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Preparation and Characterization of Targeted Drug Carrier Materials Poly-β-Cyclodextrin-PEG-Folate Acid

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Abstract: *Objective:* Targeted drug delivery can concentrate drugs at the lesion site and selectively kill lesion cells. Folate receptors have limited expression in human healthy cells but are over-expressing on the surface of cancer cells. Our study decided to develop one of site-specific intracellular delivery against the folate receptor. *Methods:* First, poly β-CD-PEG-OCH_3 and poly β-CD-PEG-FA were synthesized according to respective reaction routes and were confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR) and infrared spectroscopy (IR). Second, busulfan was selected as drug model and loaded into the carriers by self-assembly. The cytotoxicity of poly-β-CD-PEG-OCH_3, busulfan-loaded poly-β-CD-PEG-OCH_3, poly-β-CD-PEG-FA and busulfan-loaded poly-β-CD-PEG-FA was determined by a crystal violet stain assay. Last, The potential of poly-β-CD-PEG-FA for use in the targeting delivery of busulfan was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis in HepG2, which contain folate receptors on its surfaces. *Results:* poly β-CD-PEG-OCH_3 and poly β-CD-PEG-FA were successfully synthesized and proved by ^{1}H NMR and IR. Busulfan was successfully loaded into poly β-CD-PEG-FA and poly β-CD-PEG-OCH_3 and their content are 3.379 mg/g and 3.548 mg/g, respectively. Poly β-CD-PEG-OCH3 and poly β-CD-PEG-FA with the concentration between 12.5-50 μg/ml had no effect on cell survival rate of HepG2 cells but with over 100 μg/ml had a significant inhibitory effect. The MTT results revealed that in HepG2 cells, the cytotoxicity of poly-β-CD-PEG-FA loaded busulfan cells is greater than that of poly-β-CD-PEG-OCH_3 loaded busulfan. *Conclusion:* Poly-β-CD-PEG-FA can be used as a carrier for hydrophobic anticancer chemotherapeutic drugs. After being loaded into poly-β-CD-PEG-FA, busulfan still maintained its original anticancer activity and had obvious targeted drug delivery effect on tumor cells with folate receptor over-expression.

Keywords: Busulfan, Poly-β-Cyclodextrin, Folate, Targeted Delivery, Cancer

1. Introduction

The biggest drawback of intravenous administration is the low blood drug concentration at the lesion site, which lead to poor treatment effect. One of methods is to extend the treatment cycle in order to achieve the expected therapeutic effect. It is necessary for drugs with poor solubility and low bioavailability to increase the dosage to achieve the required blood drug concentration in the lesion. However, high blood drug concentrations can not only kill lesion cells but cause a some degree of damage to normal cells. In contrast, targeted drug delivery can concentrate drugs at the lesion site, selectively kill lesion cells, and avoid or reduce damage to normal cells [3]. Because the vascular permeability of tumor tissue is higher than that of normal tissue, the macromolecules or nanoparticles can pass through the gap of tumor vascular wall smoothly [4]. At the same time, the macromolecule drugs and nanomedicines that enter tumors are not easily excreted and concentrate at the tumor site, which achieves passive targeting [5].

β-cyclodextrin is a hydrophobic cavity and non-toxic [6] cyclic oligosaccharide. The volume of its hydrophobic cavity is approximately 262 nm [7]. If hydrophobic drugs are loaded into the hydrophobic cavity, it is possible to achieve passive drug targeting. Active targeting is achieved through the

interaction between specific ligands on drug carriers and their receptors on tumor cells [8]. Folate receptors have limited expression in human healthy cells, but are overexpressed on the surface of cancer cells [9]. Therefore, folic acid is very suitable as a ligand for tumor targeted molecular therapy [10]. Polyethylene glycol chains can reduce the interaction between blood and extracellular components [11], improve circulation time, and reduce toxicity [12]. This study combines folic acid with poly (ethylene glycol) through a polyethylene glycol chain β-cyclodextrin coupling to synthesize drug carrier β-CD-PEG-FA. Using busulfan as a drug model, the drug loading performance of the drug carrier and its targeting performance against tumor cells with overexpression of folate receptors was studied.

