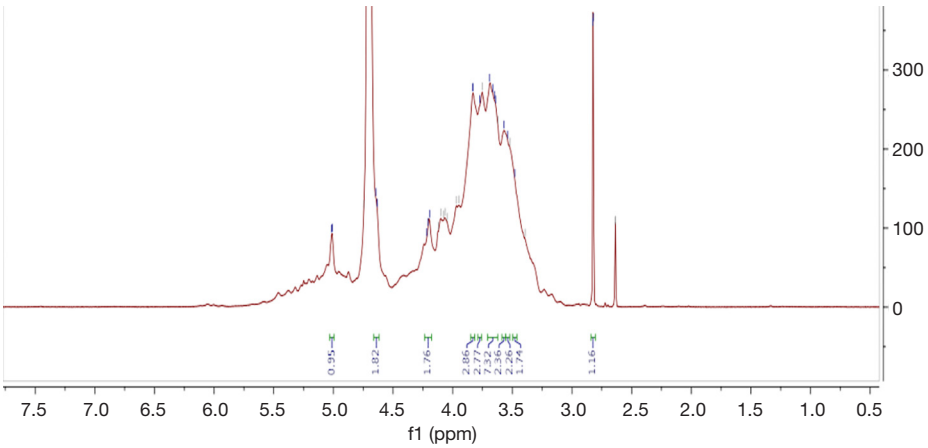

Figure 1 The ^{1}H-NMR spectra of poly-GTAC-β-CD.

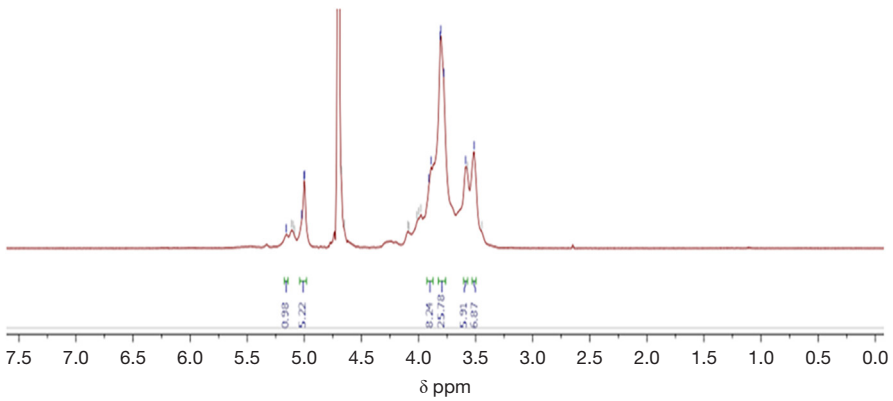

Figure 2 The ^{1}H-NMR spectra of poly-CM-β-CD.

Table 1 The chemical shifts of poly-GTAC-β-CD as well as poly-CM-β-CD and its corresponding protons

Chemical shift δ_H/ppm	Annotation
5.76–5.60	OH-2, OH-3 of poly-β-CD
4.87–4.76	OH-1 of poly-β-CD
4.52–4.36	OH-6 of poly-β-CD
4.139–4.1408	-O-CH_2-COOH
9.0–12.0	-COOH
3.3001–3.3008	-N^+ (CH_3)

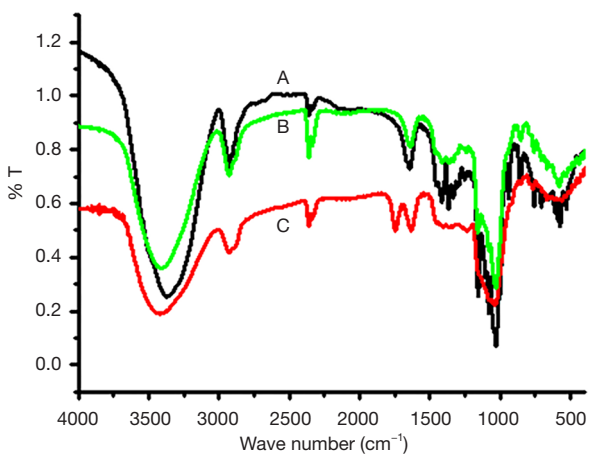

Figure 3 The IR spectra of (A) poly-β-cyclodextrin, (B) poly-GTAC-β-CD and (C) poly-CM-β-CD.

