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Synthesis and cytotoxic activity of platinum complex immobilized by branched polyethylene glycol

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Abstract—Two-arm branched mPEG (monomethoxy-polyethylene glycol) with different molecular weights (M_n = 4000, 6000, 9400) was synthesized and used as carrier for immobilization of cisplatin [*cis*-diammine(dichloro)platinum (II), CDDP]. As a contrast, CDDP modified with linear mPEGs was also synthesized. All these polymeric drugs modified with branched mPEG are water soluble and show higher cytotoxic activity against C6 human breast cancer cells than cisplatin modified with linear mPEG with the same molecular weight. All the polymeric CDDP showed a much lower toxicity than the CDDP.

Polymer–drug conjugates, that is, macromolecular prodrugs, can be expected to improve the distribution of drugs in the body and prolong their half-lives and activities in vivo. Many kinds of water-soluble polymers are used as drug carriers such as dextran,[1] chitin,[2] polypeptide,[3] and polyethylene glycol (PEG).[4] Among them, PEG has been the most widely used as antitumor drug carrier because it shows excellent water solubility, low immunogenecity, and non-toxicity. Compared with low molecular weight antitumor drugs, antitumor drugs modified with PEG can be expected to achieve high water solubility and overcome side effects. Ouchi et al.[4–7] reported several prodrugs of PEG end capped with antitumor agents such as 5-fluorouracil, CDDP, and doxorubicin, which showed high antitumor activities. Huang et al.[8] reported the PEGylation of chlorambucil. It also exhibited high antitumor activity.

Recently, the synthesis of branched PEG and its applications of modifying drugs and proteins have become one of the focuses of PEG study. Monfardini et al.[9] reported that the enzymes modified with the branched PEG presented greater stability to proteolytic digestion relative to those modified with the linear mPEG. Reddy et al. [10] reported in his review that branched-chain PEGylated protein is more stable against enzyme proteolysis than linear moieties, and may also enhance the absorption and distribution of the protein. These advantages confirm that the branched PEG may be the nice choice in protein therapeutics.

Cisplatin [*cis*-diammine(dichloro)platinum (II), CDDP] is one of the most potent antitumor platinum complexes, but the accumulation of CDDP in kidney causes severe renal toxicity. It is sparingly soluble not only in water but also in lipid. Furthermore, it often shows very short half-lives in the body and exhibits undesirable side effects. It is well known that the cytotoxic activity of platinum complex is gradually decreased in blood stream because of ligand exchange reactions with compounds having amino groups. Ohya et al.[11] reported CDDP modified with linear mPEG maintained its cytotoxic activity during the circulation in bloodstream because the steric hindrance of PEG kept the platinum complex from such deactivating factors. Since branched PEGs have much more steric hindrance, they may keep better the cytotoxic activity of CDDP.

On the basis of these facts, a novel kind of antitumor drug with branched PEG was synthesized by us. Two-arm branched mPEGs (DImPEG) with different molecular weights were used to modify CDDP. To compare with DImPEG drugs, the samples of CDDP with linear mPEG were also prepared. The preliminary investigation was focused on their in vitro antitumor activities and acute toxicity.

