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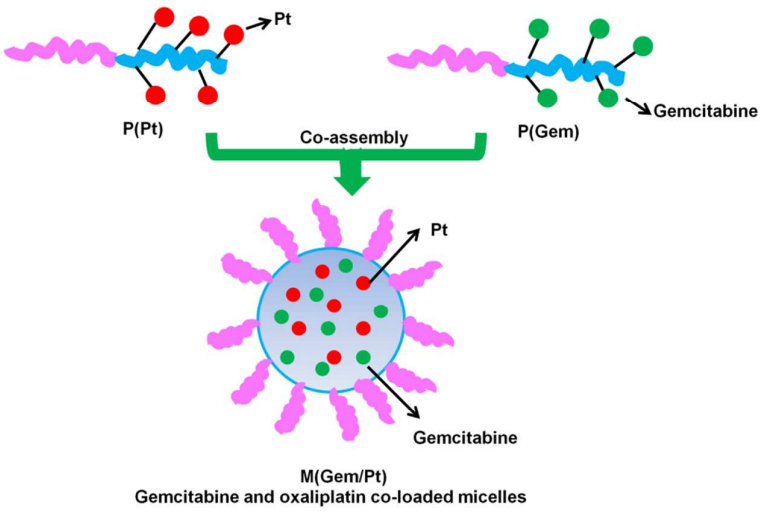

M(Gem/Pt)
Gemcitabine and oxaliplatin co-loaded micelles

A biodegradable polymer platform for co-delivery of clinically relevant oxaliplatin and gemcitabine

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Abstract: Anticancer drugs of gemcitabine and oxaliplatin combined in clinic regimen (GEMOX regimen) were co-loaded to a biodegradable polymer platform for drug delivery.

Introduction

Oxaliplatin, (trans-R,R-1,2-diaminocyclohexane) platinum(II) oxalate, is a third generation platinum drug, which is currently approved for clinic treatment of colon cancer worldwide[1,2]. Oxaliplatin generates the same type of inter- and 1,2-GG intrastrand cross-links as cisplatin but it has a spectrum of activity and mechanisms of action and resistance different from those of cisplatin and carboplatin[3,4]. However, oxaliplatin still has shown great side effects including neurotoxicity, hematological toxicity and gastrointestinal tract toxicity though the side effects are much milder than cisplatin[5,6].

Gemcitabine is a nucleoside analog of deoxycytidine which is used in various carcinomas including non-small cell lung cancer, pancreatic cancer, bladder cancer, colon and breast cancer[7-9]. Though it is very effective on several cancers, gemcitabine only has an *in vivo* half-time of 8-17 min [10]. To achieve the desirable anticancer effect, gemcitabine is typically administered in a higher dose. In clinic, extensive reports have shown that the anticancer effects of oxaliplatin are optimized when it is administered in combination with other anticancer agents, such as 5-fluorouracil, topoisomerase I inhibitors, taxanes and especially with gemcitabine [11-13].

In cilinic, gemcitabine combined with oxaliplatin is called GEMOX regimen. GEMOX regimen has a certain clinical curative effect in treatment of advanced malignant solid tumor, but the adverse side effects are still quite severe [14,15]. Selective targeting cancer cells by new generation of anticancer drugs are attracting [16-17]. Recent advances in nanotechnology open the door for drug delivery via encapsulating or conjugating anticancer drugs to a possible drug carrier for selectively targeting to the cancer cells [18-20]. Development of nanomedicine provides a possibility of co-delivering two or more drugs, enhancing the anti-tumor efficacy, overcoming drug resistance and reducing the side effects of anti-cancer drugs by prolonging the systemic circulation, improving the bio-distribution of the drugs, and passively or actively targeting the drugs to the cancerous sites [21-23].

Here, we introduce a biodegradable polymer platform for co-delivery of clinically relevant oxaliplatin and gemcitabine. As shown in Scheme 1, polymer conjugates of oxalipaltin and gemicitabine (abbreviated as P(Pt) and P(Gem), respectively) were synthesized by coupling them to biodegradable polymer carriers. Then, by simply mixing and co-assembling the two polymer-drug conjugates, hybrid micelles containing both gemcitabine and oxaliplatin (M(Gem/Pt)) can be obtained with great ease (Scheme 2). Moreover, via simply varying the weight ratio of P(Pt) to P(Gem) used for co-assembling, the dose ratio of gemcitabine to oxaliplatin in M(Gem/Pt) can be easily adjusted. The hybrid micelles were characterized via DLS and TEM. *In vitro* study revealed synergistic effect of M(Gem/Pt) similar to that of gemcitabine/oxaliplatin combination. *In vivo* animal biodsitribution study showed more accumulation of M(Gem/Pt) in the tumor site than that of the free drug combination. At last, the tumor inhibition study demonstrated that M(Gem/Pt) was much more efficacious than the single drug of oxaliplatin or gemcitabine and the combination of them. Therefore, the strategy used in this study, i.e., polymeric conjugates of different drugs and their co-assembling, provides a promising platform for co-delivering clinically relevant anticancer drugs with desired synergistic effect.

Results and Discussion

Synthesis of polymer conjugates P(Pt) and P(Gem)

Our previous work reported a biodegradable amphiphilic polymer carrier poly(ethylene glycol)-block-poly(L-lactide-co-2-methyl-2-carboxyl-propylene carbonate) (MPEG-b-P(LA-co-MCC)) used for chelating Pt(II) complexes onto its pendant carboxylic acid groups to form a polymer–Pt(II) conjugate (P(Pt))[24]. When the P(Pt) conjugate was self-assembled into micelles, the Pt species were located in the core part of the micelles because of the hydrophobic nature of the P(LA-co-MCC/Pt) segment, and the Pt species were effectively protected against the outside environment. The poly(ethylene glycol) corona of the micelles helps the micelles to resist protein adsorption and thus enables the micelles to circulate longer in the blood. These Pt-bearing micelles are expected to be internalized by the cancer cells via endocytosis. The Pt species would be released from the polymer backbones, escape from the endosomes and play their role of anti-tumor agent, while the polymer carrier itself can be degraded and excreted ultimately. Synthesis and characterization of the P(Pt) conjugates were carried out extensively in our previous work[24], and hence they will not be detailed here. The platinum content in P(Pt) was determined by ICP-MS to be 10 wt%[24].

Similarly, by simply conjugating gemcitabine with this biodegradable copolymer MPEG-b-P(LA-co-MCC) using DCC/NHS, polymer-gemcitabine conjugates (P(Gem)) can be obtained. To find out the drug content in P(Gem) conjugates, a series of stock solutions in water/acetone of gemcitabine were prepared and their UV curves from 240 nm to 340 nm were collected as shown in Figure 1a. Just by plotting the UV absorbance at 268 nm against the concentration of gemcitabine, a standard curve (Regression equation: $A = -0.0085 + 24.1333C$, $R^2 = 0.9991$) was obtained as shown in Figure 1b. By virtue of this standard curve (Assuming that it is suitable for gemcitabine in the conjugate) and UV measurement of 1 mg/ml P(Gem) solution in water/acetone (Figure 1c), the gemcitabine content in P(Gem) was determined to be ca. 10% w/w.

Preparation and characterization of polymer micelles M(P), M(Pt), and M(Gem)

As previously described [24], blank polymer micelles (M(P)) from MPEG-b-P(LA-co-MCC) and micelles M(Pt) from P(Pt) i.e., MPEG-b-P(LA-co-MCC/Pt) were prepared by adding enough water into the polymer solutions in acetone under stirring. As pointed out earlier, amphiphilicity is responsible for the micellization, and the hydrophobic P(LA-co-MCC) or P(LA-co-MCC/Pt) block forms the inner core and the hydrophilic MPEG block forms the shell. Because the platinum atom can chelate with the carboxyl groups from one or two different polymer chains, incorporation of Pt would lead to cross-linking of the micelle core. The blank polymer micelles M(P) and Pt-loaded micelles M(Pt) were characterized by TEM and DLS (Table 1, Figure 2). M(P) had a mean diameter of 70 nm by TEM and 77 nm by DLS with a zeta potential at −30 mV. M(Pt) had a mean diameter of 41 nm by DLS and 34 nm by TEM with a zeta potential of −12.8 mV[24].

