Fabrication of poly(γ-glutamic acid)-based biopolymer as the targeted drug delivery system with enhanced cytotoxicity to APN/CD13 over-expressed cells

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Abstract
Poly(γ-glutamic acid)-based targeted drug delivery system (PAMCN) targeting transmembrane metalloprotease aminopeptidase-N (APN/CD13) was fabricated and evaluated for the enhancement of targeting efficiency and cytotoxicity. The cisplatin (CDDP) loading content of PAMCN was about 36±5% and PAMCN showed a sustainable release profile with a half-maximal release time (t1/2) of 23 h. The average size of PAMCN was 132±18 nm determined by light scattering (LS) and 158±67 nm by atomic force microscopy (AFM). Flow cytometry and fluorescence microscope analysis showed that the drug carrier (PAMN) could specifically bind to human umbilical vein endothelial cells (HUVEC). PAMCN enhanced the efficacy of CDDP to HUVEC cells with the half maximal inhibitory concentration (IC50) value decreased to 90.83±33.00 μg/ml comparing with free CDDP treatment and showed less tube formation amounts (p<0.01) than free CDDP in matrigel angiogenesis inhibition assay in vitro. In vivo toxicity experiment indicated that the survival rate of KM mice in PAMCN group was 100% and PAMCN reduced the hepatic and renal toxicity significantly compared to free CDDP group. Therefore, this novel drug delivery system presents a promising potential for antiangiogenic chemotherapy.

Introduction
Angiogenesis is a critical step for tumor growth and metastasis [1]. In contrast, antiangiogenic therapies interfere with the complex processes of growth, migration and differentiation of blood vessels [2], leading to the reduction of tumor blood supply, reducing tumor growth and even eliminating the tumor in some experiments [3,4]. Therefore, drug delivery system targeting tumor vasculature has emerged as one of the most promising approaches for the treatment of malignant tumor [5,6]. It has been reported that the metalloprotease aminopeptidase-N (APN/CD13) is an important regulator of angiogenesis where its expression on activated blood vessels is induced by angiogenic signals, playing multiple roles in cell functions and invasion angiogenesis [7]. Therefore, APN/CD13 can serve as a marker and a potential therapeutic target on dividing human umbilical vein endothelial cell (HUVEC). Furthermore, in vivo screening of phage libraries led to the discovery of the Asn–Gly–Arg (NGR) peptide motif that binds primarily to the APN/CD13 receptor expressed in the endothelium of angiogenic blood vessels [8,9]. In addition, NGR-bearing peptide, GNGRAHA [10], also showed significant targeting effect. Therefore, NGR and APN/CD13 can serve as a ligand–receptor pair as far as specific targeting is concerned.

As for drug delivery system, poly-γ-glutamic acid (γ-PGA) is a very promising drug carrier. It is nontoxic, hydrophilic, biodegradable and with high drug loading efficiency. Several studies have reported the use of γ-PGA as a drug carrier to load antitumor drugs, including paclitaxel, camptothecin and cisplatin (CDDP) [11,12]. Previous studies in our laboratory also showed that these CDDP loaded to γ-PGA based biopolymers exhibited very good stability in vitro and were stable during storage for prolonged periods [13,14]. In this report, we described the fabrication of the APN/CD13 targeted γ-PGA biopolymer drug delivery system (PGA-Asp-Mal-CDDP-mNGR, PAMCN) using APN/CD13 targeting peptide GNGRAHAC (mNGR, to which a cysteine was added at the carboxyl terminal of the peptide). Fluorescence imaging and flow cytometry analysis were performed to address the potent targeting effect of the drug carrier (PGA-Asp-Mal-mNGR, PAMN). The sustained release profile of CDDP from PAMCN, its cytotoxicity against the HUVEC cells and matrigel angiogenesis inhibition were further investigated. Finally, in vivo toxicity experiments were performed to confirm the safety of PAMCN.
Materials and methods

