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# **PEGylated graphene oxide for tumor-targeted delivery of paclitaxel**

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## Abstract

Graphene has been considered one of the most promising carbon derivatives in material science for the past few years. Graphene oxide (GO) sheet has shown excellent tumor-targeting ability in mice as well as biocompatibility, and little toxicity. In our work, we conjugated the anticancer drug paclitaxel (PTX) to aminated polyethylene glycol (PEG) chains on GO sheets via a cleavable ester bond to obtain GO-PEG-PTX conjugate. The PEGylated GO was water-soluble and exhibited high biocompatibility. More importantly, compared to Taxol<sup>®</sup>, GO-PEG-PTX showed prolonged blood circulation and higher efficacy in suppressing the tumor growth as well as increased accumulation rate of PTX in tumor using a murine B16 melanoma cancer model. In addition, GO-PEG-PTX demonstrated to be safe with no obvious tissue toxicity to the main organs. Overall, compared to Taxol<sup>®</sup>, GO-PEG-PTX exhibited prolonged blood circulation time and higher tumor suppressing efficacy.

## Introduction

Graphene has a hexagonal packed structure formed by a two-dimensional single layer of carbon atoms<sup>1,2</sup>. Owing to its unique properties, graphene and its derivatives have attracted tremendous attention in the fields of electronics, energy, materials and biomedical applications<sup>3-6</sup>. For biomedical application in physiological environments, proper surface functionalization is essential to design graphene-based nanocarrier systems with high solubility in the physiological fluids and acceptable biocompatibility. Various types of hydrophilic polymers have been utilized to functionalize graphene oxide (GO) using covalent or non-covalent bonding<sup>7</sup>. Functionalized GO has been used for drug and gene delivery<sup>7-19</sup>, as well as in biosensor platforms, photothermal therapy and in tumor and cell imaging<sup>9, 20-30</sup>. Numerous types of polymers and molecules are possible to use for modifying the surfaces of GO via hydrogen bonding or  $\pi$ - $\pi$  interactions<sup>7, 31</sup>. Many aromatic chemotherapy drug molecules can be conjugated onto the surface of GO through physical adsorption, resulting in changing GO from poorly soluble sheets to water-soluble conjugates<sup>8-10, 32</sup>. Dai et al. first loaded a water insoluble anticancer analogue of camptothecin (CPT) onto GO surface through non-covalent conjugation via  $\pi$ - $\pi$  interaction. The GO loaded with SN38 exhibited acceptable biocompatibility and excellent solubility both in aqueous and physiological solutions<sup>8</sup>. Several research groups have studied targeted delivery of doxorubicin (DOX) and CPT attached to surface modified GO, which exhibited superior anticancer efficacy<sup>10, 14, 17, 19, 32</sup>.

Liu et al. have first reported that GO modified by coating its surfaces with polyethylene glycol (GO-PEG) can become highly aqueous soluble and stable in physiological fluids such as serum<sup>8</sup>. These findings can make the *in vivo* intravenous application of GO feasible. Moreover, GO-PEG has shown significant passive tumor targeting ability, which might be ascribed to the enhanced permeability and retention (EPR) effect of tumor tissue<sup>33</sup>. The long-term *in vivo* biodistribution and toxicity investigations have demonstrated that GO-PEG exhibited no obvious tissue toxicity, and no organ damage or inflammation symptoms<sup>33, 34</sup>, which revealed the superior characteristics of GO-PEG compared to un-coated GO<sup>35</sup>. According to Liu and co-workers, following

intravenous injection of GO-PEG, the formulation has accumulated in the reticuloendothelial systems (RES) such as liver and spleen and were gradually cleared out via renal excretion and biliary pathway into feces over time<sup>34, 36, 37</sup>.