Similarly, M(Gem) from P(Gem), i.e., MPEG-b-P(LA-co-MCC/Gem) were prepared by replacing P(Gem) for P(Pt) in the above procedure. The M(Gem) obtained had a mean diameter of 45 nm by DLS and 38 nm by TEM with a zeta potential of −5.6 mV (Table 1).

Preparation of M(Gem/Pt) at different Gem/Pt ratios

Because P(Pt) and P(Gem) have the identical polymer backbones and differ from each other only in the two drug molecules attached, it is possible to co-assemble them to form hybrid micelles M(Gem/Pt) with each micelle containing the two components. The practical operation is very simple, just to use their mixture solution instead of individual conjugates as the starting material of self-assembling. Because of the conjugation of gemcitabine and oxaliplatin with the hydrophobic segments of the polymers, both of them are expected to reside in the core part of the micelles along with the hydrophobic polymer block (Scheme 2). Considering that combination therapy requires definite dose ratio of the two drugs, three typical molar ratios of gemcitabine and oxaliplatin were designed, i.e., 0.5:1, 1:1, and 2:1. Approach to these ratios is realized via calculating the weights of P(Gem) and P(Pt) according to the above ratios and drug content in each polymer-drug conjugate and dissolving them together in acetone. After the co-assembling procedure, hybrid micelles M(Gem/Pt) were obtained. They are coded as M(Gem/Pt=0.5:1), M(Gem/Pt=1:1), and M(Gem/Pt=2:1), correspondingly.

Figure 2a and Figure 2b showed the particle sizes of M(Pt) and M(Gem) to be 30-40 nm by TEM. Figure 2c shows a spherical morphology of M(Gem/Pt) micelles. The mean particle size and zeta potentials of M(Gem/Pt) determined by TEM and DLS were collected in Table 1. Results showed that M(Gem/Pt=0.5:1), M(Gem/Pt=1:1) and M(Gem/Pt=2:1) had mean diameters of 42 nm, 73 nm and 115 nm, respectively, increasing with the Gem/Pt ratio. Keeping in mind that the particle sizes of M(Pt) and M(Gem) are only 34-38 nm, the bigger size of M(Gem/Pt) is attributed to the contribution of gemcitabine. The zeta potential of M(Gem/Pt=0.5:1), M(Gem/Pt=1:1), and M(Gem/Pt=2:1) were −8.6 mV, −6.8 mV and −4.3 mV, respectively, somewhat increasing with the Gem/Pt ratio. This may be because gemcitabine has more NH units than oxaliplatin moieties.

Drug release profiles of gemcibatine and platinum from M(Gem/Pt)

It is important that the hybrid micelles M(Gem/Pt) should release both drugs at a desirable kinetics so as to ensure their anticancer efficacy. To study this, drug release experiments were performed via dialysis method against buffered solution at pH5.0 and pH7.4, respectively. ICP-OES was used to determine the amount of platinum outside the dialysis bag and UV-vis spectroscopy was used to determine the gemcitabine released. For each drug, the relative accumulative release weight percentage with respect to the total drug payload in the sample was measured as a function of release time.

Figure 3a shows the drug release profiles at different pH values. From the release profiles, we can find following features: 1) both gemcitabine and Pt are released in a pH dependent way. They are released faster at pH5.0 than at pH7.4. For example, at 12 h, the cumulative release percentages of Pt at pH 5.0 and pH 7.4 are 68% and 33%, respectively, and those of gemciatbine are 56% and 49%, respectively. Obviously, this pH dependence is due to the hydrolysis of drug linkage with the polymeric backbone or the breakage of the polymer backbone as the polymer is biodegradable. 2) Among the four drug release curves, the one of Pt at pH7.4 is the lowest and that of Pt at pH5.0 is the highest. In other words, P(Pt) displays more pH dependence than P(Gem) does. This probably implies that the COO-Pt linkage in P(Pt) is more susceptible to acidolysis than the amide linkage in P(Gem) as depicted in Figure 3b.

The above pH dependence of both Gem and Pt release is of significance. It is well known that the pH of human blood is 7.4 and there is an acidic environment within cancer cells. The release rates of Pt and Gem are relatively low during blood circulation so that they may cause less side effects on healthy organs or tissues. Once the M(Gem/Pt) micelles get internalized, pharmaceutical Gem and Pt species can be released quickly and serve as anticancer agents. Given a M(Gem/Pt) composition, i.e., an initial P(Gem)/P(Pt) ratio, the release kinetics of Gem and Pt will determine the real dose ratio of Gem to Pt, that is usually a function of release time. As shown in Figure 3, Pt is released more rapidly than Gem at pH5.0 so that relatively Pt species is predominant at the earlier stage and Gem becomes predominant gradually.

***In vitro* MTT assay of single drugs**

Cytotoxicities of various drugs were evaluated by MTT assay with human breast cancer MCF7 cells as test cells. Firstly, MTT was performed for gemcitabine, oxaliplatin, M(Gem), and M(Pt). As our previous study[24] has shown very low cytotoxicity of M(P) i.e., MPEG-b-P(LA-co-MCC), all cytotoxicity observed is attributed to the Pt or Gem species existing in or released from the formulations, not to the polymer carrier itself. As shown in Figure 4, the cells treated with the four drugs give lower cell viability values at 72 h than 48 h; correspondingly, two sets of IC_{50} values are obtained (Table 2). Gemcitabine is less cytotoxic than oxaliplatin so that the IC_{50} of gemcitabine is at least 3 times of that of oxaliplatin (Figure 4a, Table 2). At a drug concentration of 100 μmol/L, the cells treated with gemcitabine had a cell viability of 37.8% at 48 h and 29.5% at 72 h, while these data for oxaliplatin treated cells are 2.51% and 0.22%, respectively. To a first approximation, cytotoxicities of M(Gem) and M(Pt) are comparable with those of gemcitabine and oxaliplatin, correspondingly. But the IC_{50} difference between M(Gem) and M(Pt) is smaller than that between gemcitabine and oxaliplatin (Table 2), indicating that micellarization of gemcitabine and oxaliplatin do affect their cell uptake and intracellular kinetics to a certain extent.

***In vitro* MTT assay of Gem/Pt combinations and M(Gem/Pt)s at different ratios**

To evaluate our strategy of using one biodegradable polymer to co-deliver both gemcitabine and oxaliplatin, we compared the free Gem/Pt combinations and micellar M(Gem/Pt)s at three different ratios,

namely, Gem/Pt = 0.5, 1 and 2 on MCF7 cells at 48 h and 72 h. The results were collected in Figure 5 and Table 3, with the viability as a function of Pt concentration, because, as shown in the previous section, oxaliplatin has lower IC_{50} than gemcitabine and Pt species is released more rapidly than Gemcitabine from M(Gem/Pt). For the same reason, only the IC_{50} of oxaliplatin is discussed hereafter.

For small molecule Gem/Pt combinations, IC_{50} of the three formulations (Gem/Pt = 0.5:1, 1:1, and =2:1) is 3.6, 3.0 and 0.73 μM of Pt, respectively (Table 3) for the cells cultured for 48 h, and 0.89, 0.69 and 0.55 μM, respectively, for 72 h. Bearing in mind that the IC_{50} of oxaliplatin alone is 11.0 and 3.6 μM at 48 and 72 h, respectively, the synergistic effect between oxaliplatin and gemcitabine is obvious. Especially for the Gem/Pt=0.5:1 formulation, the Pt concentration of 3.6 μM corresponds to a gemcitabine concentration of 1.8 μM, much lower than the IC_{50} of gemcitabine alone (34.5 μM at 48 h, Table 2). In other words, incorporation of small amount of gemcitabine in the formulation greatly enhances the cytotoxicity of oxaliplatin. This is in agreement with the clinic practice.