Materials

Cis-dichlorodiammineplatinum (II) (cisplatin, CDDP) (Sigma-Aldrich, Saint Louis, MO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT, Ameresco, Solon, OH), 4′,6-diamidino-2-phenylindole (DAPI, Roche, Basle, Switzerland), trypsin (Ameresco), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, Medpep Co. Ltd., Shanghai, China) and 1-hydroxybenzotriazole (HOBt, Medpep Co. Ltd., Shanghai, China), and PE-labeled anti-human CD13 antibody (Biolegend, San Diego, CA) and BCA-based protein quantification kit (Biocolor, Bioscience and Technology, Shanghai, China) were used as received. The dialysis bags (MWCO, 10,000 Da, Spectrum, Rancho Dominguez, CA) and BCA-based protein quantification kit (Biocolor, Bioscience and Technology, Shanghai, China) were used as received. FITC labeled Peptide GNGRAHAC (mNGR) and FITC labeled peptide HEGITFTSDLKQMEEEAVRLFIEWLKNГГPSSGAPPSC (P40, irrelevant peptide sequence used as the control) were synthesized by China peptides Ltd., Shanghai. HUVEC cells and human cancer cell line HeLa were purchased from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All chemicals and reagents used were of analytical grade. HUVEC cells were cultivated using ECM (ScienCell, San Diego, CA) with 100 IU/ml penicillin and 100 IU/ml streptomycin. HeLa cells were cultured in RPMI-1640 with glutamine, containing 10% FBS (Gibco, Grand Island, CA), 100 IU/ml penicillin and 100 IU/ml streptomycin. Both cell lines were cultured in 37°C incubator supplemented with 5% CO2.

Preparation of PAMCN

The PGA-Asp-Mal-CDDP-mNGR (PAMCN) biopolymer was synthesized as previously described by Geng et al. [15] with some modifications. Briefly, H-Asp(OtBu)-OtBu-HCl was linked to low molecular weight γ-PGA through amide reaction with the catalyst EDC·HCl to synthesis aspartylated PGA (PA). Then N-(maleimidohexanoyl)-ethylene diamine (NME) was added into PA to produce PGA-Asp-Mal (PAM). The structure of PA and PAM was identified by FT-IR spectrometry (Nexus 670, Nicolet, Madison, WI), respectively. PGA-Asp-Mal-CDDP (PAMC) was obtained by adding CDDP to the PAM solution by complexation with pendant carboxylic ions [16,17] and shook for 48 h in the dark at 37°C, then purified by dialysis against ultra-pure water for 48 h (MWCO, 10,000 Da) to remove free CDDP and freeze-dried in vacuum. PGA-Asp-CDDP (PAC) complex was obtained according to the similar procedure of PAMC to be used as the control. At last, mNGR peptide was added into PAMC solution and the reaction was carried out at 4°C for 12 h to yield PGA-Asp-Mal-CDDP-mNGR (PAMCN). The final product was purified by dialysis against ultra-pure water for 48 h (MWCO, 10,000 Da) to remove unbinding peptide and then freeze-dried in vacuum. The CDDP concentration in PAC and PAMCN was determined by a colorimetric o-phenylenediamine diame method [18].

The drug carrier PAMN was fabricated by coupling mNGR peptide to PAM. PAMN conjugation was analyzed by SDS-PAGE. The conjugate ability was detected by staining with coomassie brilliant blue R250 (CBB-R250) and also confirmed by fluorescence labeling technology [19,20].

Particle size and morphological analysis

The mean particle size of PAMCN was determined by Light scattering (Malvern Zetasizer Nano-ZS, Worcestershire, UK) at 25°C in triplicates. Biopolymer dispersion samples were stored at 4°C. The mean diameter and polydispersity index (PI) were used to present particle size distribution. Morphological evaluation of PAMCN was performed with atomic force microscopy (Nanoscope Multiode 8, Bruker, Billerica, MA). Mica disks used as supporting media were cleaved before use for sample preparation. Aqueous samples were deposited on a freshly cleaved mica surface at room temperature to allow passive absorption. Allowing the sample to dry, the mica was used for AFM analysis. The images were collected in low amplitude tapping mode.