2. Materials and Methods

2.1. Materials

Poly β-cyclodextrin (Mw=5000-10000 Da) was bought from Shandong Binzhou Zhiyuan Biotechnology Co., Ltd. Polyethylene glycol monomethyl ether (Mw=1000 Da) was bought from *Aladdin Reagent* (Shanghai) Co., Ltd. 4-Nitrobenzenesulfonyl chloride, dimethyl sulfoxide (DMSO), hydroiodic acid and folic acid dehydrated product were purchased from Shanghai Guoyao Reagent Group Co., Ltd; Cell culture medium and supplements, fetal bovine serum, alama blue and busulfan were bought from Sigma Aldrich; Dialysis bag with interception molecular weight=1500-2000 Da was purchased from Xiamen Aolan Biotechnology Co., Ltd.

2.2. Preparation of Poly β-CD-PEG-OCH_3 and Poly β-CD-PEG-FA

After 10.00 g polyethylene glycol monomethyl ether, 2.22 g p-nitrobenzenesulfonyl chloride and 10 ml of DMSO were added into a 50 ml round bottom flask, the reaction at 80°C continued for 48 hours when stirring. Crude ploy β-CD-PEG-OCH_3 was obtained if 11.34g of poly β-cyclodextrin was further added into the mixture and the reaction continued for other 48 hours. Crude poly β-CD-PEG-FA was obtained if 4.41g folic acid, 1.28g ohydroiodic acid, 2.22g p-nitrobenzenesulfonyl chloride and 11.34g of poly β-cyclodextrin were sequentially added into the mixture and the reaction continued for 48 hours, respectively. After crude ploy β-CD-PEG-OCH_3 and poly β-CD-PEG-FA were dialyzed with pure water for 48 hours in order to separate DMSO and remove unnecessary products, respectively, the pure products were lyophilized to following experiments.

When KBr compression method with wavelength range between 400 and 4000 cm^{-1}, ploy β-CD-PEG-FA and poly β-CD-PEG-OCH_3 were detected, collected data and plotted, respectively. Further, the structure of ploy β- CD-PEG-FA and poly β-CD-PEG-OCH_3 were confirmed by nuclear magnetic resonance hydrogen (NMR) spectroscopy using tetramethylsilane as the internal standard and D2O as the solvent.

2.3. Poly β-CD-PEG-OCH_3 and Poly β-CD-PEG-FA Coupled with Busulfan

After 4.00 g poly β-CD-PEG-OCH_3, 1.0 g busulfan and 10 ml of DMSO were added into a 50 ml round bottom flask, the mixture was stirring and the reaction lasted at 80 ° C for 72 hours. The reaction solution was dialyzed with pure water for 48 hours in order that DMSO was separated and unwanted products were removed, and the resulting product was collected through freeze-drying. The protocol of poly β-CD-PEG-FA coupled with busulfan was the same as poly β-CD-PEG-OCH_3 except 4.00 g poly β-CD-PEG-FA instead of 4.00 g poly β-CD-PEG-OCH_3.

The surface element analysis method was used to evaluate the content of busulfan from poly β- CD-PEG-FA and poly-β-CD-PEG-OCH_3. Briefly, 100 mg samples were wrapped in ashless paper, respectively, and then placed into a 500 ml oxygen bottle containing 5 ml of absorption solution for combustion. The sulfate in the obtained absorption solution was separated using IonPac AS14-AG14 as a separation column and rinsed with a solution containing 0.001 M $NaHCO_3$ and 0.0035 M Na_2CO_3, and its conductivity was detected.

2.4. Cytotoxicity Test in Vitro

First, $HepG_2$ cells were spread in culture medium containing 10% FBS, 1.0 mM sodium pyruvate, 0.1 mM non essential amino acids, and 1.5 g/l NaHCO3, and cultured at 37˚C and fully humid air containing 5% CO_2. $HepG_2$ cells with 5.0 $\times 10^4$ cells/ml were laid in a 24-well plate and continue growing overnight at the same condition. Second, different concentrations of poly β-CD-PEG-OCH_3 coupled with or without busulfan, poly β-CD-PEG-FA coupled with or without busulfan was added to each well. Last, after three days, the cells in each well were exposed to 0.4 ml of 2% crystal violet and 20% methanol at room temperature for 30 minutes, washed with distilled water, and photographed.