The IC_{50} values of Pt for the three M(Gem/Pt) formulations M(Gem/Pt=0.5:1), M(Gem/Pt=1:1), and M(Gem/Pt=2:1) were 4.4, 2.7, and 1.3 μM, respectively, at 48 h (Table 3) and 3.6, 3.4 and 2.6, respectively, at 72 h. Comparison to the IC_{50} of oxaliplatin alone of 16.8 μM reveals the synergistic effect between P(Gem) and P(Pt) in M(Gem/Pt). It is noticed that the IC_{50} values of M(Gem/Pt)s were not low enough at 72 h compared to those at 48, but the synergistic effect is still significant (IC_{50}: 3.6 vs. 8.4 μM, Table 3 and Table 2).

Combination index analysis of drug combinations

To further quantify the synergistic effect, combination index (CI) was calculated from the IC_{50} data obtained according to previously published work [26,27]. The CI values lower than, equal to, and higher than 1 denote synergism, additivity, and antagonism, respectively. The CI values for Gem/Pt and M(Gem/Pt) at various ratios both at 48 h and 72 h were depicted in Figure 6. It can be clearly found that for both Gem/Pt and M(Gem/Pt) combinations at 48 h and 72 h, the CI values are well below 1 with the only exception of small molecule formulation Gem/Pt=1:1, the CI of which is near 1.0. This convincingly means great synergy in these combinations. Moreover, as analyzed earlier, taking the fact into consideration that gemcitabine is released from M(Gem/Pt) relatively more slowly than Pt species so that the real dose ratio of Gem/Pt in cancer cells is lower that the apparent one, the synergy observed in M(Gem/Pt)s would have more significance compared to that in small molecule Gem/Pt combinations.

***In vivo* biodistribution study of Gem/Pt and M(Gem/Pt)**

An attracting advantage of co-delivering both gemcitabine and oxalipaltin in this biodegradable polymer platform is to potentially prolong the blood circulation of the drugs, and hence to enhance drug accumulation at the tumor site, to improve anti-cancer efficacy, and to reduce side effects [28,29]. Therefore, it is vital to get some insight into the drug biodsitribution of Gem/Pt and M(Gem/Pt). The test

mice were given Gem/Pt or M(Gem/Pt) at a dose of 5 mg/kg of Pt and 10 mg gemcitabine/kg body weight. One hour after drug administration via intravenous injection, mice were sacrificed and their blood, cancer tissue, and main visera were collected, and platinum contents in these organs were detected by ICP-MS, expressed as "μg Pt per g of tissue or blood", and were considered to stand for "drug content" of Gem/Pt or M(Gem/Pt). The results are shown in Figure 7. Several features of the drug biodistribution can be seen: (1) At 1 h, Gem/Pt accumulates much more in the kidney (8.1 μg Pt/g), while less M(Gem/Pt) was found in the kidney(5.1 μg Pt/g). It is known that drug content in kidneys is associated with kidney clearance and nephrotoxicity of the both oxaliplatin and gemcitabine. This pair of data implies reduction of nephrotoxicity for M(Gem/Pt). (2) The drug contents in blood and cancer tissue of M(Gem/Pt) are over two times those of Gem/Pt (4.4 and 2.9 μg Pt/g vs. 2.8 and 1.3 μg Pt/g). These differences may result in enhanced efficacy and slower blood clearance of M(Gem/Pt). (3) Quite a fraction of M(Gem/Pt) exists in liver (9.8 μg Pt/g), spleen (7.3 μg Pt/g) and lung (8.3 μg Pt/g), while corresponding gem/Pt contents are 5.0, 4.3, and 3.3 μg Pt/g, respectively). This feature may cause damages in these organs on one hand, and may be used to target tumors in these organs on the other hand.

In vivo antitumor study

At last, the efficacy of M(Gem/Pt) combinations were evaluated using a xenograft model of H22 cancer developed by subcutaneous injection of H22 cells in the anterior limb of KM mice. When tumors were ca. 50-100 mm^3 in size, 5 days after inoculation of the cancer cells, the mice were randomly divided into 7 groups with 10 mice in each group and were given the drugs intravenously on day 0, 2 and 4, with the day of the first injection counted as day 0. The tumor size and body weight were then monitored every two days for 17 days. The results are shown in Figure 8 along with drug doses for each group.

As shown in Figure 8a, compared with the PBS control group, gemcitabine alone group (10 mg/kg), oxaliplatinon alone group (5 mg/kg), and Gem/Pt combination group (10 mg/kg of Gem plus 5 mg/kg of Pt) displayed considerable tumor inhibition effect. The tumor volumes were in the order of Gem/Pt combination group < oxaliplatinon alone group < gemcitabine alone group in the first week, but 8 out of 10 mice of the oxaliplatin alone group and all mice of the Gem/Pt group died at the end of the first week, obviously due to great systemic toxicity of oxaliplatin.

In contrary, there were no death events in three micellar groups, i.e., M(Gem) group (10 mg/kg), and M(Pt) group (5 mg/kg), and M(Gem/Pt) group (10 mg/kg of Gem plus 5 mg/kg of Pt). This indicates great reduction of the systemic toxicity caused by the micellarization of oxaliplatin and provides a possibility of using a higher dose of oxaliplatin to achieve a higher efficacy. Among the three micellar groups, M(Gem) exhibited less efficacy than gemcitabine alone, probably because M(Gem) has to undergo a process of cell uptake and intracellular release. The other two groups exhibited much better tumor volume inhibition, especially the M(Gem/Pt) group. It was the best among the 7 groups tested. Its difference from M(Pt)

group was significant although both of them had the same doses of gem and Pt. This is ascribed to the synergistic effect between the released species *in vivo* of gemcitabine and oxaliplatin from M(Gem/Pt).

The relative body weight changes were collected in Figure 8b. It can be clearly found that the oxaliplatin alone group (5 mg/kg) and Gem/Pt combination group (10 mg /kg of Gem plus 5 mg/kg of Pt) showed serous weight loss and immediately after the period of drug injection (from day 0 to day 4) and almost all mice died in one week. On the contrary, the M(Pt) and M(Gem/Pt) group mice showed considerable weight loss, but recovered from the damage caused by drug injection, gradually gained body weight after one week, and finally survived the test for 17 days, denoting lower toxicity and safety of the micellar nanoparticles of M(Gem/Pt) compared to the free gemcitabine/oxaliplatin combination.

Pt-DNA adducts formation

An effective drug delivery system is required to deliver the drug, gene or protein to the cancer cells and also to ensure the interaction of the drug, gene or protei with the proper organelles in the cancer cells [30-32]. As DNA is supposed to be the final intracellular target of platinum drugs, the amount of platinum-DNA adducts formed in the cancer cells is believed to be a measure of their efficacy [33]. Therefore, following experiment was carried out to detect the Pt-DNA formation in the animal tumors after treatment of drugs: The tumor bearing mice (with tumors of ca. 1000 mm^3) were randomly divided into 7 groups with 3 mice in each group and given 7 different formulations via intravenous injection. 24 h post drug injection, the mice were sacrificed, the tumor tissues were harvested, the tumor cells were collected, and all DNAs in the tumor cells were separated and purified. Finally the platinum content in the “total DNA” was determined by inductively coupled plasma mass spectroscopy (ICP-MS) as a measure of the Pt-DNA adducts formed.