Release profiles of CDDP from PAMCN

The release profiles of CDDP from PAMCN and PAMC were studied in sodium chloride solution (NaCl, 0.9%) with a dialysis method (MWCO, 10,000 Da) at room temperature and CDDP in the same solution was used as the control. Briefly, 5 ml PAMCN and PAMC solution was placed in dialysis bags, then immersed in 100 ml NaCl solution (0.9%) with constant horizontal shaking. At predetermined intervals, 1 ml of NaCl solution was removed to measure CDDP concentration and 1 ml NaCl solution (0.9%) was replenished. The amount of CDDP in the samples at each time point in the solution was measured by the colorimetric o-phenylenediamine method using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA) at 702 nm.

In vitro cell targeting assay

HUVEC cells and HeLa cells were seeded in a 24-well plate (5×10^4 cells per well) and incubated with mNGR (30 µg/ml), PAMN (peptide concentration equaled to 30 µg/ml) and P40 (peptide concentration equaled to 30 µg/ml) was used as an irrelevant control peptide to incubate with HUVEC cells. Concentration of mNGR was quantified by BCA protein assay kit according to the manufacturer’s instructions (Biocolor, Shanghai, China). After 30 min of incubation at 37°C, cells were washed three times with PBS solution and visualized under a fluorescence microscope (Olympus, IX2-ILL100, Tokyo, Japan) and recorded by camera (Olympus, U-LH100L-3, Tokyo, Japan).

APN-mediated targeting of PAMN to HUVEC or HeLa cells was further confirmed by flow cytometry (FACScan Becton Dickinson, Franklin Lakes, NJ), which could be used to quantify the cellular binding of biopolymer. HUVEC cells or HeLa cells were digested by 0.25% trypsin and the suspensions were centrifuged at 1000 rpm for 5 min. The cell pellets were re-suspended in 2 ml PBS and mixed with mNGR (50 µg/ml), P40 (50 µg/ml), PAMN (peptide concentration is equivalent to 50 µg/ml), respectively. The mixtures were
incubated at 37 °C for 30 min. Cells treated with PBS were used as control. After incubation, cells were washed three times with PBS solution and suspended in 500 µl FACS buffer. The mean fluorescence intensity was measured to evaluate the targeting effect. For each cell line, 10,000 events were analyzed to generate diagrams using CellQuest Pro 3.3 Software (Becton Dickinson, Franklin Lakes, NJ).

**In vitro cytotoxicity assay**

HUVEC cells were seeded in 96-well plates at the density of \(1 \times 10^4\) cells/well. CDDP or PAMCN was added at the equivalent CDDP concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/ml, respectively, in both general and specific cytotoxicity assays. In the general cytotoxicity assay, HUVEC cells were exposed to either CDDP or PAMCN contained culture medium for 24 or 48 h without removing the drugs in the whole incubation period. In the specific cytotoxicity assay, cells were exposed to either CDDP or PAMCN contained culture medium for only 10 min, and drugs were removed and cells were washed with PBS. Fresh medium was added to the cells and the cells were cultured continuously for 24 or 48 h. At the end of the experiment, the cell surviving rate of two kinds of treatment (drugs retained or removed) was quantitated by MTT assay. Briefly, 20 µl of 5 mg/ml MTT was added to each well and incubated for 3 h. The supernatant was removed and 200 µl DMSO was added to each well. The absorbance at a wave-length of 570 nm of each well was measured using a multi-well scanning spectrophotometer (Multiskan GO, Thermo Scientific). The cell surviving rate was evaluated by the OD values.