Table 2 The wave number of poly-GTAC-β-CD as well as poly-CM-β-CD and its corresponding IR vibration

Wave number/cm^{-1}	Annotation
1,770–1,750	the C=O stretching vibration of -COOH
1,190–1,001	C-O-C stretching vibration of ether in β-CD, demonstrating that FA binds chemically to poly-β-CD
3,500–3,250	O-H stretch
1282.6	O-H deflection
2,960–2,850	the stretching vibration of C-H
1,380	the δ_{C-H} of CH_3
1,200–1,050	the stretching vibration of C-O

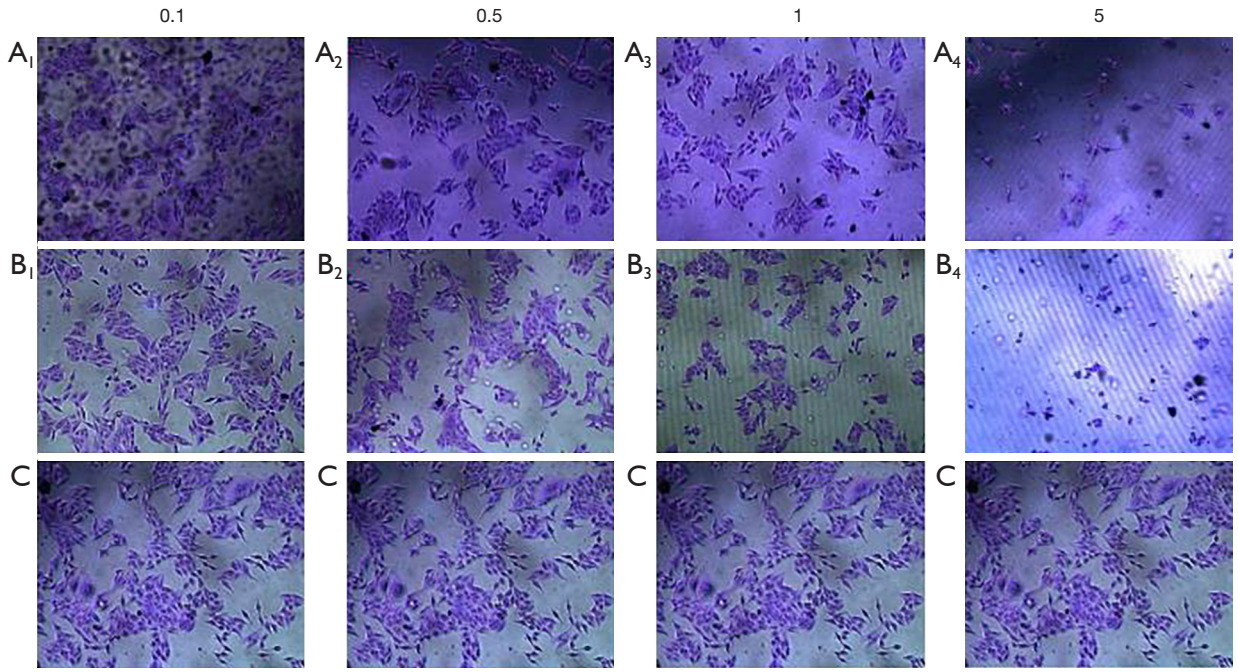

Figure 4 The cytopathic effect of poly-β-cyclodextrin, poly-CM-β-cyclodextrin and poly-GTAC-β-cyclodextrin on tumor cell HepG2. (A1, A2, A3 and A4) Represented the cytopathic effect of poly-CM-β-cyclodextrin. (B1, B2, B3 and B4) Represented the cytopathic effect of poly-GTAC-β-cyclodextrin. (C) was a blank control experiment. Tumor cell lines were seeded in 24-well plates at a density of 5×10^4 cells for each well and incubated with poly-β-cyclodextrin, poly-CM-β-cyclodextrin or poly-GTAC-β-cyclodextrin at the indicated concentration (mg/mL). After 72 h incubation, cells in each well were exposed to 0.4 mL 2% crystal violet in 20% methanol for 30 min at room temperature and rinsed with distilled water in preparation for image analysis. magnification 100×.