As shown in Figure 9, the amount of Pt-DNA adducts formed is dose dependent, from 2.62 to 3.49 (pg Pt)/(μg DNA) with increasing Pt dose from 5 to 20 mg/kg. Two gemcitabine/oxaliplatin combinations also showed Pt-dose dependence. Notably, Pt-DNA adducts detected in M(Pt) group (5 mg/kg) and M(Gem/Pt) group (10 mg/kg of Gem plus 5 mg/kg of Pt) was at a level of 17.7 and 25.3 (pg Pt)/(μg DNA), respectively, much higher than the small molecule drug groups. This is ascribed to the micellarization of the drugs. These measured levels of Pt-DNA adducts provide convincing evidence or explanation for the above MTT results (Figures 4 and 5) and *in vivo* tumor inhibition data (Figure 8).

Conclusion

In summary, a polymer-oxaliplatin conjugate and a polymer–gemcitabine conjugate were prepared by attaching oxaliplatin and gemcitabine to a biodegradable amphiphilic block copolymer containing pendant carboxylic acid groups, respectively. The two conjugates were further co-assembled into hybrid micelles, M(Gem/Pt) at different gemcitabine/oxaliplatin ratios. Representative micelles which had a molar ratio of Gem to Pt equal to 2:1 possessed a spherical shape with a mean diameter of 132 nm and with a surface potential of −4.3 mV. The hybrid micelles could release oxaliplatin and gemcitabine under the intracellular

conditions. The *in vivo* study showed that M(Gem/Pt) had much lower systemic toxicity and enhanced efficacy against xenograft cancer model than gemcitabine alone, oxaliplatin alone or even Gem/Pt combinations.

Materials and Methods

Materials

N,N-dicyclohexylcarbodiimide(DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich. Gemcitabine was purchased from Aladdin (Shanghai). 1,1-diamine-cyclohexane-platinum(II) dichloride (DAHPt(II)) was prepared as previously described in our published paper[24]. The block copolymers poly(ethylene glycol)-block-poly(L-lactide-co-2-methyl-2-carboxyl-propylene carbonate) (MPEG-b-P(LA-co-MCC)) was synthesized in our laboratory as previously described[24]. The molecular formula of all the polymers used in this paper was $MPEG_{5000}$-b-P(LA_{1000}-co-MCC_{960}) determined by proton nuclear magnetic resonance (^1H NMR). Other chemicals and solvents were obtained commercially and used without further purification.

General methods

An inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the total platinum contents in the MPEG-b-P(LA-co-MCC/Pt) conjugate (P(Pt), Scheme 1) and samples obtained outside of the dialysis bags in drug release experiments. An inductively coupled plasma mass spectrometer (ICP-MS, Xseries II, Thermoscientific, USA) was used for quantitative determination of trace levels of platinum. The morphology of the polymer micelles was measured on a JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS90. UV-vis spetra of the stock solutions of gemcitabine in H_2O/acetone(50%v/50%v) were recorded on a UV-2400 spectrophotometer (2100, Shimadzu). For testing the drug content in the polymer conjugates, polymer drug conjugates were dissolved in H_2O/acetone(50%v/50%v) and tested on the UV-2400 spectrophotometer.

Synthesis of MPEG-b-P(LA-co-MCC/Pt) conjugates (P(Pt))

P(Pt) was prepared as previously described in our published paper [24].

Synthesis of MPEG-b-P(LA-co-MCC/Gem) conjugates (P(Gem))

Gemcitabine was conjugated to the polymer MPEG-b-P(LA-co-MCC) with the help of DCC and DMAP. Briefly, gemcitabine (0.182 g, 0.5 mmol) was dissolved in 20 ml dried dichloromethane (DCM) under stirring in a round flask, to which MPEG-b-P(LA-co-MCC) (0.33 g, 0.282 mmol carboxyl group) dissolved in 20 ml CH_2Cl_2 was added. Thereafter, DCC (0.103 g, 0.3 mmol), DMAP (0.030 g, 0.25 mmol)

were added into the mixed solution. The reaction was carried out in ice bath for 24 hours. And then the reaction solution was filtered to remove DCU formed and precipitated by ethyl ether. The solid product was collected by filtration and vacuum-dried to get white powders. Then the product was dissolved in dimethyl sulfoxide (DMSO) and the solution was dialyzed against water to remove un-reacted gemcitabine, and finally lyophilized to obtain P(Gem) conjugate.

Preparation of P(Pt) micelles (M(Pt)), P(Gem) micelles (M(Gem)), and hybrid micelles of P(Gem) and P(Pt) (M(Gem/Pt=0.5:1), M(Gem/Pt=1:1) and M(Gem/Pt=2:1))

The hybrid micelles M(Gem/Pt) were prepared by co-precipitation method with a molar ratio of Gem/Pt = 0.5:1,1:1 and 2:1. Taking the ratio of Gem/Pt=1:1 as an example, briefly, P(Gem) and P(Pt) with a molar ratio of Gem/Pt equal to 1:1 were mixed and dissolved in a flask containing an prescribed amount of acetone (total polymer concentration 10% w/v), and then water of double volume of the acetone used was added drop-wise into the flask under stirring to form a micellar solution. The solution was rotary evaporated to remove acetone and then freeze-dried to obtain the M(Gem/Pt). The individual micelles of P(Gem) and P(Pt) were prepared in a similar way. To simplify the nomination of all the micelles, “M” is used to stand for micelles. Therefore, P(Gem) micelles can be written as “M(Gem)” and M(Gem/Pt) stands for combination of P(Gem) and P(Pt).

Drug Release from hybrid micelles M(Gem/Pt=0.5:1)

50 mg of M(Gem/Pt=0.5:1) was dissolved in 5 ml of phosphate buffer solution (PBS, 0.01 M, pH 7.4). The solution was then placed into a pre-swollen dialysis bag (molecular weight cutoff of 3.5 kDa) and immersed into 45 ml of PBS. The dialysis was conducted at 37 °C in a shaking culture incubator. 1 milliliters of aliquot was withdrawn from the incubation medium at specified time intervals. After sampling, equal volume of fresh PBS was immediately added into the incubation medium. The same drug release procedure was performed in acetate buffer solution at pH 5.0. Platinum and gemcitabine released from the hybrid micelles were measured by ICP-OES and UV-vis (wavelength: 268 nm), respectively. Each of the drugs released from the micelles was expressed as cumulative percentage of the drug outside the dialysis bag to the total drug in the original micelles.

MTT (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

MCF7 (human breast cancer) cells were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and cultured in DMEM (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Life Technologies), 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C.

MCF7 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 10^4 cells/well and incubated in DMEM overnight. The medium was then replaced with various drug formulations of oxaliplatin, M(Pt), gemcitabine, M(Gem), free gemcitabine/oxaliplatin (Gem/Pt)

combinations with Gem/Pt molar ratio of 0.5:1, 1:1 and 2:1, and micellar combinations M(Gem/Pt)s with Gem/Pt molar ratio of 0.5:1, 1:1 and 2:1,abbreviated as M(Gem/Pt=0.5:1), M(Gem/Pt=1:1), and M(Gem/Pt=2:1), respectively. All of the drugs containing platinum were modulated to a final equivalent Pt concentration from 0.0064 to 100 μM (5× dilution). Various gemcitabine concentration for gemcitabine alone and M(Gem) group was also adjusted by 5× dilution, ranging from 0.0064 to 100 μM. The incubation of each drug was continued for 48 h and 72 h. Then, 20 μL of MTT solution in PBS at a concentration of 5 mg/ml was added and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT and addition of 150 μL of DMSO to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 minutes, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

Mice use

Chinese KM mice (6–8 week old, female, 18–25 g) were purchased from Jilin University (Changchun, China) and maintained under pathogen-free conditions and they had free access to food and water throughout the experiments. The animal use protocol was approved by the Animal Ethics Committee of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences.