Paclitaxel (PTX) is a widely used chemotherapeutic drug which promotes tubulin polymerization and formation of extraordinarily dysfunctional and stable microtubules, disrupting the normal tubule dynamics<sup>38</sup>. However, its poor aqueous solubility is a serious limitation that prevents proper formulation and clinical application of the drug<sup>39</sup>. In order to overcome low aqueous solubility of PTX, formulations based on Cremophor EL (e.g. Taxol<sup>®</sup>) have been prepared and used via slow intravenous infusion following dilution with NaCl (0.9%) or dextrose (5%) solutions. However, the solvent system mainly Cremophor EL in Taxol<sup>®</sup> causes serious toxicological effects<sup>40</sup> such neurotoxicity and nephrotoxicity, which may significantly reduce the overall therapeutic benefit of PTX. Therefore, Cremophor EL-free delivery systems of PTX have been proposed, including those based on polymeric nanoparticles, polymeric micelles, liposomes and many prodrug formulations<sup>41-45</sup>. Owing to the advantages and unique performance of GO as a targeting system, formulations of PTX loaded onto modified GO are worth exploring.

Unlike DOX and CPT, PTX has no extend  $\pi$ -structure larger than one aromatic ring<sup>46</sup>, hence, it is difficult to load PTX onto GO by physical adsorption. Thus, we have investigated the possibility of loading PTX onto GO-PEG through covalent conjugation, aiming to improve the drug physiological solubility, and enhance the formulation compatibility and cancer targeting ability. In this work we used GO-PEG as a drug carrier to load PTX via an ester bond, which is cleavable after *in vivo* injection. The covalent conjugation has been reported to be successful at attaching PTX to single-walled nanotubes (SWNTs) and  $\alpha,\beta$ -Poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA)<sup>39, 47</sup>. The influence of GO-PEG-PTX conjugation on drug solubility in water and mouse serum was investigated. Moreover, further *in vivo* investigations were carried out to evaluate the anti-cancer efficacy of GO-PEG-PTX, the blood circulation time, drug bioavailability, tissue distribution and tumor uptake rate. The *in vivo* biodistribution of GO-PEG-PTX was also investigated and compared to that of Taxol<sup>®</sup>. To the best of our knowledge, this is the first study that demonstrated the feasibility of using PEGylated graphene oxide as nanocarriers for PTX and investigated the anticancer activity of the resultant complex compared to Taxol<sup>®</sup>.

## Results and discussions

To improve the solubility and *in vivo* properties of GO, a variety of modifiers were investigated to conjugate to the surface of GO, such as PEG, folic acid, polyethyleneimine, chitosan, etc<sup>10, 11, 15, 17</sup>. Because PEG has been widely used for improving the water solubility and biocompatibility of many nanomaterials in biomedicine, in this study we chose PEG4000 to improve the solubility and *in vivo* behavior of GO. The synthesis route of GO-PEG was outlined in Fig 1B. In brief, GO was first converted to GO-COOH through sonication and carboxylation at the surface of GO. Then NH<sub>2</sub>-PEG4k-NH<sub>2</sub> was conjugated with carboxylate groups of GO-COOH via amide bond. The carboxylation was done to convert the esters and epoxides into carboxyl group, by which GO could conjugate with PEG-NH<sub>2</sub>. The decoration of GO by PEG significantly improved its solubility both in water and physiological solutions. FT-IR spectra were used to confirm the

assigned structure of GO-PEG. The conjugation of PEG with GO-COOH through amide bond formation could be verified by the peak at  $\sim 2850\text{ cm}^{-1}$  (C-H bond),  $\sim 1650\text{ cm}^{-1}$  (C=O bond) and  $\sim 1100\text{ cm}^{-1}$  (C-O bond) (Fig. 2B). The size of GO-PEG particles was below 100 nm as observed by atomic force microscopy (AFM) (Fig. 2A). According to the UV-vis spectra, GO-PEG possessed both higher near-infrared and visible absorption than GO did (Fig. 2C). The increased optical absorption might be caused by hydrolysis of ester bond and ring-opening of epoxide groups on the surface of GO molecule under the basic synthesis condition during the carboxylation step.