Keywords: *cis*-Platinum; Branched polyethylene glycol; Immobilization.
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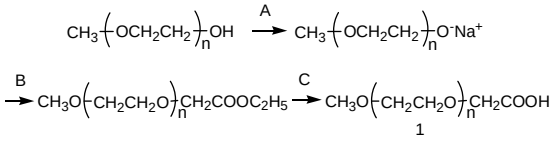
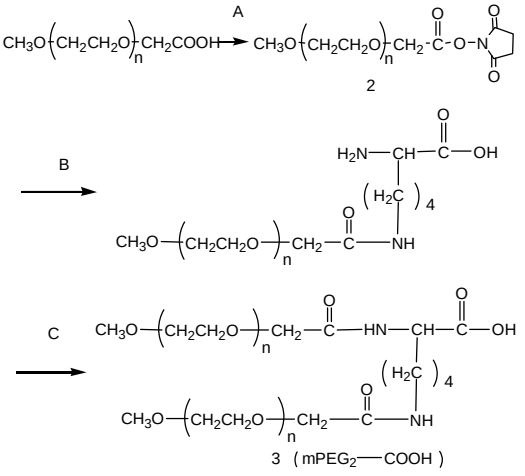
Monomethoxy-polyethylene glycols (mPEGs, M_n = 2000, 3000, 4700) were synthesized in our laboratory. The polydispersity values were about 1.08–1.10. As Scheme 1 showed that the linear mPEGs with carboxyl group (mPEG-COOH, compound 1)[12] were synthesized by the method of Bückmann et al.[13] The preparation of DImPEGs was carried out according to Scheme 2. mPEG-COOH (M_n = 2000, 9.5 g, 4.6 mmol), *N*-hydroxysuccinimide (NHS) (1.06 g, 9.2 mmol), and dicyclohexyl carbodiimide (DCC) (2.86 g, 13.8 mmol) were used to prepare activated ester of mPEG (mPEG-NHS) in yield of 95%. Then, the activated ester reacted with the amino group of lysine. Because lysine hardly dissolves in organic solvents and its α-amino group is less active, a two-step procedure was used. First, mPEG-NHS (4.3 g, 2 mmol) was coupled to the ω-amino group of excessive lysine (1.46 g, 10 mmol) in water. After purification, the α-amino group of above-mentioned product reacted with another part of mPEG-NHS (4.7 g, 2.2 mmol) in CH_2Cl_2. The weight of purified DImPEG was 8.2 g The whole procedure was monitored by gel permeation chromatography (GPC.Fig. 1)

This is a new method to prepare branched PEG. Monfardini et al.[9] prepared branched PEG in his work, but the linear PEG was coupled to lysine through carbamate bond. Guiotto et al.[14] proved that the carbamate bond formed by mPEG-NHS with α-amino group of lysine is unstable and might hydrolyze in basic solution; then the branched PEG would lose one arm. Since in following experiments, the hydrolysis of ester bonds in basic solution was a necessary step, a more stable amide bond was used to substitute the carbamate in the preparation of branched PEGs.



Scheme 1. Reagents and conditions: (A) sodium naphthalene, THF, 60 °C; (B) ethyl bromoacetate 4 h; (C) NaOH (0.1 mol/L), 50 °C, 24 h.



Scheme 2. Reagents and conditions: (A) NHS and DCC, CH_2Cl_2, 12 h; (B) lysine, water, pH 8, 4 h; (C) mPEG-NHS, CH_2Cl_2, 40 °C, 24 h.

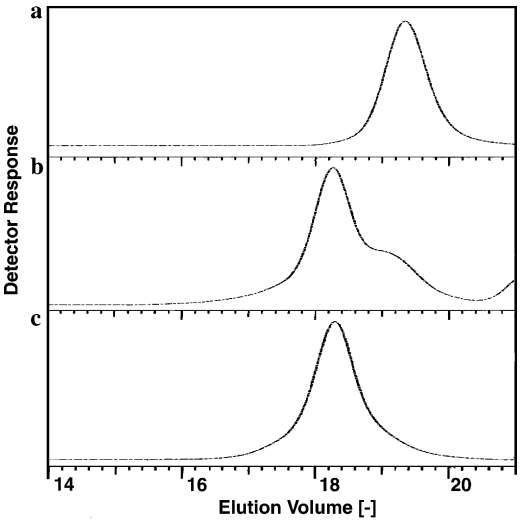
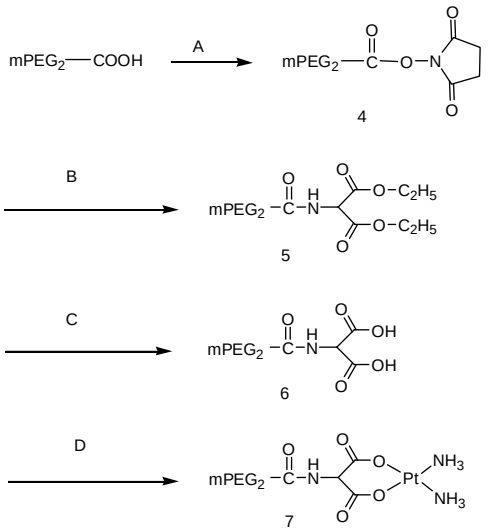


Figure 1. GPC traces of (a) mPEG (M_n = 2000), (b) crude product of DImPEG-4000, and (c) DImPEG-4000.