Tumor model establishment

H22 cells (murine liver cancer cell lines) were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and cultured in DMEM (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies), 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C. The mouse H22 xenograft tumor model was developed by injecting 1×10^6 cells in 0.1 ml of H22 cell/PBS suspension into the right flank of a KM mouse. The tumor nodules were allowed to grow to desirable volume before use.

Biodistribution

The tumor model was established as described above. When the tumor nodules grew to ca. 1000 mm^3, tumor-bearing KM mice were randomly assigned to 2 groups with 3 mice for each group and were intravenously injected with free gemcitabine/oxaliplatin combination or M(Gem/Pt) (both 5 mg/kg of Pt plus10 mg/kg of Gem). After 1 h, the mice were sacrificed. The Pt contents in the collected blood, cancer tissues and organs were measured by ICP-MS. Data were shown as mean value ± S.D.

In vivo antitumor efficacy

The tumor model was established as described above. When the tumor nodules grew to ca. 50-100 mm^3, tumor-bearing KM mice were randomly assigned to 7 groups with 10 mice in each group and mice were injected with gemcitabine (10 mg/kg), oxaliplatin (5 mg/kg of Pt), gemcitabine/oxaliplatin

combination (10 mg/kg of Gem plus 5 mg/kg of Pt), M(Pt) (5 mg/kg), M(Gem) (10 mg/kg), and M(Gem/Pt) (10 mg/kg of Gem plus 5 mg/kg of Pt) and PBS, respectively. Mice were intravenously injected three times on day 0, 2, and 4, respectively.

Tumor length (major axis of the tumor) and width (minor axis of the tumor) were measured with calipers. Body weight and tumor volume of each mouse were measured every two days over a period of 17 d. The tumor volume was calculated using the following equation: Tumor volume (V) = length × width^2/2, as previously described [26,27]. Tumor growth and relative body weight curves were plotted using the average tumor volume and mean relative body weight in each group.

In vivo Pt-DNA adducts formation

The tumor model was established as described above. When the tumor nodules got a size of ca. 1000 mm^3, tumor-bearing KM mice were randomly assigned to 7 groups with 3 mice in each group and mice were injected once with oxaliplatin (5 mg/kg of Pt,10 mg/kg of Pt and 10 mg/kg of Pt), M(Pt) (5 mg/kg of Pt), gemcitabine/oxaliplatin combinations (10 mg/kg of Gem plus 5 mg/kg of Pt; 10 mg/kg of Gem plus 10 mg/kg of Pt), and M(Gem/Pt) (10 mg/kg of Gem plus 5 mg/kg of Pt; 10 mg kg of Gem plus 10 mg/kg of Pt). At 24 h post drug injection, the mice were sacrificed and the tumors were collected, washed several times with cold PBS and dried. Genomic DNA was separated from the collected solid tumor samples and purified using DNAZOL (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instruction. The final DNA pellet was air-dried and then dissolved in 0.1 mL distilled water overnight. The next day, the DNA concentration and purity was determined by measuring absorbance at 260/280 nm with a nanodrop UV spectrometer (NanoDrop Technologies, Inc., Wilmington, DE). An aliquot of DNA (60 μL) was digested with 70% nitric acid (64 μL) in a 65 °C water bath overnight. This was diluted with water (776 μL) containing indium and Triton X-100 to achieve a final concentration of 5% acid (final concentration of 1 ppb for indium and 0.05% Triton X-100). The Pt concentration was then determined by inductively coupled plasma mass spectroscopy (ICP-MS).

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Student's t-test was used to determine the statistical difference between various experimental and control groups. Differences were considered statistically significant at a level of $P < 0.05$.

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Table 1. Physical parameters of various micelles prepared

Micelles	Particle diameter (nm)		Zeta potential (mV)[a]
	TEM	DLS	
Blank micelles	70	77	−30
M(Pt)	34	41	−12.8
M(Gem)	38	45	−5.6
M(Gem/Pt=0.5:1)	42	50	−8.6
M(Gem/Pt=1:1)	73	89	−6.8
M(Gem/Pt=2:1)	115	132	−4.3

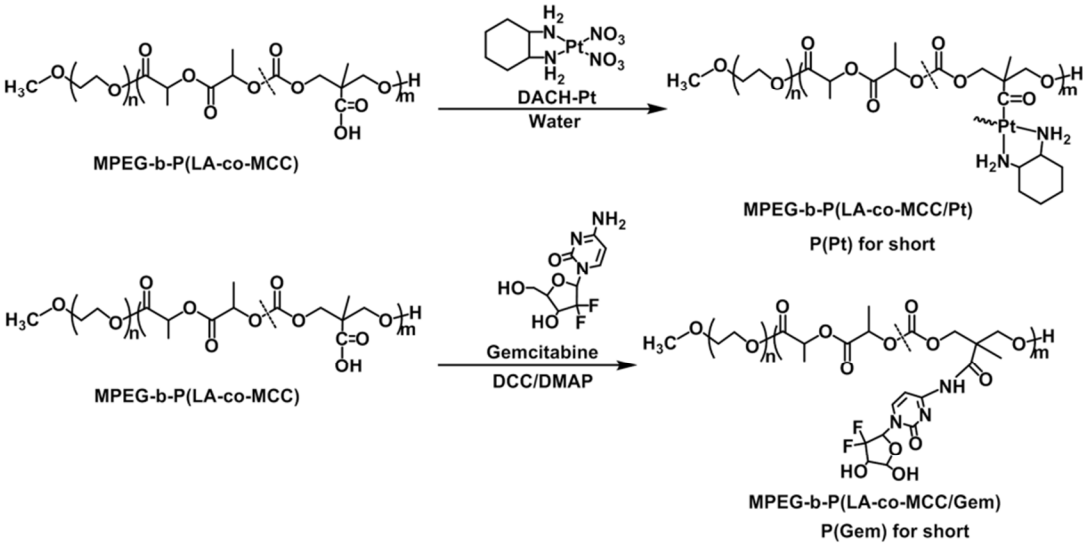
a: Zeta potential data for blank micelles and M(Pt) were extracted from our previous publication[24].

Table 2. IC_{50} values of single drugs on MCF7 cells at 48 h and 72 h

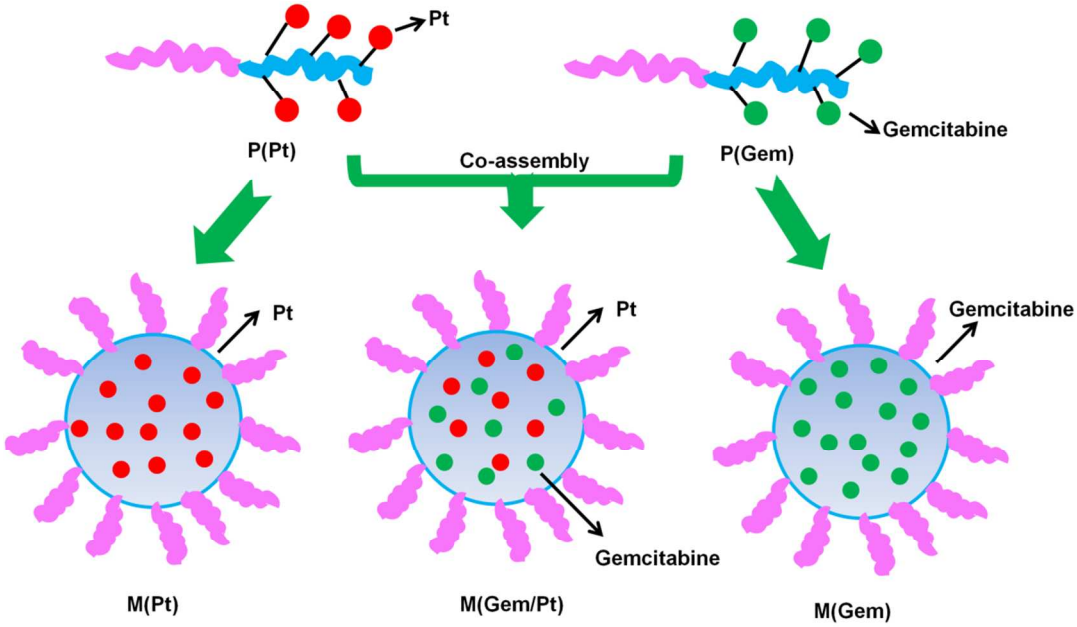
Drug	IC_{50}(μmol/L)	
	48 h	72 h
Gemcitabine	34.5	2.7
Oxaliplatin	11.0	3.6
M(Gem)	19.9	8.4
M(Pt)	16.8	8.4