To ensure a proper releasing profile of PTX after intravenous injection, PTX was conjugated to GO-PEG via a cleavable ester bond, which can be hydrolyzed by both chemical and enzymatic pathways (Fig 1A). GO-PEG-PTX complex was synthesized by a two-step reaction. Firstly, 2'-O-succinyl-PTX was synthesized, and then 2'-O-succinyl-PTX was conjugated to GO-PEG. The synthesis of 2'-O-succinyl-PTX was carried out according to the previously reported methods<sup>39, 47-49</sup>. As described in Fig. 1A, the PTX molecule was first linked by its 2'-hydroxyl terminus with succinic anhydride. TLC analysis was used for detecting whether free PTX was fully converted to the resultant. The unreacted succinic anhydride turned into water soluble succinic acid after stirring at 60°C for 1 hour. The desired compound was obtained by purification using silica gel column. After that, modified PTX was coupled to the terminal amine group of the PEG previously being attached on the surface of GO molecule through amide bond<sup>47</sup>. As a standard reaction of forming amide bond, 2'-O-succinyl-PTX and GO-PEG were dissolved in water/DMSO (1:1) mixed solvent, and EDC-HCL and NHS were added as catalysts. After dialysis in DMSO and water respectively, the purified GO-PEG-PTX was analyzed by HPLC method to confirm the successful synthesis. Different from blank PTX, GO-PEG-PTX exhibited improved solubility both in water and in saline. *In vitro* release study indicated that GO-PEG-PTX displayed excellent stability in PBS solution, in which only very low proportion of PTX was released from GO-PEG-PTX complex within 48 h (Fig. 3). The release rate of PTX in C57 mice serum was faster than that in PBS (about 30% of total PTX was released within 24 h) (Fig. 3). The cleavage of ester bond in serum could be ascribed to the existence of esterase enzyme. Importantly, the relatively moderate release rate might ensure that PTX loaded onto GO-PEG would reach the target tissue and then release PTX to exert therapeutic effect.

Cytotoxicity of GO-PEG-PTX was investigated using MTT assay with A549 and B16 cancer cell lines. As indicated in Fig. 4, GO-PEG-PTX exhibited approximately similar cytotoxic efficacy to that exhibited by Taxol<sup>®</sup> at relatively high concentrations. No obvious cytotoxicity of plain GO-PEG was found during the experiments, even at high concentrations. These results indicated that loading PTX in GO-PEG did not significantly interfere or reduce cytotoxicity of PTX against cancer cells.

We then further investigated the cellular uptake of GO-PEG-PTX using A549 and B16 cell lines. Some previous research investigations have employed fluorescent or radioactive materials to tag GO molecules in order to monitor their dynamics following cellular uptake<sup>32, 47</sup>. In this study, we used HPLC to investigate the cellular uptake rate because the linear chain of NH<sub>2</sub>-PEG4k-NH<sub>2</sub> did not have much room for conjugating the fluorescent material, especially when two terminals of PEG were occupied by both GO and PTX. HPLC method was established to monitor the intracellular drug contents. It was shown in Fig. 5 that the intracellular concentration of drug using GO-PEG-PTX was slightly lower than that of Taxol<sup>®</sup> at low doses. However, when the drug

concentration was increased the intracellular drug content almost reached the same level of that seen by Taxol<sup>®</sup>. Both A549 and B16 cell lines shared the same tendency. The cellular uptake results and the cytotoxicity investigation suggested that the *in vitro* anti-cancer ability of GO-PEG-PTX was comparable to that of Taxol<sup>®</sup> at the relatively high concentrations used. However, the *in vitro* anti-cancer ability of GO-PEG-PTX was lower than that of Taxol<sup>®</sup> at the relatively low doses. The previous study of Liu et al. showed that cytotoxicity of PTX conjugated to SWNTs was relatively lower than that of Taxol<sup>®</sup> at certain drug dose range (10~100 µg/mL)<sup>47</sup>. The low cellular uptake rate of the drug used as GO-PEG-PTX might be attributed to the use of GO molecules which seemed to interfere with the cellular uptake of the drug. This was confirmed previously by Chang and co-workers who have reported that GO alone could hardly be taken up by cancer cells<sup>50</sup>. Thus when conjugated with GO, PTX was difficult to enter the cancer cells if the ester bond between GO and PTX did not cleave. The other reason was PTX releasing from GO took time as the PBS buffer and cell culture medium did not contain any enzyme that may facilitate the cleavage of the ester bond. The releasing behavior of GO-PEG-PTX was carried out as shown in Fig. 3. The linkage between GO and PTX was highly stable in PBS solution, and it could not be easily cleaved even in enzymatic serum condition (<25% within 24 hour). Although the conjugation with GO hindered the immediate uptake of PTX by the cells, the long blood circulation time offered by conjugating the drug to GO-PEG after *in vivo* treatment was highly advantageous at enhancing the anticancer efficacy of PTX. Using this strategy, PTX administration as GO-PEG-PTX may result in higher drug bioavailability and enhanced antitumor effect.