HepG2 cell viabilities were ~100% which indicated that no cytotoxicity of poly-GTAC-β-CD and poly-CM-β-CD at the concentration of 100–500 μg/mL was found. The results were consistent with the previous report, demonstrating that cyclodextrin exhibited no toxicity *in vitro* (23) and *in vivo* (24). However, poly-CM-β-CD and poly-GTAC-β-CD exhibited a slightly inhibitive effect on HepG2 cell lines at a concentration of 1,000 μg/mL.

Evaluation the potential of poly-GTAC-β-CD-5-FU/poly-5-FU inclusion compound for HepG2 cancer cells

To determine the impact of negative and positive charges of inclusion compound on its antitumor efficient, the potential of poly-β-CD-5-FU, poly-GTAC-β-CD-5-FU, and poly-5-FU inclusion compound for HepG2 cancer cells were investigated by MTT assay. The phosphate-buffered saline

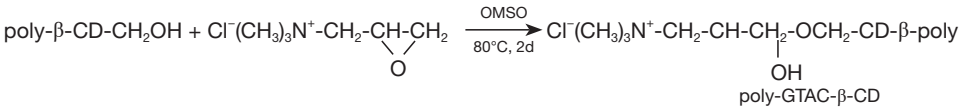

Figure 5 The preparation route and schematic diagram of poly-GTAC-β-CD.

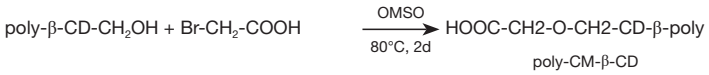

Figure 6 The preparation route and schematic diagram of poly-CM-β-CD.

was utilized as control. *Figure 4* indicated that inclusion of model drug 5-FU into poly-GTAC-β-CD or poly-CM-β-CD had no impact on the potential of model drug 5-FU for HepG2 cells and there was a significant difference in cytotoxicity among poly-β-CD-5-FU, poly-GTAC-β-CD-5-FU and poly-CM-β-CD inclusion compound.

Discussion

Preparation Principle of poly-GTAM-β-CD

The preparation route for poly-GTAC-β-CD is shown in the schematic diagram of *Figure 5*.

There was an ethylene oxide structural unit in GTMAC. Because of the large tension of the ternary ring, GTMAC was in high chemical activity, which can react with hydroxyl groups on poly-β-CD. There were primary hydroxyl and secondary hydroxyl groups in poly-β-CD but the chemical activity of primary hydroxyl was larger than that of secondary hydroxyl. Therefore, the groups of poly-β-CD which reacted with GTMAC were mainly primary hydroxyl. The ring-opening reaction between hydroxyl and ethylene oxide can be regarded as a special nucleophilic substitution reaction. GTMAC was soluble in H_2O and DMSO. However, poly-β-CD was poorly soluble in water and well soluble in DMSO. Both GTMAC and poly-β-CD were polar compounds and the polar aprotic solvent is favorable for nucleophilic substitution reaction. Therefore, DMSO was used as a solvent in this reaction.

Preparation Principle of poly-CM-β-CD

The preparation route for poly-CM-β-CD is shown in the schematic diagram of *Figure 6*. The reaction of hydroxyl with dibromoacetic acid was belonging to the nucleophilic substitution reaction. Bromoacetic acid was well soluble in H_2O or DMSO. But poly-β-CD was insoluble in H_2O instead. Furthermore, due to hydrogen bonding, water molecules can form a hydration shell around the hydroxyl group on poly-β-CD, blocking the substitution reaction of hydroxyl with bromoacetic acid. Because the polar characteristic of GTMAC and poly-β-CD was observed, DMSO was utilized as a solvent in this reaction.