Table 3. IC_{50} values of combined drugs on MCF7 cells at 48 h and 72 h

Drug	Gem/Pt (mol/mol)	$IC_{50}$of Pt(μmol/L)	
		48 h	72 h
Gem/Pt=0.5:1	0.5:1	3.6	0.89
Gem/Pt=1:1	1:1	3.0	0.69
Gem/Pt=2:1	2:1	0.73	0.55
M(Gem/Pt=0.5:1)	0.5:1	4.4	3.6
M(Gem/Pt=1;1)	1:1	2.7	3.4
M(Gem/Pt=2:1)	2:1	1.3	2.6



Scheme 1. Preparation of biodegradable polymer conjugates of oxaliplatin (P(Pt)) and gemcitabine (P(Gem)). The active species of oxaliplatin, 1,2-diamine-cyclohexane platinum(II) (DACHPt) was attached to the polymer chains.



Scheme 2. Self-assembly and co-assembly of P(Pt) and P(Gem) to form single drug loaded micelles M(Pt) and M(Gem) and dual drug loaded micelles M(Gem/Pt).

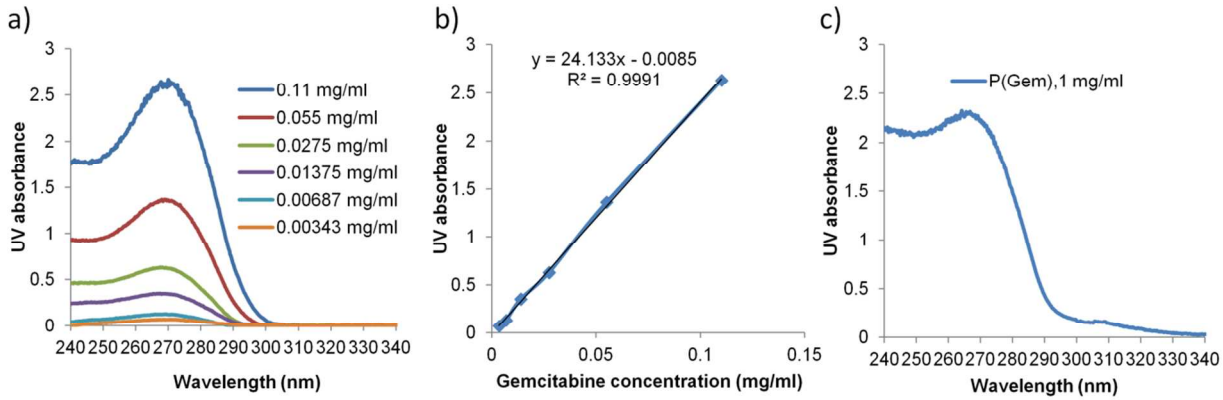


Figure 1. Determination of the gemcitabine content in P(Gem) conjugates.(a) UV-vis spectra of stock solutions of gemcitabine in water;(b) standard curve of gemcitabine stock solutions; (c) UV-vis spectra of P(Gem) at 1 mg/ml.

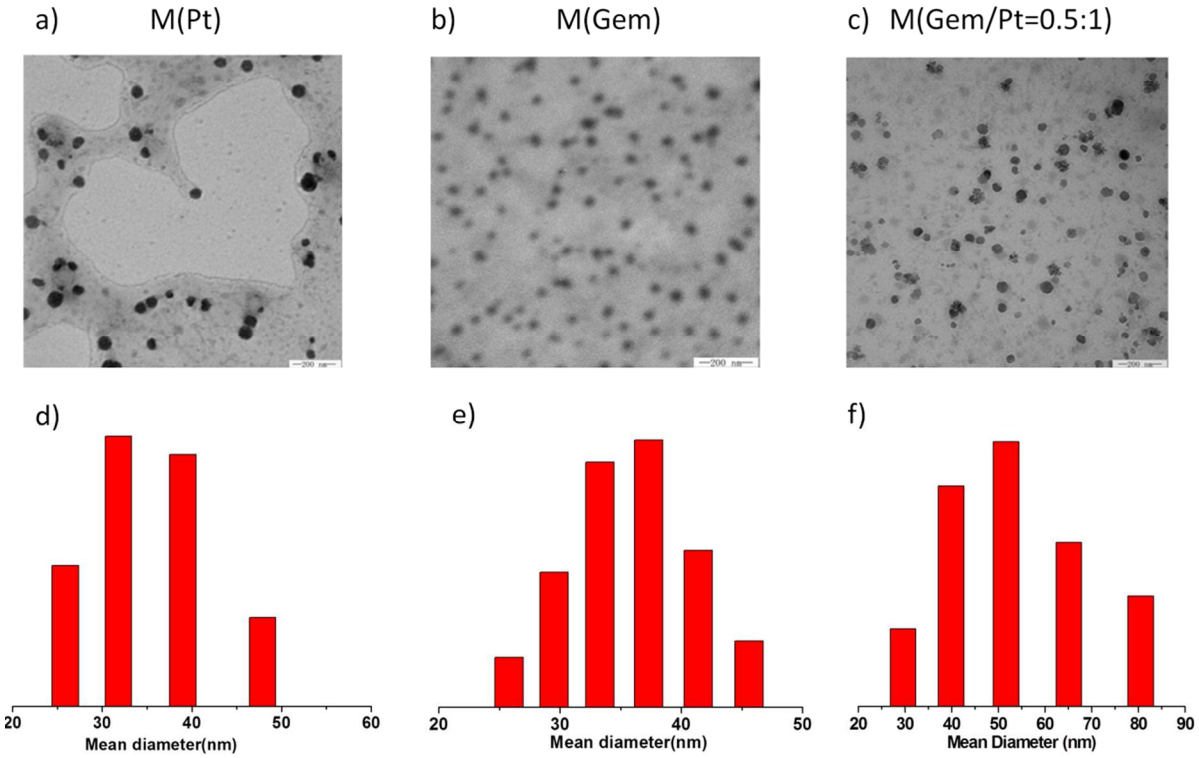


Figure 2. Representative TEM (a-c)and DLS images(d-f) of M(Pt) (a,d), M(Gem) (b,e) and M(Gem/Pt=0.5:1) (d,f).