2.5. Evaluation of Tumor Cell Targeting Ability of Poly β-CD-PEG-FA

The 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) staining method was used to evaluate the efficacy of poly β-CD-PEG-FA coupled with busulfan which has the ability to target $HepG_2$ cells overexpressing folate receptors.

$HepG_2$ cells were seed with 1×10^4 cells/well in 100μl culture medium in 96-well plate, incubated overnight to obtain a fusion rate of 75% -80%. The culture medium was then replaced with fresh serum-free medium and added with a series of different concentrations of poly β-CD-PEG-OCH_3 or poly β-CD-PEG-FA coupled with busulfan. The cells were further cultured for 72 hours at 37˚C and 10 μ MTT solution (25 μ 100 added to each well (g/ml) before terminating the cultivation for 4 hours. Finally, the culture medium containing MTT was replaced with 100 μL DMSO solution,

and the absorbance was measured at 650 nm and 595 nm using an ELISA reader. Supposing the survival rate of untreated control cells is 100%, the cell survival rate is calculated using the following formula:

$$\text{Cell Viability (\%)} = \frac{[OD595(sample) - OD650(sample)]}{[OD595(control) - OD650(control)]} \times 100\%$$

OD595 (sample) and OD650 (sample) represent the value of poly β-CD-PEG-OCH_3 or poly β-CD-PEG-FA coupled with busulfan, but OD595 (control) and OD650 (control) representing measurements taken from wells treated with DMEM containing only 10% fetal bovine serum. Each sample was repeated four times.

2.6. Statistic Analysis

All experiments were repeated four times and the measurement results were made in quadruplicate. The data were represented as the mean ± standard deviation based on four measurements. Student t-test was used for statistical analysis and $P<0.05$ was considered to indicate a statistically significant difference.

3. Results and Discussion

3.1. Preparation of Poly β-CD-PEG-OCH_3 and Poly β-CD-PEG-FA

First, 4-nitrobenzenesulfonyl chloride can react at 80℃ with the hydroxyl group of CH_3O-PEG-OH to generate polyethylene glycol monomethyl ether p-nitrobenzenesulfonate. Second, the reaction occured between polyethylene glycol monomethyl ether p-nitrobenzenesulfonate and poly β-CDs. Because each poly β-CD molecule has many hydroxyl groups and hydroxymethyl groups, poly β-CD can be grafted onto polyethylene glycol monomethyl ether p-nitrobenzene sulfonate by nucleophilic substitution under mild conditions, which produced poly β-cyclodextrin polyethylene glycol monomethyl ether.