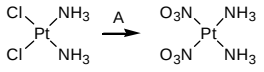
Purifying DImPEG was a difficult procedure. It is impossible to separate DImPEG from the mixture of linear mPEG and DImPEG by dissolve–precipitate method due to their similar physicochemical properties. Ultrafiltration is a peculiar method in separation of proteins, and the proteins with different molecular weights could be separated. By means of this method, DImPEG could be isolated from the unreacted mPEG. Aqueous (10%) of PEG mixture (8.2 g) was ultrafiltrated under 1.5 atmospheric pressure, and 5.0 g of pure DImPEG was obtained. Figure 1 described the purification results; it showed that no mPEG was detected after ultrafiltration.

Cisplatin [*cis*-diammine(dichloro)platinum (II), CDDP] modified with DImPEG was synthesized according to Scheme 3. First, the activated ester of DImPEG (DImPEG-NHS) was prepared using the similar way of the first step in Scheme 2. Second, the DImPEG-NHS (4.5 g, 1.1 mmol) was coupled to the amino group of diethyl aminomalonate (0.7 g, 3.3 mmol) and compound 5[15] was obtained. After saponification, DImPEG with two carboxyl groups (DImPEG-DA) was formed. By ligand exchange reactions of nitrato complex of CDDP (4.5 mmol) with DImPEG-DA (0.92 mmol), cisplatin modified with DImPEG (DImPEG-Pt) was obtained and the yield was 3.4 g. And nitrato complex of CDDP [diammine(dinitrato)platinum(II)) was synthesized by the method of Ohya et al.[16] (Scheme 4). At last, three polymeric drugs (DImPEG-4000-Pt, DImPEG-6000-Pt, and DImPEG-9400-Pt) with the molecular weight of 4000, 6000, and 9400 were obtained, respectively.

To compare the difference between the cisplatin modified with linear and branched PEG, we also prepared the cisplatin with linear mPEG by the similar method according to Scheme 3. Linear mPEG-COOH with dif-



Scheme 3. Reagents and conditions: (A) NHS and DCC, CH_2Cl_2, 12 h; (B) diethyl aminomalonate, triethylamine (TEA), CH_2Cl_2, 12 h; (C) a mixture of methanol and 1 mol/L NaOH (9:1, v/v), 2 h; (D) diammine(dinitrato)platinum(II), 60 °C, 24 h.



Scheme 4. Reagents and conditions: (A) silver nitrate, water, 60 °C, 6 h.

ferent molecular weights (M_n = 4000, 6000, and 9400) were synthesized and their polydispersity was in the range of 1.08–1.10. Three polymer drugs (mPEG-4000-Pt, mPEG-6000-Pt, and mPEG-9400-Pt) with different molecular weights were also obtained.

The contents of cisplatin modified by PEG were determined by inductively coupled plasma (ICP) and listed in Table 1. From the data, it was found that in some samples, the cisplatin contents modified by mPEG exceed 100%. This may be caused by diol by-product, which was always present in the synthesis of mPEG. Its separation was very difficult and time consuming, and its content and polydispersity value depend on the molecular weight (higher for high mass PEG).[17] However, as we indicated in the following discussion, this phenomenon did not affect the reliability of our final conclusion.

In vitro, the cytotoxic assays of DImPEG-4000-Pt, DImPEG-6000-Pt, DImPEG-9400-Pt, mPEG-4000-Pt, mPEG-6000-Pt, and mPEG-9400-Pt were performed on the C6 human breast cancer cells.[18] According to the contents of cisplatin in the conjugates, IC_{50} values for the polymeric drugs were determined from Figures 2 and 3 and listed in Table 2. Compared with that of CDDP, their IC_{50} value against C6 human breast cancer cells was in the range of 10^{-4}–10^{-5} mol/L. As shown in Figures 2 and 3, the cytotoxic activities of these conjugates were relatively lower than that of free CDDP. Ohya et al.[11] proved that the reduction of cytotoxic activities of these polymeric drugs may attribute to the fixing of cisplatin on the PEG terminal and the activity could be recovered after releasing from the conjugates. Thus, the immobilization of CDDP to PEG did not have fatal effect on the cytotoxic activity of CDDP.