The analysis by ^{1}H NMR and IR spectroscopy of poly-CM-β-CD and poly-GTAC-β-CD

For the introduction of the quaternary ammonium group into β-CD, 2,3-epoxypropyl trimethylammonium chloride (Mw 152 Da) was selected as a reactant which reacted with methylol groups in β-CD, providing a physiologically stable ether bond. There were hydroxyl groups and methylol groups in β-CDs. The nucleophilic ability of methylol was stronger than that of hydroxyl. Therefore, 3-epoxypropyltrimethylammonium chloride was selected to favor the linkage of the methylol, resulting in poly-GTAC-β-CD. To introduce carboxymethyl into β-CD, bromoacetic acid (Mw 139 Da) was used as a reactant which reacted with methylol groups of β-CD, providing a physiologically stable ether bond. There were hydroxyl groups and methylol groups in β-CDs. The nucleophilic ability of methylol was stronger than that of hydroxyl. Therefore, bromoacetic acid was selected to favor the linkage of the methylol, resulting in poly-CM-β-CD. All the unwanted residual and free reactants were removed by dialysis in later step. The ^{1}H NMR spectrum of poly-GTAC-β-CD showed all the characterization peaks of 2,3-epoxypropyltrimethylammonium chloride, and poly-β-CD, demonstrating that 2,3-epoxypropyltrimethylammonium chloride bound chemically

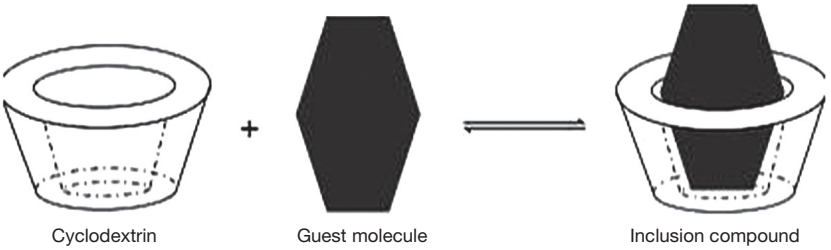

Figure 7 The schematic diagram for the formation of cyclodextrin-5-FU inclusion compound.

to poly-β-CD through an ether linkage. By the same token, the ^{1}H NMR spectrum of poly-CM-β-CD showed all the characterization peaks of Br-CH_2COOH and poly-β-CD, suggesting that Br-CH_2COOH bound chemically to poly-β-CD through an ether linkage. The relevant signals of 2,3-epoxypropyltrimethylammonium chloride, and Br-CH_2COOH were too weak to be observed in ^{1}H NMR because there were overwhelming majority protons of poly-β-CD than those of 2,3-epoxypropyltrimethylammonium chloride and Br-CH_2COOH.

Preparation principle of inclusion compound

Figure 7 is a schematic diagram for the principle of inclusion compound. The lipophilic cavity of cyclodextrin molecules provided a microenvironment into which appropriately sized non-polar 5-FU can enter and form β-CD-5-FU inclusion complexes (25). The hydrophobic drug 5-FU could induce the β-CD conjugates to self-assemble and form nanoparticles (26). The main driving force for the formation of β-CD-5-FU inclusion complex was the release of enthalpy-rich water molecules from the hydrophobic cavity. Water molecules were displaced by the highly hydrophobic 5-FU molecules presenting in the solution to attain an apolar-apolar association and decrease of cyclodextrin ring strain, resulting in a stable lower energy state (27). Water was the most commonly used solvent in which complexation reactions were performed. The more soluble β-CD existed in the solvent, the more molecules became available for complexation. poly-CM-β-CD and poly-GTAC-β-CD are well soluble in water but 5-FU is poorly soluble. However, poly-CM-β-CD, poly-GTAC-β-CD and 5-FU are all well soluble in DMSO. Therefore, DMSO was used as a solvent in this reaction to improve the formation of inclusion host-guest complexes.

Cytotoxicity and uptake by tumor cells

In vitro cytotoxicity of poly-CM-β-CD and poly-GTAC-β-CD

With the increase of the concentration, the osmotic pressure of the solution would increase accordingly, which had a certain impact on cell growth. With the increase of the concentration of poly-CM-β-CD or poly-GTAC-β-CD, HepG2 cell viabilities were seriously affected. In conclusion, the low concentration of poly-GTAC-β-CD and poly-CM-β-CD did not affect HepG2 cell growth, while the high concentration of poly-GTAC-β-CD or poly-CM-β-CD had an obvious effect on HepG2 cell growth. These results demonstrated that poly-GTAC-β-CD and poly-CM-β-CD can be used as drug carriers.