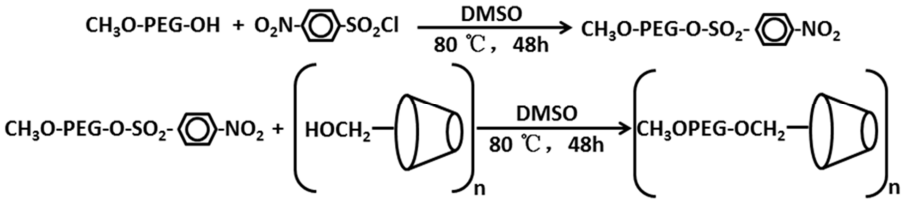

Figure 1. *The reaction generating poly-β-CD-PEG-OCH_3.*

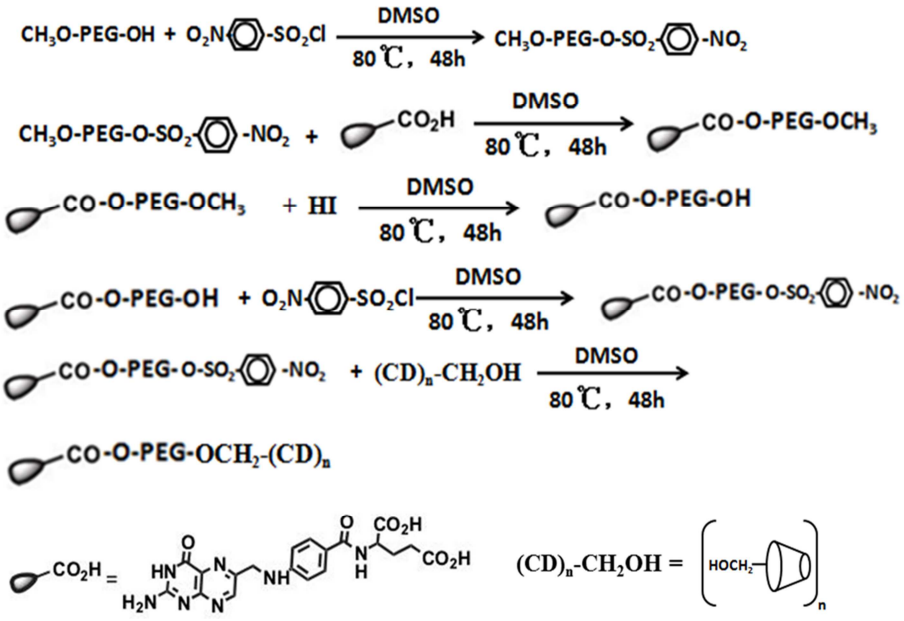

Figure 2. *The preparation schematic diagramof poly-β-CD-PEG-FA conjugates.*

The production of poly β-CD-PEG-FA conjugates could be divided into five reactions. The first reaction was the same as the first step of poly β-CD-PEG-OCH_3. The second reaction occured between folate and nitrobenzene sulfonate with polyethylene glycol monomethyl ether. Each folate molecule has many carboxyl and amino groups. As the nucleophilic ability of amino groups is stronger than that of carboxyl groups, the amino group in theory takes priority over the carboxyl group in the reaction with polyethylene glycol monomethyl ether p-nitrobenzenesulfonate. However, because the lone pair electrons on the amino group being conjugated with the p-π of the pterin ring, the nucleophilic

ability of the amino group is too weak to react with polyethylene glycol monomethyl ether p-nitrobenzene sulfonate before the carboxyl group. In addition, due to the steric hindrance imposed by the folate chain, folate derivatives are expected to be mainly composed of γ-composed of connecting isomers. Therefore, the product selected in the second step is beneficial for the distal end the connection of γ-carboxyl residues. The third step is the reaction between polyethylene glycol monomethyl ether folate and HI, which released free hydroxyl groups and formed polyethylene glycol folate monoester. The fourth step is the reaction between polyethylene glycol folate monoester and 4-nitrobenzenesulfonyl chloride to form polyethylene glycol folate p-nitrobenzenesulfonic acid diester. Both folic acid and p-nitrobenzenesulfonic acid can react with poly β-hydroxymethyl reaction on cyclodextrin. However, the departure ability of p-nitrobenzenesulfonic acid ions is much greater than that of folate ions, which result that the product of the fifth step is polymerization β- cyclodextrin polyethylene glycol monoether folate.