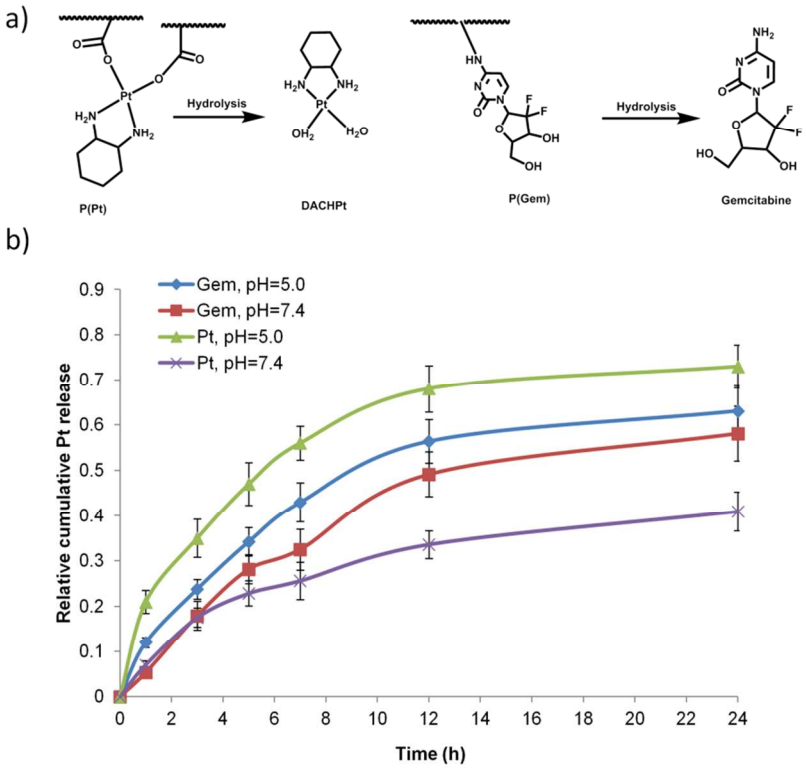


Figure 3. Possible gemcitabine and Pt drug release pathways from M(Gem/Pt) (a) and drug release profiles of M(Gem/Pt) at pH=5.0 and pH=7.4 (b).

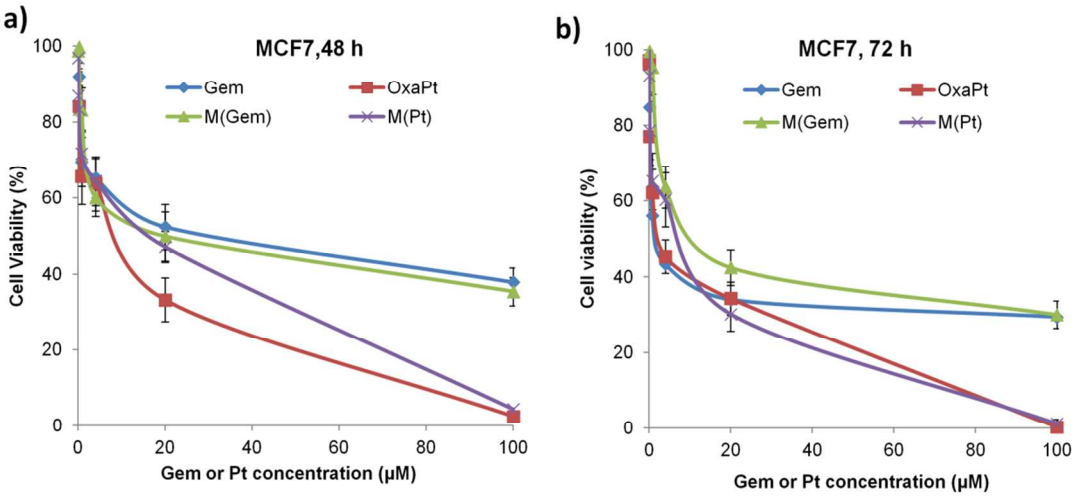


Figure 4. *In vitro* evaluation of single drug gemcitabine, oxaliplatin, M(Gem) and M(Pt) on MCF7 cancer cells at 48 h(a) and 72 h(b).

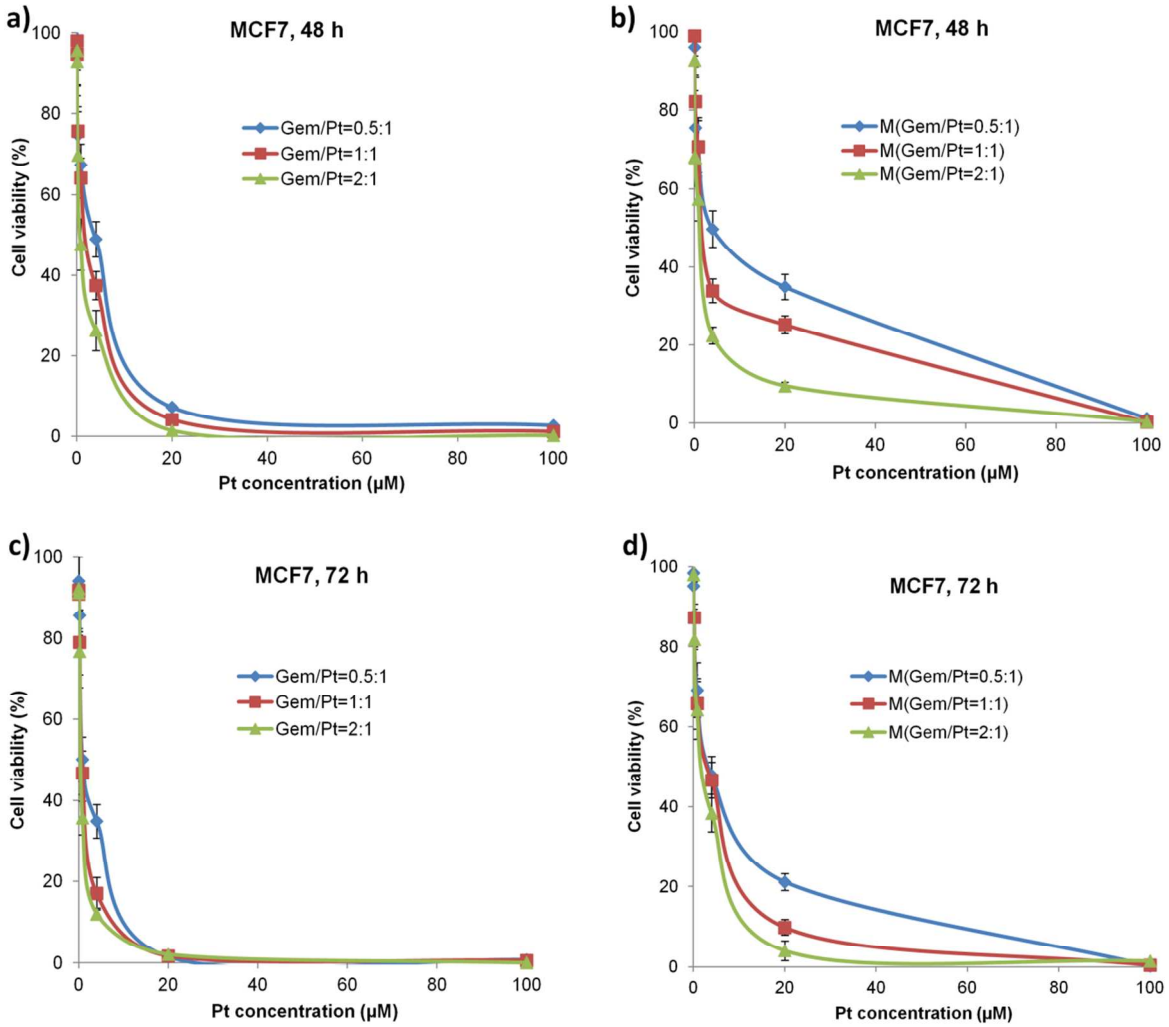


Figure 5. *In vitro* evaluation of gemcitabine/oxaliplatin combination (a, c) and hybrid M(Gem/Pt) (b, d) at various ratios at 48 h (a, b) and 72 h(c, d).