**In vitro matrigel angiogenesis inhibition assay**

Matrigel is an assortment of extracellular matrix proteins extracted from Engelbreth–Holm–Swarm tumors in mice [21–24], which primarily consists of laminin, collagen IV and enactin considered to be a reconstituted basement membrane preparation. The matrigel angiogenesis assay can be used to screen compounds for angiogenic activity or to determine if it has an effect on angiogenesis. In order to test whether PAMCN can selectively inhibit endothelial cells undergoing angiogenesis, HUVEC cells were seeded in matrigel. HUVEC capillary tube formation was performed as follows. The 24-well tissue culture plates were coated with matrigel basement membrane matrix (200 µl per well, Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to polymerize at 37 °C for 1 h. HUVEC cells (4 × 10^5 cells/well) were grown in a final volume of 300 µl ECM. After 4 h incubation, 300 µl PAMCN (CDDP concentration 200 µg/ml) was added into each well for 10 min incubation. Same volume of PBS or CDDP (200 µg/ml) was used as negative and positive controls, respectively. Then all wells were washed with PBS for three times, and then fresh culture medium was added. After 12 h, the angiogenesis was observed and counted through an inverted phase-contrast photomicroscope (Olympus, IX2-ILL100) and recorded by photograph (Olympus, U-LH100L-3). The number of angiogenesis was measured by counting the number of tube like structures formed by connected endothelial cells in five randomly-selected microscopic fields.

**In vivo acute toxicity of PAMCN**

**In vivo acute toxicity of PAMCN** was studied in normal KM mice. According to Feng et al. [13], female KM mice (5–7 weeks old, 17–19 g) were divided into four groups of 10 mice each according to their body weight. All the groups were treated for a total of three administrations every two days. Two groups were treated intravenously with either free CDDP (4 mg/kg) or PAMCN at a CDDP equivalent dose of 4 mg/kg; two groups were treated with drug carrier PAMN (equal to PAMCN) or PBS (200 µl) as control groups. The survival rate was examined for 16 days after the first day of injection. Body weight was measured each day as an indicator of systemic toxicity. On the last day of the experiment, mice were sacrificed and blood samples were collected. White blood cells (WBC) and red blood cells (RBC) were counted by hemocytometer for hematological assessment. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CRE) were measured using an autoanalyzer (Hitachi, BM704, Tokyo, Japan) for renal function (BUN and CRE) and hepatic function (ALT, AST) toxicity test. All the animal experiments throughout this study were conducted in full compliance with local and national ethical and regulatory principles for animal care.

**Statistical analysis**

Statistical analysis to identify differences between groups was performed by Student’s t-test using Graph Pad Prism 5 software. A \(p < 0.05\) was considered statistically significant. All data are presented as mean ± SD.

**Results and discussion**

**Characterization of PAMCN**

The FT-IR spectra of PA and PAM are shown in Figure 1(A) and (B). The PAM was synthesized by the modification of PA (PGA-Asp). In brief, we introduced the maleimides into PA materials [15]. The double bonds in maleimides are specific to thiols in the pH range of 6.5–7.5 to form stable thioether materials [15]. The maleimide functionalized carrier can be comprehensively used to bind to specific peptides with thiols [27]. The absorption peaks at \(\sim 1700\) cm\(^{-1}\) (\(\nu_{C=O}\)) and \(\sim 3100\) cm\(^{-1}\) (\(\nu_{OH}\)) were attributed to the carboxylic acid and the absorption peaks at \(\sim 1630\) cm\(^{-1}\) (amide I) and \(\sim 3400\) cm\(^{-1}\) (\(\nu_{NH}\)) were assigned to the amide group. The characteristic peak of amide (1615 cm\(^{-1}\)) was higher and the characteristic peak of carboxyl (1733 cm\(^{-1}\)) was lower in the infrared spectrum of PAM compared to that of PA. These results indicated that the amidation reaction successfully conjugated the maleimide group to PA. The drug carrier PAMN was analyzed by SDS-PAGE. The successful coupling of PAM to mNGR peptide was proved by SDS-PAGE analysis (data is shown in Supplementary Figure 1).

In this study, the CDDP loading content in PAMCN was 36 ± 5% (Table 1). No great difference between PAMCN and PAMC suggested the conjugation of mNGR peptide to PAMC had few influence on the structure of PAMN. The poor solubility of inorganic CDDP in water markedly limits its application. CDDP carried by novel PGA-based biopolymers
improve its solubility and implement satisfactory drug loading content.