To evaluate the toxicity of the polymer drugs, LD_{50} values were measured and listed in Table 3.[19] It shows that

Table 1. The content of cisplatin in the conjugates

Drug	Content of cisplatin (%)
DImPEG-4000-Pt	98.1
DImPEG-6000-Pt	99.4
DImPEG-9400-Pt	90.3
mPEG-4000-Pt	98.5
mPEG-6000-Pt	102.5
mPEG-9400-Pt	109.3

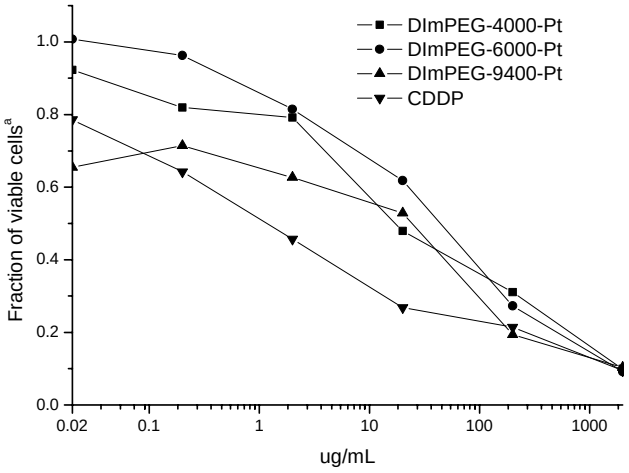


Figure 2. Semi-log plots of viability of cells versus DImPEG drug concentration. [a]The fraction of viable cells = OD $values_{treated}$/OD $values_{control}$.

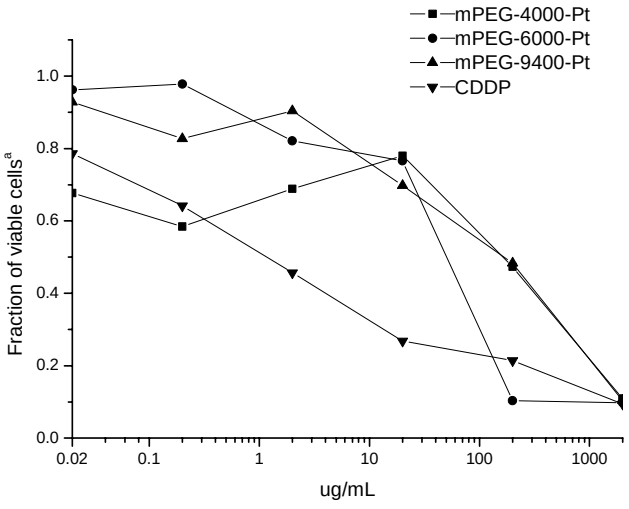


Figure 3. Semi-log plots of viability of cells versus mPEG drug concentration. [a]The fraction of viable cells = OD $values_{treated}$/OD $values_{control}$.

Table 2. IC_{50} values of the polymer drugs

Drug	IC_{50}, 10^{-5} mol/L
CDDP	0.527
DImPEG-4000-Pt	6.27
DImPEG-6000-Pt	27.2
DImPEG-9400-Pt	11.9
mPEG-4000-Pt	61. 4
mPEG-6000-Pt	30.7
mPEG-9400-Pt	61.9

Table 3. LD_{50} of the polymer drugs and CDDP

Drug	LD_{50}, mg/kg
CDDP	25.8
DImPEG-4000-Pt	901.5
DImPEG-6000-Pt	988.1
DImPEG-9400-Pt	1013.7
mPEG-4000-Pt	895.6
mPEG-6000-Pt	976.0
mPEG-9400-Pt	1095.3

the toxicity of the polymer drugs whether modified with linear PEGs or with branched PEGs was dropped greatly. The LD_{50} values of them were 34–42 times greater than that of CDDP. When the molecular weights of the polymer drugs increased, the LD_{50} increased too.