In order to understand the pharmacokinetics of GO-PEG-PTX, we used HPLC assay to measure PTX concentration in plasma at different time intervals after i.v. injection of the formulations into Wistar rats via tail vein (2 mg/kg). Blood was collected at different time points after injection. The time-concentration curves of GO-PTX-PEG and Taxol<sup>®</sup> both exhibited a standard two-compartment model. As shown in Fig. 6, the elimination speed of PTX was relatively rapid both in GO-PEG-PTX and Taxol<sup>®</sup> group. Compared with Taxol<sup>®</sup>, longer second phase blood circulation half-time, higher bioavailability and lower clearance rate were observed for GO-PEG-PTX. The underlying reason was that some time is needed for the cleavage of ester linkage between PTX and GO-PEG to occur<sup>51</sup>.

To further evaluate the tumor targeting ability of GO-PEG-PTX, we studied tissue distribution of GO-PEG-PTX and Taxol<sup>®</sup> in B16 melanoma cancer bearing C57 mice. The tissue distribution of GO-PEG-PTX and Taxol<sup>®</sup> were measured at 30 min, 1 h, 4 h, 8 h, 12 h and 24 h after injecting GO-PEG-PTX or Taxol<sup>®</sup> via tail veins of B16 tumor bearing mice. At predetermined time points, the blood samples were collected and the animals were sacrificed. Tissues including hearts, livers, spleens, lungs and kidneys were isolated immediately. Plasma or tissue homogenates were extracted by methanol, and then quantitative measurement of PTX was done by HPLC. As shown in Fig. 8, after i.v. administration of Taxol<sup>®</sup>, peak concentrations of PTX in tumor were achieved within 8 h (4%), and PTX decreased significantly to a very low concentration in tumor at 24 h post-injection. In the case of other organs (Fig. 7), at 0.5 h, PTX was widely distributed in liver (56%), kidney and lung with negligible concentrations being detected in the tumor tissue (0.08%). The drug concentration in liver, lung and kidney reached the highest within 0.5 h, and then decreased gradually over time. The concentration of PTX in spleen remained relatively low throughout the detecting period, which reached the highest amount at 12 h (2%). The highest

blood concentration of PTX was achieved within 12 h (8%). The PTX in all tissues were almost dispelled completely at 24 h except liver (4%).

By contrast, after *i.v.* administration of GO-PEG-PTX, the highest level of PTX in tumor was obtained within 4 h (14.2%), which was significantly higher than that of Taxol<sup>®</sup> group. The concentration of PTX remained relatively high even at 24 h (2%) after administration. For other tissues, the liver and kidney reached the highest concentration within 0.5 h (28% and 16%), decreasing gradually over time. The highest concentration in spleen, lung and blood were achieved within 1 h, 1 h and 12 h, respectively (32%, 6%, 10.5%). After 24 h of treatment, PTX in liver and spleen remained at moderate levels (7% and 6% respectively).