3.2. Poly β-CD-PEG Folic Acid Copolymer with 1H NMR and IR Spectral Analysis

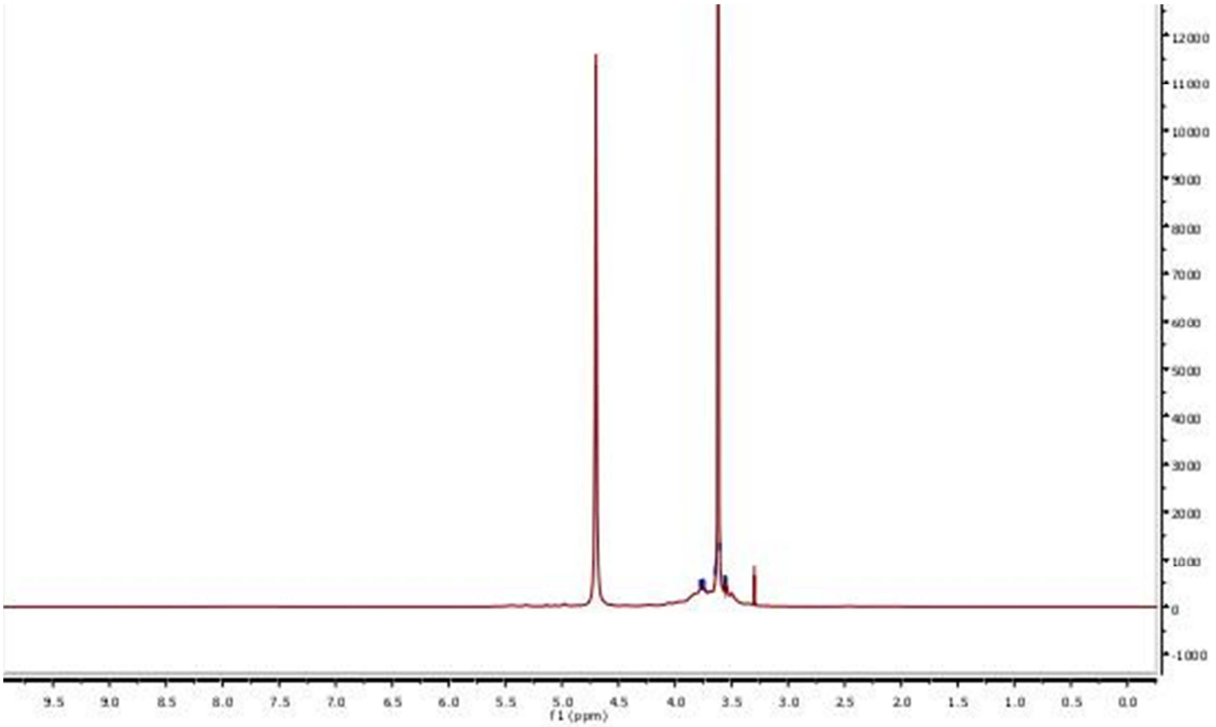

Figure 3. *The [1]H-NMR spectra of poly β-CD-PEG-FA.*

MPEG-OCH3 used as the linker reacted with the carboxyl group of folic acid and the hydroxymethyl of β-cyclodextrin in order to connect folic acid to polymer β-cyclodextrin, which provides physiologically stable ester bond and ether bond (Figure 2). Because of the steric hindrance imposed by the folic acid chain, polyethylene glycol monomethyl ether p-nitrobenzenesulfonate was chosen to promote the connection of the distal end γ carboxyl residues. Poly β-cyclodextrin contains hydroxyl and hydroxymethyl groups and the nucleophilic ability of hydroxymethyl is stronger than that of hydroxyl. Therefore, polyethylene glycol folate p-nitrobenzenesulfonic acid diester was chosen to promote the connection of hydroxymethyl groups, thereby obtaining poly β- cyclodextrin-PEG p-nitrobenzenesulfonic acid diester monoether folate. All unnecessary products and free reactants can be removed through dialysis in subsequent steps. NMR spectroscopy confirmed that the successful synthesis of poly β-cyclodextrin PEG-FA (Figure 3), whose NMR hydrogen spectrum is as follows:

Table 1. *The chemical shifts of poly-β-cyclodextrin-PEG-FA 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
6.0-8.1	the aromatic protons of folate acid
2.45-2.60	the protons in ethylene groups of the PEGunits -O-CH_2CH_2O-

The [1]H NMR spectrum of CD-PEG-FA shows that all characteristic peaks of CD, PEG and FA which indicate that FA interacts with poly β-CD through PEG. In NMR spectroscopy, the peak area of hydrogen is proportional to the number of hydrogen. Since the protons of PEG and poly (cyclodextrin) are overwhelmingly more than the aromatic protons of folic acid, the related signals of folic acid are too weak to be observed in [1]H NMR spectra. Therefore, further study was done to analyze poly β-CD-PEG-FA by infrared spectroscopy to confirm the formation of poly β-CD-PEG-FA (Figure 4).