The effect of chain length of PEG on the cytotoxic activity was investigated. From Table 2, it was observed that with the variation of molecular weights of DImPEGs, the IC_{50} of corresponding polymer drugs was changed too. The IC_{50} of DImPEG-4000-Pt was lower than that of DImPEG-6000-Pt and DImPEG-9400-Pt. That means DImPEG-4000 affects the cytotoxic activity of cisplatin least and was the most suitable one for modifying cisplatin.

The effect of the different type of PEG drugs on the cytotoxic activity was also investigated. Table 2 showed that the cytotoxic activity of DImPEG-CDDP is higher than that of the drug modified by linear PEG with the same molecular weight. For example, the IC_{50} value of mPEG-4000-CDDP is about 10 times more than that of DImPEG-4000-CDDP. The releases of platinum moieties from two types of PEG are similar because they all are connected with PEGs through the same chelate-type coordination bond. Hence, the difference of cytotoxic activities between the two kinds of polymer drugs is attributed to the difference of steric hindrance of these PEGs. Guiotto et al.[14] also reported that 'Branched' PEG analogues are superior with respect to the linear ones in creating an 'umbrella-like' surface coverage of the protein, thus protecting it from proteolysis and reducing its inactivation during conjugation.

In conclusion, we reported the synthesis of CDDP modified with branched PEG. It is a new polymer drug. The cytotoxic activity of DImPEG-CDDP is higher than that of linear mPEG-CDDP. That means branched PEGs is a better drug carrier than linear ones.

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12. Selected data for compound 1: IR ν_{max} (KBr, cm^{-1}) 1735 (O═C); ^1H NMR (500 MHz, $CDCl_3$) δ 4.16 (s, 2H, OCH_2COOH), 3.38 (s, 3H, CH_3O), 3.49–3.79 (CH_2CH_2O of PEG backbone).
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15. Selected data for compound 5: IR ν_{max} (KBr, cm^{-1}) 1741 (O═CO), 1665 (O═CNH); ^1H NMR (500 MHz, $CDCl_3$) δ 1.30 (t, 6H, CH_2CH_3), 3.38 (s, 6H, CH_3O), 1.35–1.80 (6H, $HNCHCH_2CH_2CH_2CH_2NH$), 3.28 (2H, $HNCHCH_2CH_2CH_2CH_2NH$), 3.79–4.28 (4H, OCH_2CH_3, 4H, OCH_2CONH, 1H $CONHCHCONH$) 3.49–3.79 (CH_2CH_2O of PEG backbone).
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18. In vitro cytotoxicity of these polymer drugs was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The C6 human breast cancer cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% inactivated calf serum at 37 °C in an atmosphere of 5% CO_2. The cells harvested from log phase were digested by 0.25% trypsin and diluted to 70,000 cells/mL by DMEM culture solution containing serum. Then, the cells were seeded onto a 96-well plate for 100 μL per well. Then, there were 7000 cells per well. The plate was kept in CO_2 incubator at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The polymer drugs were dissolved in the DMEM culture solution. The solution was filtrated. Then, six sample solutions with the CDDP concentration of 0.02, 0.2, 2, 20, 200, and 2000 μg/mL, respectively, were prepared. The drug solutions were added to each well for 100 μL. The cells were cultured for 3 days. Then, the cultured cells in each well were mixed with 20 μL of MTT solution of 5 mg/mL in DMEM culture solution without serum and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. The top clear solution was got away and 150 μL dimethylsulfoxide (DMSO) was added to dissolve the formazan for each well. After shaken for

10 min by plate shaker, the OD value of each well was measured on an ELISA spectrophotometer at 570 nm.

19. For the assessment of the acute toxicity, the TA1 mice were randomly divided into five groups (20/group and female/male = 1:1); polymer drugs and CDDP were injected ip × 1 into TA1 mice at five different dose levels on day 0. Then, the behavior and death distribution of the test mice were recorded. The highest death rate appeared on day 1 and the condition of the survivals was good after 2 weeks. LD_{50} was calculated by using Bliss method.