The results of tissue distribution of GO-PEG-PTX and Taxol<sup>®</sup> reflected the tumor treatment efficacy and main organ accumulation of the two formulations. By conjugating with GO-PEG, PTX exhibited a significantly different *in vivo* distribution. PTX given as GO-PEG-PTX showed higher blood concentration than Taxol<sup>®</sup> over the whole time course examined, which was consistent with the pharmacokinetic investigation. The distinct difference between GO-PEG-PTX and plain PTX in physiological solutions could be attributed to the prolonged blood circulation. Another obvious difference in tissue distribution of PTX was the concentration in spleen. Much higher concentration was found in GO-PEG-PTX group compared with that in the Taxol<sup>®</sup> group. After injection of GO-PEG-PTX, the PTX concentrations were 27, 25 and 14 times higher than that of Taxol<sup>®</sup> at 1 h, 4 h and 8 h respectively. It was previously reported that graphene as well as SWNTs have higher accumulating capacity in RES organs such as liver and spleen<sup>36, 37, 47, 52, 53</sup>. Liu et al. proved that the uptake of drug-macromolecular complex such as PTX-SWNTs by RES organs could serve as a scavenger system to metabolize and eliminate toxic drugs as well as carriers. Therefore, when we utilize GO as a drug carrier, the toxicity in those organs must be considered. Some reports announced that unmodified GO may induce some adverse effects<sup>35, 54, 55</sup>. However, Liu and co-workers demonstrated that no noticeable organ damage or inflammation was observed after *i.v.* injection of GO-PEG into mice at a dose of 20 mg/kg<sup>56</sup>. In this study, we have also investigated the *in vivo* toxicity of GO-PEG-PTX and made a comparison with that of Taxol<sup>®</sup>, plain GO-PEG and saline. After *i.v.* administration of these four formulations, the weight of each C57 mouse was recorded every day. According to the results obtained, no significant body weight change was observed in each group 30 days after the treatment (Fig. 9B). Liver damaged related blood chemistry analysis was performed 30 days after the first injection of formulation. No obvious differences between the four groups were found (Fig. 9A), indicating that GO-PEG-PTX did not cause any detectable physiological damage to the liver at the given dose. Meanwhile, as displayed in Fig. 9C, the immunohistochemistry analysis of liver and spleen separated from the mouse in each group 30 days after the treatment did not show any inflammation or pathological changes. According to these results, GO-PEG-PTX has shown to be safe for *in vivo* administration at the dose of 2 mg/kg, and its accumulation in RES organs such as liver and spleen did not seem to affect the health condition of C57 mice within the time course of the investigation.

It was clearly shown in our data that GO-PEG-PTX exhibited much higher PTX uptake rate in tumor tissue than that of Taxol<sup>®</sup> by about 5.5 times (1h), 3.5 times (4h), 3.3 times (8h) and 2.8 times (12h) (Fig. 6.). Even at 24 h after injection, PTX concentration of GO-PEG-PTX in tumor remained above 2% (i.e. 12 times higher than that of Taxol<sup>®</sup>). The EPR effects of GO could be the main reason of the significantly increased PTX distribution in tumor using GO-PEG-PTX. It was believed that the leaky and tortuous vasculatures of tumor tissue might prefer to withhold nanosize

materials such as graphene and SWNTs. The tumor targeting ability of GO has been shown to be stronger than that of SWNTs because of their difference in geometrical structure<sup>52, 57, 58</sup>. Even though the biodistribution of GO-PEG, of which had a long-term accumulation process lasting for several weeks<sup>36, 47</sup>, PTX loading on GO-PEG was metabolized and excreted quickly. The difference between the tissue distribution of GO-PEG and GO-PEG-PTX were caused by the cleavage of the ester linkage between PTX and GO-PEG after injection into the animals. The same result has also been reported in the biodistribution study of PTX-SWNTs<sup>47</sup>. It was believed that the ester linkage was cleaved by carboxylesterases mostly in the liver<sup>47, 51, 59, 60</sup>. In previous studies, water solubility of PTX was improved by using the vehicle Cremophor EL, enabling PTX to be clinically used. However, our study indicated that GO-PEG-PTX exhibited much longer blood circulation time and thus much better tumor uptake rate than the formulation of Taxol<sup>®</sup>. These results implied that a lower dosage of GO-PEG-PTX might achieve the same anti-cancer efficacy as Taxol<sup>®</sup> did. As a consequence, our novel conjugate would reduce the toxicity and side effects of PTX in normal organs, hence possibly offering more desirable characteristics and better safety profile compared to the market available formulation of PTX (Taxol<sup>®</sup>).

In order to investigate the *in vivo* cancer suppressing efficacy of GO-PEG-PTX and compare to that of Taxol<sup>®</sup>, B16 murine melanoma cancer animal model was established for the experiments. Female C57 mice bearing subcutaneously implanted B16 tumors were intravenously injected with GO-PEG-PTX or Taxol<sup>®</sup> at the equivalent dosage of PTX for 3 times (0, 3, 6 days after injection), where 0 day refers to the day of injecting PTX formulations for the first time. Two weeks after injection, the tumor volume and survival condition were recorded every day. The results of tumor growth speed showed that Taxol<sup>®</sup> can suppress tumor growth at a moderate rate (Fig. 10A). Moreover, the injection of GO-PEG-PTX induced significant tumor inhibition as compared to that of Taxol<sup>®</sup>. The tumor growth speed showed no difference between blank GO-PEG group and saline group, which indicated that the blank GO-PEG itself did not have any anticancer efficacy. The survival time of each group also shared the same tendency with the result of tumor growth speed (Fig. 10B). Mice in GO-PEG-PTX group had the longest survival time. The survival time of mice in Taxol<sup>®</sup> group was less than that of GO-PEG-PTX group, but was longer than those of GO-PEG and saline groups.