Evaluation the potential of poly-GTAC-β-CD-5-FU/ poly-CM-β-CD-5-FU inclusion compound for HepG2 cancer cells

Significant difference in cytotoxicity among poly-β-CD-5-FU, poly-GTAC-β-CD-5-FU and poly-CM-β-CD inclusion compound was observed in *Figure 8*. These results may be attributed to the involvement of charge in HepG2 cellular association and endocytosis of inclusion compounds. Zelepukin *et al.* reported that the effect of charge was a crucial factor when testing over eight NP formulations with different surface characteristics (28). The cell membrane is composed of the phospholipid bilayer with the negative charge on its surface. Thus, poly-β-CD-5-FU can be swallowed by HepG2 cells through the weak electrostatic attraction hydrogen bond between hydroxyl and the negative charge on the cell membrane surface. The quaternary ammonium groups on poly-GTAC-β-CD-5-FU were positively charged (Zeta potential +40.3 mV), which can form a strong electrostatic attraction with the negative charged on the HepG2 membrane. Consequently, poly-GTAC-β-CD-5-FU was more easily swallowed by HepG2 cells. However, the large steric hindrance

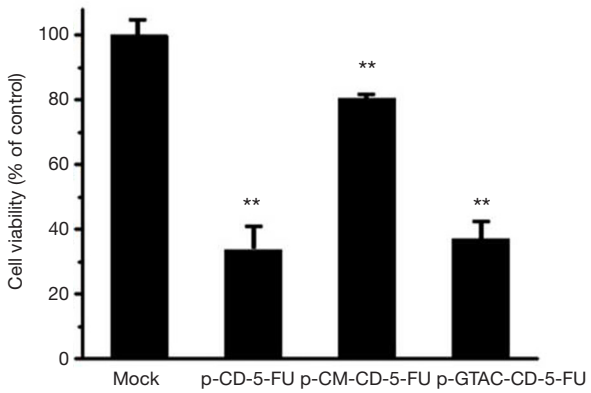

Figure 8 Cytotoxicity of poly-β-cyclodextrin loaded 5-FU, poly-GTAC-β-cyclodextrin loaded 5-FU, and poly-CM-β-cyclodextrin loaded 5-FU. HepG2 cells were incubated with poly-β-cyclodextrin loaded 5-FU, poly-GTAC-β-cyclodextrin loaded 5-FU and poly-CM-β-cyclodextrin loaded 5-FU at a 5-FU concentration of 5 μg/mL. After 24 h incubation, the survival ratios of HePG2 cells were determinate by MTT assay. Results were expressed as a relative percentage to untreated control HepG2 cells and represented the mean of four repetitive experiments. Significance is indicated by **, P<0.005.

of the quaternary ammonium group blocked the endocytosis of poly-GTAC-β-CD-5-FU. Therefore, the killing effect of poly-β-CD-5-FU on HePG2 was similar to that of poly-GTAC-β-CD-5-FU. For poly-CM-β-CD-5-FU, the dissociated carboxyl was negatively charged (Zeta potential –56.3 mV), which can form electrostatic repulsion force with the negative charge on the HepG2 membrane surface. This made poly-CM-β-CD-5-FU difficult to approach cells and block the endocytosis of poly-CM-β-CD. Hence, poly-CM-β-CD-5-FU had the worst killing effect on HePG2 cells. These results were consistent with the result reported by Frohlich (29). Charge is a key determinant of cellular localization, where highly positively charged NPs tend to show higher cellular uptake compared to negatively charged particles. It is worth pointing out that this high rate of accumulation also leads to increased non-specific binding to normal cells, and to cytotoxicity combined with a short half-life. In contrast, negatively charged NPs have very limited uptake in cells (29).

Conclusions

The current study demonstrated that the killing effect of poly-β-CD-5-FU and poly-GTAC-β-CD-5-FU on HePG2 cells was similar. By contrast, poly-β-CD-5-FU revealed the worst killing effect on HePG2 cells. The feasibility of using poly-β-CD derivatives *t* deliver 5-FU was confirmed. β-CD derivatives poly-GTAC-β-CD and poly-CM-β-CD had no cytotoxicity. The formation of inclusion complex between 5-FU and poly-GTAC-β-CD or poly-CM-β-CD did not affect the antitumor activity of 5-FU. Charge of nanoparticles played an important role in their cellular uptake. Poly-β-CD derivatives have potential applications in sustained release and targeted drugs for tumor. Further studies in progress will study this novel poly-β-CD derivatives drug delivery system *in vivo*.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-1118). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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