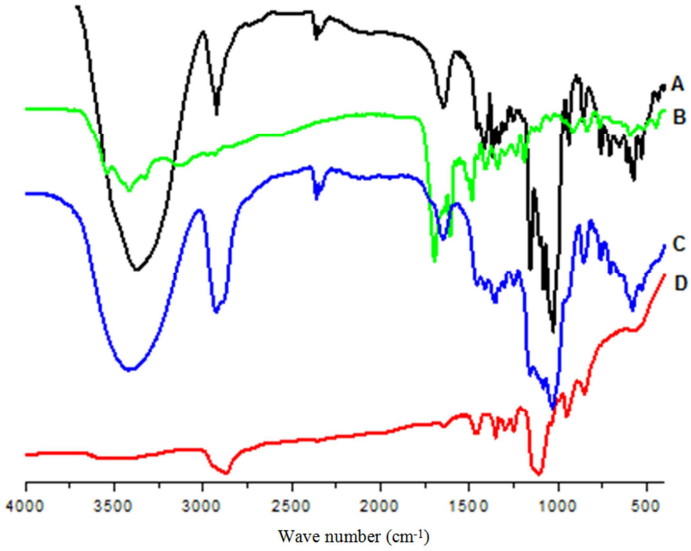

Figure 4. *The IR spectra of (A) poly-cyclodextrin, (B) folate acid, (C) poly cyclodextrin-PEG-FA and (D) HO-PEG-OCH$_3$.*

The FT-IR spectrum of poly cyclodextrin, folate acid, poly β-CD and HO-PEG-OCH$_3$ was shown in figure 4 and each component was as follows (table 2):

Table 2. *The wave number of poly β-cyclodextrin-PEG-FA and its corresponding IR vibration.*

Wave Number/cm^{-1}	Annotation
1701	theC=O stretching vibration of carboxylic acid in FA.
1190 ~ 1001	C-O-C stretching vibration of ether in β-CD, demonstrating that FAbinds chemically to poly-β-CD.
3431.2	O-Hstretch
1282.6	O-H deflection
2887.3	C-H stretch
1244	C-O deflection
1112.8	C-O stretch of ether

As shown in table 2, it can be seen that the FT-IR spectrum of the copolymer β-CD-PEG-FA can reproduce the characteristic absorption peaks of folate, poly β-CD and HO-PEG-OCH$_3$, Which was consistent with the expected chemical structure of poly β-CD-PEG-FA.

3.3. Poly β-CD-PEG-OCH$_3$ and Poly β-CD-PEG-FA Coupled with Busulfan

The most remarkable feature of cyclodextrin is that it can form complexes of solid inclusion complexes with various solid, liquid and gas compounds through molecular complexation [13]. The poly β-CD-PEG-OCH$_3$ or poly β-CD-PEG-FA with busulfan were dissolved in DMSO in order to facilitate the formation of inclusion complexes. The lipophilic cavity of cyclodextrin molecule provides a microenvironment in which nonpolar busulfan of appropriate size can enter to form a cyclodextrin busulfan complex [14]. The main driving force for the formation of poly cyclodextrin-busulfan inclusion complex is the release of water molecules from the cavity. Water molecules are replaced by more hydrophobic bisulfan molecules in the solution to achieve the association between nonpolar and reduce the ring tension of cyclodextrin, thus forming a more stable low-energy state [15]. The more soluble cyclodextrin is in solvent, the more molecules can be used for complexation. As both poly β-cyclodextrin and busulfan are slightly soluble in water, DMSO was used as the solvent in this reaction instead of water although water is the most commonly used solvent for complexation reactions. Sulfur element was further analyzed to determine if it had formed the inclusion complex of β-cyclodextrin and busulfan.