It was shown that PTX loading on GO-PEG possessed significantly improved anticancer efficacy compared with Taxol<sup>®</sup>, which was proved by slowing down the tumor growth and the prolonging survival time. The significantly higher tumor suppressing rate of GO-PEG-PTX might be ascribed to its much higher tumor uptake compared with that of Taxol<sup>®</sup>, which was verified in our biodistribution study. No enhanced tumor suppressing effect or longer survival time of mice after blank GO-PEG treatment was observed, indicating that the GO-PEG material only played a role of drug carrier without eliciting any noticeable anticancer efficacy. However, Arya et al. have reported that carbon nanostructures such as GO and SWNTs can enhance the sensitivity of lung cancer cells to PTX when carbon nanostructures and PTX were incubated with cells together. Their *in vitro* study indicated that GO and SWNTs had the ability to generate reactive oxygen species, which was crucial for PTX induced cell death, as potential co-therapeutics for PTX<sup>61</sup>. We believe that further *in vivo* investigations are needed to illuminate the anticancer ability of blank GO-PEG using different cancer models, and more prolonged course of therapy and different experimental conditions.

To the best of our knowledge, using GO as a carrier for PTX through covalent conjugation to

achieve *in vivo* therapeutic effects is a novel approach to improve the physiological solubility, bioavailability and tumor targeting ability of PTX. The unique features of the surface of GO facilitates the loading of other anticancer drugs or photosensitizers<sup>8, 12, 62</sup>, which might cause additive or synergistic effects along with the anticancer agent, and the GO-drug complex can also absorb near infrared light to achieve photothermal therapy over tumor tissue<sup>20, 32</sup>. Those investigations can be carried out in the future to improve GO-PTX system starting from the highly promising findings established in our present investigation.

## Conclusion

In summary, we loaded PTX on GO-PEG carrier system through covalent conjugation, and studied the therapeutic effects of GO-PEG-PTX *in vitro* and *in vivo*. Compared with Taxol<sup>®</sup>, GO-PEG-PTX exhibited better tumor suppressing efficacy due to the prolonged blood circulation time and higher uptake rate in tumor tissue. No obvious *in vivo* toxicity or tissue damage was found in mice after *i.v.* injection of GO-PEG-PTX for 30 days. All findings in this study indicated that PEGylated graphene oxide is an excellent nanocarrier for paclitaxel for cancer targeting. Further investigations in the future will include other anticancer drugs and differently surface engineered graphene oxide.

## Materials and methods

### 1. Materials and animals

Graphene oxide (GO) was purchased from Tianjin Plannano Technology Co., Ltd. Chloroacetic acid was purchased from Tianjin Fuchen Chemical Reagents Factory. N-(3-dimethylamino propyl-N'-ethylcarbodiimide) hydrochloride (EDC-HCl), N-hydroxysuccinimide (NHS), Succinic anhydride and pyridine were purchased from Chengdu Kelong Chemical Reagents Factory. Paclitaxel and docetaxel were purchased from Xi'an Hao-xuan Biological Technology Co., Ltd. Aminated polyethylene glycol (NH<sub>2</sub>-PEG-NH<sub>2</sub>, Mw = 4000). DMEM cell culture medium was bought from Thermo Scientific and fetal bovine serum was supplied by Fumeng Gene Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied by Sigma. BCA protein assay reagent kit was purchased from Pierce, USA. C57 mouse and Wistar rats were provided by Chengdu Dashuo Biotechnology Co., Ltd. All procedures with animals were conducted in accordance with institutional animal care and use guidelines.