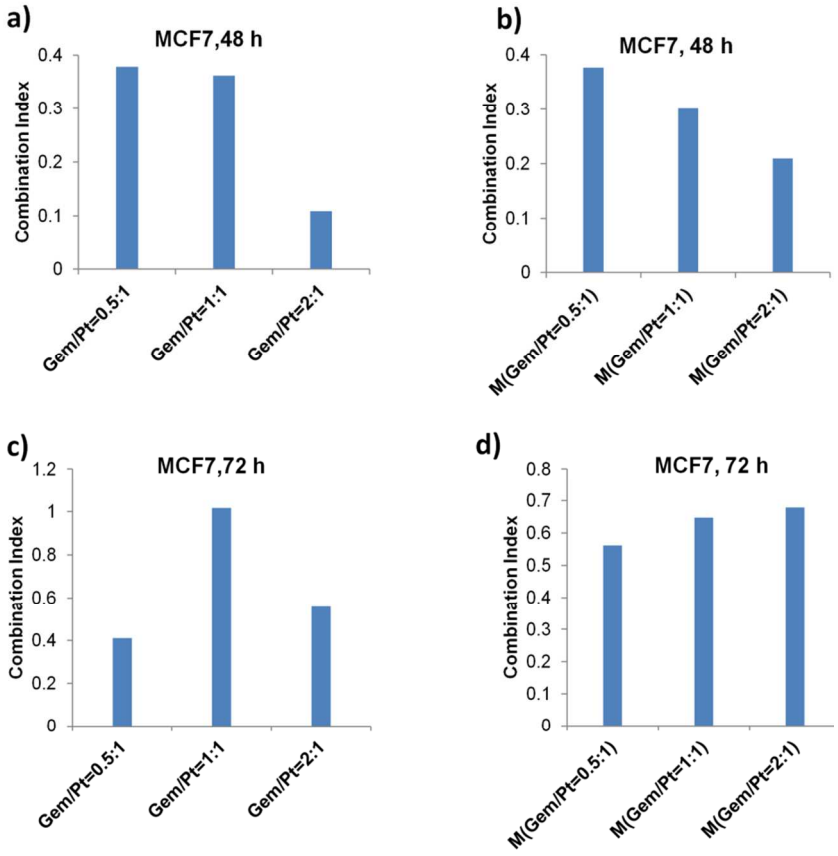


Figure 6. Combination Index of gemcitabine and oxaliplatin combination (a, c) and hybrid M(Gem/Pt) (b, d) at 48 h (a, b) and 72 h (c, d).

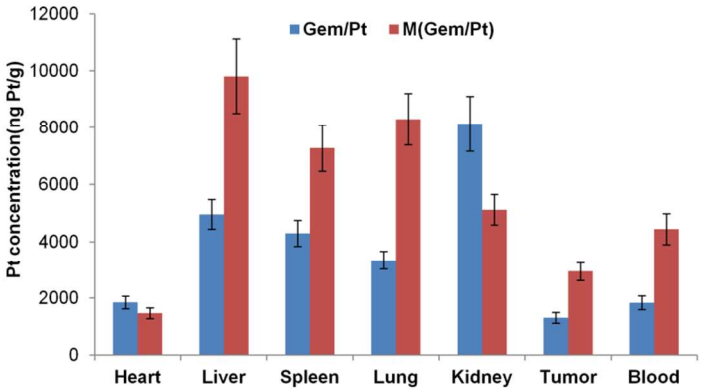


Figure 7. Biodistribution of free combination of gemcitabine and oxaliplatin as well as the nanoformulation of M(Gem/Pt) at 1 h. The drug dose for the two combinations was set the same (10 mg gemcitabine/kg plus 5 mg Pt/kg). Only the Pt contents in the collected tissues or organs were tested via ICP-MS. Each group consisted of 3 mice. Data were shown as mean value ± S.D.

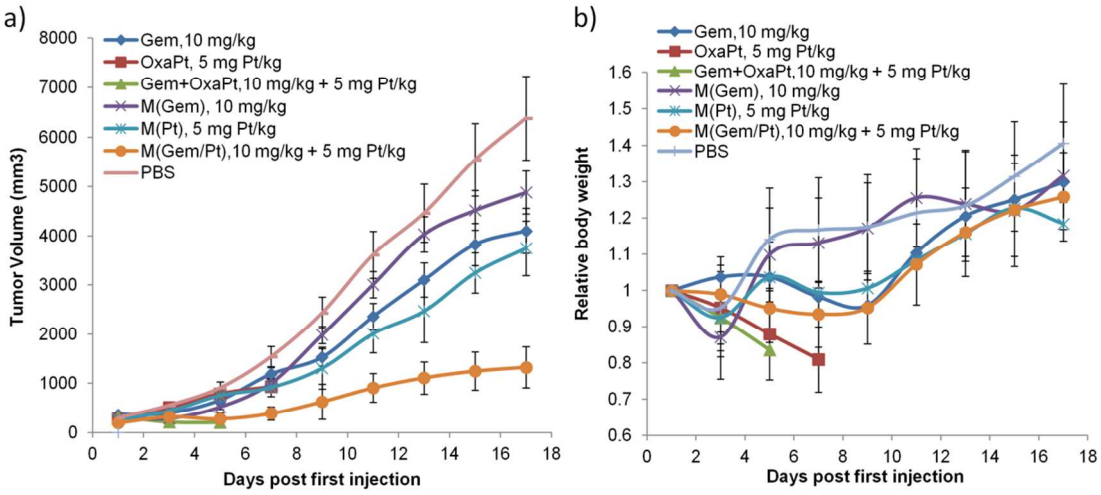


Figure 8. *In vivo* evaluation of various drugs on tumor models. Mice were injected with gemcitabine (10 mg/kg), oxaliplatin (5 mg Pt/kg), gemcitabine (10 mg/kg) and oxaliplatin (5 mg Pt/kg), M(Pt) (5 mg Pt/kg), M(Gem) (10 mg/kg), and M(Gem/Pt) (gemcitabine: 10 mg/kg; Pt: 5 mg/kg) and PBS. Gem and OxaPt were used to indicate gemcitabine and oxaliplatin respectively. Ten mice were in each group. (a) Tumor volume versus the days post first injection; (b) Relative body weight versus the days post first injection.

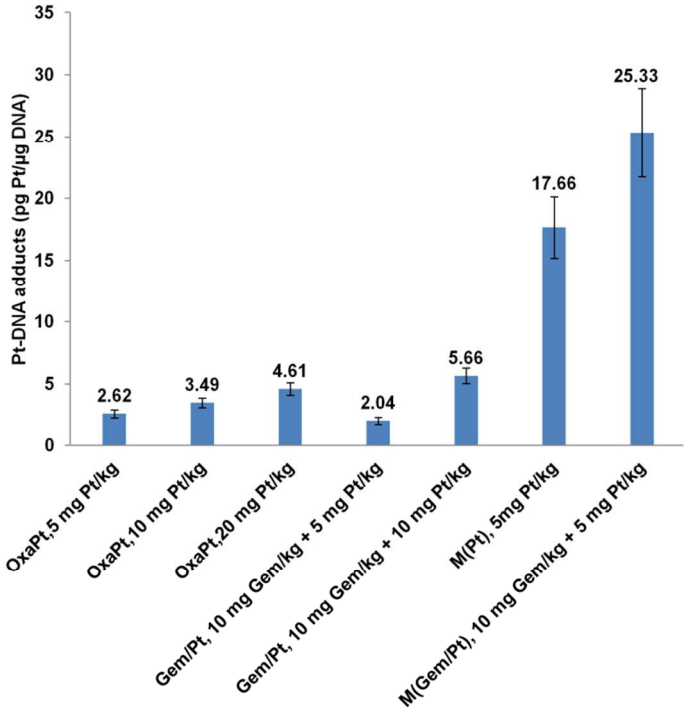


Figure 9. Pt-DNA adducts formed after 24 h treatment of various drugs. Gem and OxaPt were used to indicate gemcitabine and oxaliplatin respectively. There were 3 mice in each drug group. Data were shown as mean value ± S.D. After drug treatment, mice were sacrificed and the tumors were collected, washed by PBS and dried. Then, the genomic DNA was extracted and the Pt contents in the DNA were measured by ICP-MS.