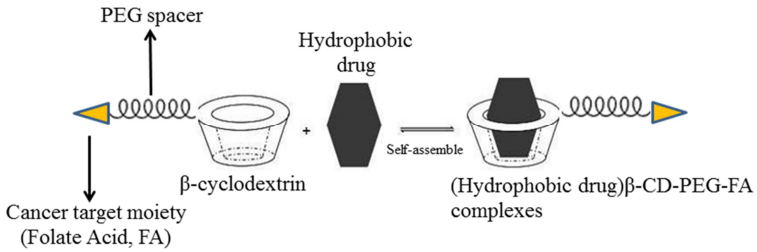

Figure 5. *The schematic diagram for the formation of cyclodextrin-busulfan inclusion compound.*

3.4. Determination of the Content of Busulfan from Poly β-CD-PEG-OCH$_3$ or Ploy β-CD-PEG-FA

Because β-CD-PEG-OCH3 and poly β-CD-PEG-FA contains no sulfur elements and the free busulfan and DMSO have been completely removed during dialysis, the study used sulfur element analysis to determine the content of loaded busulfan. The results show that the content of busulfan in 1g poly β-CD-PEG-FA and poly β-CD-PEG-OCH$_3$ was 3.379 mg and 3.548 mg, respectively, which indicated that it was successfully coupled with poly β-CD-PEG-FA and poly β- CD-PEG-OCH$_3$ and inclusion complexes were formed.

3.5. Cytotoxicity in Vitro of Poly β-CD-PEG-OCH3 and Poly β-CD-PEG-FA

To verify the effective drug loading, biocompatibility, and cytotoxicity of poly β-CD-PEG-OCH$_3$ and poly β-CD-PEG-FA, this study conducted in vitro cytotoxicity studies by crystal violet method using the HepG2 cell line as model. When the cells were used as samples or control treated with or without poly β-CD-PEG-OCH$_3$ or poly β-CD-PEG-FA incubates in culture medium for 72 hours, respectively, the crystal violet staining was used to determine the cell survival rate in the presence of β-CD-PEG-OCH$_3$ or poly β-CD-PEG-FA.

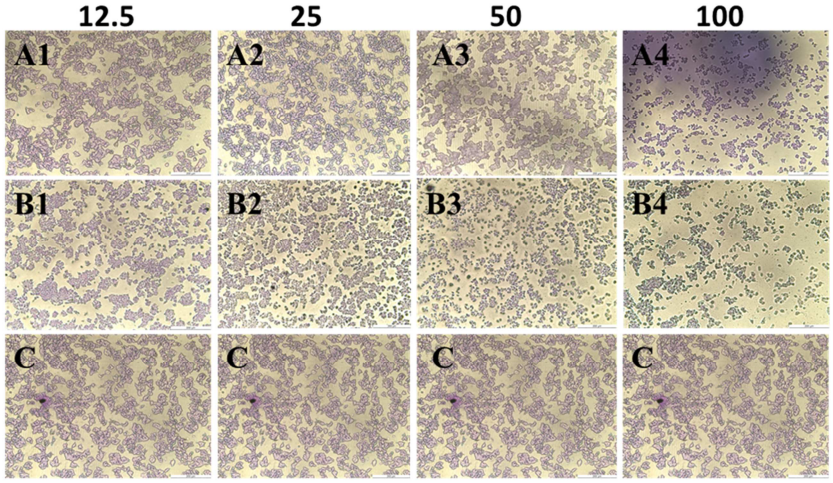

Figure 6. *A1, A2, A3 and A4 are cytopathic effect of poly-β-cyclodextrin-PEG-OCH$_3$ on tumor cell HepG2. B$_1$, B$_2$, B$_3$ and B$_4$ are cytopathic effect of poly-cyclodextrin-PEG-FA. C is a blank control experoment. Tumor cell lines were seeded in 24-well plates at a density of 5×10^{4}cells for each well and incubated with poly-β-cyclodextrin-PEG-OCH$_3$ or poly-β-cyclodextrin-PEG-FA at the indicated concentration (μg/ml). Following 72 h, cells were stained with crystal violet.*