### 2. Carboxylation of GO

To carboxylate Graphene oxide sheet, GO (10 mg) was added to water and sonicated to form an aqueous suspension. NaOH (0.12 g/mL) and chloroacetic acid (0.5 g) were then added, and the resultant mixture was sonicated for 3 h to form carboxyl group on the surface of GO. GO-COOH suspension was neutralized and purified by centrifugation at 10,000 rpm. The supernatant was discarded and the residue was washed with water twice.

### **3. Modification of GO with aminated polyethylene glycol**

Carboxylated GO was diluted by water (10 mL) and then bath sonicated with amino-terminated PEG4000 (100 mg) for 30 min, followed by addition of N-(3-dimethylaminopropyl-N'-ethylcarbodiimide) hydrochloride (EDC-HCl) to reach a concentration of 40 mmol/L. Then the mixture was allowed to react overnight. The final reactants were dialyzed in dialysis bags (MWCO = 10,000) for 4 days to obtain the PEGylated GO.

FT-IR absorbance spectrum, atomic force microscopy (AFM) and UV-vis spectrum were used to identify whether the carboxylation and PEGylation were successful.

### **4. Synthesis of 2'-O-succinyl-paclitaxel derivative**

Paclitaxel (200 mg) and succinic anhydride (0.3 g) were added to 5ml of anhydrous pyridine. The solution was stirred for 24 h at room temperature. The progress of reaction was detected by TLC (CH<sub>3</sub>OH/CHCl<sub>2</sub> 1:20 v/v). After that, 6 mL of water was added and stirred for 1 h at 60°C. The solvent (pyridine and water) was then evaporated under vacuum at 55°C using a rotary evaporator (BUCHI Rotavapor R-3). The resultant modified PTX was retrieved via extraction using ethyl acetate as solvent. Purification of the desired compounds was carried out by column chromatography on silica gel (the elution phase was CH<sub>3</sub>OH/CHCl<sub>2</sub> 1:50 v/v). <sup>1</sup>H-NMR (CDCl<sub>3</sub>) and LC-MS were employed to confirm the chemical structures.

### **5. Conjugation between GO-PEG and 2'-O-succinyl-PTX**

To synthesize GO-PEG-PTX, 2'-O-succinyl-PTX (20 mg) and GO-PEG aqueous solution (~0.4 mg/mL) were dissolved in DMSO-water component solvent (1:1 v/v) followed by addition of EDC-HCl (50 mg) and NHS (50 mg). The resultant solution was stirred at 25°C for 6 h, followed by 2 days of dialysis in DMSO and another 2 days in water. HPLC was used for identification of the conjugation of GO-PEG-PTX.

### **6. HPLC analysis**

HPLC assay methods were established for the detection of PTX concentration in cellular uptake, pharmacokinetics and biodistribution studies. Analysis was performed using Shimadzu instruments (Chiyoda-Ku, Kyoto, Japan) consisting of a 50 µL injector loop, a CTO-10A column thermostat, two LC-10AT pumps and an Diamonsil C18 reverse phase column. The column effluent was monitored at 227 nm with a flow rate of 1 mL/min at 35°C. The mobile phase was composed of acetonitrile and water (45: 55 v/v). The total run time for each sample was 15 min.

### **7. *In vitro* release behavior of PTX from GO-PEG-PTX**

GO-PEG-PTX was dissolved in PBS (pH 7.4) or C57 mouse serum and incubated for 48 hours at 37°C. The released PTX was separated via ultrafiltration using 100k Da MWCO filters, and the retained PTX was detected by HPLC at 4, 12, 24 and 48 hours incubation.

### **8. Cell culture and *in vitro* toxicity**

A549 and B16 melanoma cancer cell lines were cultured in DMEM with high glucose supplemented with 10% fetal bovine serum, 100 µg/mL of streptomycin and 100 µg/mL of

penicillin. Cells were placed in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. The cell medium was changed every other day. For the *in vitro* cell toxicity study, cells were seeded onto a 96 well bottom plate and incubated at 37°C overnight. The cells were then incubated with different concentrations of GO-PEG, GO-PEG-PTX or Taxol<sup>®</sup> (all dissolved in DMEM with high glucose) and blank DMEM with high glucose. After 24 hours of incubation, the relative cell viability was measured by MTT assay with Fluoroskan Ascent FL microplate fluorometer and luminometer (Thermo Scientific).