As shown A$_1$, A$_2$, A$_3$, B$_1$, B$_2$ and B$_3$ in figure 6, the cells treated with poly β-CD-PEG-OCH3 and poly β-CD-PEG-FA with the concentration between 12.5-50 μg/ml had a cell survival rate of 100%, which indicated they had no cytotoxicity to HepG2 cells. The previous reports also proved that cyclodextrin was not toxic in vitro [16] and in vivo [17]. However, they has a significant inhibitory effect on HepG2 cells when their concentration went up to 100 μg/ml maybe because the osmotic pressure of the solution will also increased, which has a certain impact on cell growth. These results indicate that both poly β-CD-PEG-OCH3 and poly β-CD-PEG-FA can be used as drug carriers. The experiment on the targeting ability of poly β-CD-PEG-FA was performed in order to verify if the drug loaded into poly β-CD-PEG-OCH3 or poly β-CD-PEG-FA still have drug activity and can target tumor cells.

3.6. Evaluation of the of Targeting of Poly β-CD-PEG-FA in Tumor Cells

This study used phosphate buffered saline (PBS) as a control to investigate the targeting effect of the poly β-CD-PEG-FA on tumor cells with overexpression of folate receptors.

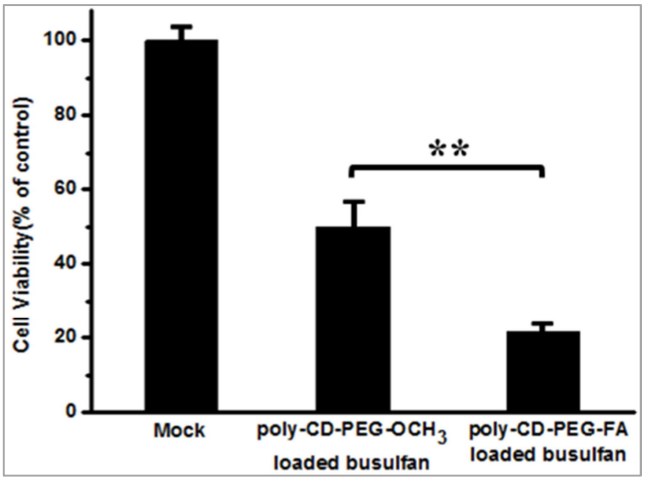

Figure 7. *Cytotoxicity of poly β-cyclodextrin-PEG-OCH$_3$ loaded busulfan and poly β-cyclodextrin-PEG-FA loaded busulfan.*

HepG2 cells were infected with poly β-cyclodextrin-PEG-OCH$_3$ loaded busulfan or poly β-cyclodextrin-PEG-FA loaded at a busulfan concentration

of 25μg/ml. After 72 h, the HepG2 cell survival ratios were analyzed by MTT assay. Results were expressed as a relative percentage to untreated control HepG2 cells and represent the mean of four repetitive experiments. Errors bar, standard deviation. **P<0.005, vs. poly-β-cyclodextrin-PEG-FA loaded busulfan.

The MTT test showed that the cytotoxicity of β-CD-PEG-FA loaded by busulfan on HepG2 cells with overexpression of folate receptors was significantly higher than that of β-CD-PEG-OCH_3 (Figure 7).

Poly β-CD-PEG-FA and poly β-CD-PEG-OCH_3 loaded by busulfan does whether or not contain folic acid, which decides if there is binding or endocytosis between folic acid and the overexpressed folic acid receptor on the surface of HepG2 cells. The endocytosis of HepG2 cells added the poly β-CD-PEG-FA loaded by busulfan was much higher than that of β-CD-PEG-OCH_3, which result that cytotoxicity caused by the former was much higher than that of the latter. These results showd that poly β-CD-PEG-FA has targeting effect on HepG2 cells with overexpression of folate receptors. What's more, it also indicates that busulfan the anti-cancer chemotherapy loaded onto the poly β-CD-PEG-FA carrier material still maintains its original anticancer drug activity.

4. Conclusion

Busulfan one of hydrophobic anticancer chemotherapy drugs could be loaded onto poly β-CD-PEG-FA at 3.379 mg/g carrying capacity and maintain its original anticancer activity that have a clear targeting effect on HepG2 tumor cells with overexpression of folate receptors. Poly β-CD-PEG-FA could be used for drug loaded hydrophobic anticancer chemotherapy drugs.

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