### **9. Cellular uptake assay**

Both A549 and B16 cells were seeded in 6 well plates. The cells were then exposed to GO-PEG-PTX or Taxol<sup>®</sup> at a range of concentrations (10, 25, 50, 100 µg/mL) for 2 h at 37°C. Cold PBS (20 °C pH 7.4) was used to wash the cells in order to remove the drug molecules which were not taken up by those cells. The cells were then digested and collected as the cellular uptake sample, which were centrifuged at 5,000 rpm for 5 min. The cell residue was lysed with RIPA buffer in order to release the intracellular drug. Methanol and 20% of trichloroacetic acid were added to the cell digests, followed by centrifugation at 12,000 rpm for 10 min. Supernatants were collected and analyzed by HPLC. The intracellular concentrations of PTX were investigated by HPLC assay using the method described earlier. 20 µL of the cell lysate from each sample was taken to determine the total cell protein content using reagent kit (Pierce, USA). The uptake rate was expressed as the amount of PTX associated with a unit weight of cellular protein.

### **10. Pharmacokinetic study**

Female Wistar rats (220±20 g) were randomly divided into two groups. The rats received GO-PEG-PTX (GO-PEG-PTX group) or Taxol<sup>®</sup> (Taxol<sup>®</sup> group) via tail vein at a dose equivalent to 2 mg/kg of PTX (n=5). At 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h intervals after injection, blood samples were taken and the plasma was separated by centrifugation at 5,000 rpm for 5 min. Docetaxel were added to each sample as the internal standard and methanol was used to precipitate the protein followed by centrifugation at 13,000 rpm for 10 min. Supernatants were collected and analyzed by HPLC following the method described above.

### **11. Animal model and drug efficacy study**

Murine B16 melanoma cancer model was established by subcutaneous injection of about 1.5×10<sup>6</sup> cells in PBS under the right arm of female C57 mice. The mice were used for experiments 14 days after injection (the tumor volume was about 100 mm<sup>3</sup>). For the treatment, 4 mg/mL of GO-PEG-PTX, Taxol<sup>®</sup>, GO-PEG and the same volume of saline were injected via caudal vein for 3 times (0, 3, 6 days after injection). The tumor size was measured by a caliper every day and was calculated as  $V = (\text{tumor length}) \times (\text{tumor width})^2/2$ . Relative tumor volumes were calculated as  $V/V_0$  ( $V_0$  was the tumor volume before mice were injected with the formulation). The survival rates of each group were then calculated to construct the survival rate curve.

### **12. Biodistribution study**

Female C57 mice bearing B16 tumor (tumor size was about 200 mm<sup>3</sup>) were intravenously injected with GO-PEG-PTX or Taxol<sup>®</sup> at a dose equivalent to 50 mg/kg of PTX. The mice were sacrificed at 30 min, 1 h, 4 h, 8 h, 12 h or 24 h after injection. Samples of blood, liver, spleen, lung, kidney

and tumor were then collected. The samples of the animals' blood were collected in heparinized tubes. Tissues were isolated, washed with saline and homogenized with 2 fold volume of 0.9% sodium chloride (g/mL). The blood samples and tissues homogenates were processed and measured by HPLC as described in the pharmacokinetic study.

### 13. *In vivo* toxicity study

Healthy female C57 mice (18~20 g) were randomly assigned into 4 groups for drug administration. GO-PEG-PTX or Taxol® with a dose equivalent to 5 mg/kg of PTX was intravenously injected into the mice in each group, and the mice in the other two groups were injected with the same volume of GO-PEG or saline via tail vein. The same dose of drug was injected every 5 days after the initial treatment, 6 times total. The weight of each mouse was recorded every day and the curve of the body weight change was established after the treatment. For histological evaluation, livers and spleens of mice were collected 30 days after the initial treatment, and were fixed in 4% v/v paraformaldehyde for 4 days. The samples were taken and stained with hematoxylin and eosin (H&E) for examination by light microscopy (Axiovert 40 CFL). For blood chemistry evaluation, blood samples were taken as soon as mice were sacrificed. The blood samples were centrifuged at 5,000 rpm and the serum was collected for chemistry analysis.

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