Size distribution of the PAMCN was observed by both LS and AFM. AFM also provided the morphology information of PAMCN. As shown in Figure 1(C), LS results revealed that PAMCN was well-dispersed particles and the size distribution of the PAMCN was almost homologous, with average diameter of 132 ± 18 nm (PDI = 0.309). The particle morphology and size distribution of PAMCN determined by AFM method showed that the morphology was anomalous and the average size was of 158 ± 67 nm (Figure 1D) which was consistent with the LS result. Tumor growth induces the development of neovasculature characterized by discontinuous endothelium with large fenestrations of 200–780 nm [28], Matsumura and Maeda [29] discovered the enhanced permeability and retention effects (EPRs) which enhanced the accumulation of the nanomedicine at the tumor site. It has been reported that small carriers (70–200 nm) [30] can have deeper penetration into tumors tissue and increased accumulation. Since the size of PAMCN fell right into this range, it is reasonable to expect an optimal result to penetrate tumor neovasculature.

Table 1. Drug-loading properties of PAMC and PAMCN.

| Groups  | Drug concentration (μg/ml) | Drug-loading contents (%) |
|---------|---------------------------|---------------------------|
| PAMCN   | 565 ± 53                  | 36 ± 5                    |
| PAMC    | 580 ± 45                  | 37 ± 6                    |

Drug-loading content was calculated by the equation: total loaded CDDP weight/total CDDP complexes × 100%.

Figure 2. CDDP release curves of CDDP, PAMC and PAMCN in physiological saline (0.9% NaCl) at room temperature. The amount of CDDP in the solution was measured by the colorimetric o-phenylenediamine method.
In vitro sustained-release profile

The release of drugs from a drug carrier system must be sustainable rather than a burst release way. The in vitro release profile is shown in Figure 2. The in vitro release profile of CDDP is a burst release profile while CDDP release from both PAMC and PAMCN is in sustainable manner. Compared to free CDDP, CDDP in PAMCN was released much more slowly. The half-maximal release time ($t_{1/2}$) of free CDDP was 1.5 h, but for PAMC and PAMCN the $t_{1/2}$ is all about 23 h. After 6 h, the CDDP released from free CDDP was about 60%, while CDDP in PAMC and PAMCN was about 12% and 13%, respectively. Seventy-nine percent of CDDP in PAMCN released appearing at 100 h. PAMC and PAMCN have sustained-release property and no obvious difference between PAMC and PAMCN release profile was observed which indicated that the link of mNGR peptide without any effects on the sustainable release of PAMC. As small molecule, free CDDP can be rapidly cleared up through the kidneys while PAMCN with an extended circulating half-life is potential to reduce dosing frequency and improve patient compliance.

In vitro cell targeting

The flow cytometry results confirmed that HUVEC was APN/CD13 positive and HeLa was APN/CD13 negative (Supplementary Figure 2) cell which was consistent with previous report [31]. In this study, we use irrelevant peptide P40 and CD13 lowly expressed cell line HeLa as negative controls. The affinity between targeting peptides and the receptor is the major effects for the cell targeting. The targeting peptides have high affinity to the targets while the irrelevant peptide has no obvious targeting effects [32,33]. Under inverted fluorescence microscope, APN/CD13 positive cell line HUVEC incubated with mNGR and PAMN showed a green fluorescence signal (Figure 3A and B) while no green fluorescence was detected when HUVEC cells were incubated with irrelevant control peptide P40 (Figure 3C), which indicated a specific interaction between HUVEC cell and targeting peptide mNGR or the targeting drug carrier PAMN. While APN/CD13 negative cell line HeLa incubated with mNGR and PAMN (Figure 3D), no green fluorescence signals were detected, meaning there was no non-specific interaction between APN/CD13 negative cells and mNGR or PAMN.
The APN-mediated targeting of PAMN to cells was further confirmed by flow cytometry, which could be used to quantify the cellular binding of the biopolymer. HeLa cells incubated with mNGR peptide showed much weaker fluorescence intensity (26.87) (Figure 4C), which was most probably due to the poor expression of APN/CD13 in HeLa cell lines. The fluorescence intensity of HUVEC incubated with irrelevant peptide P40 (10.40) was almost similar to PBS (3.75) treatment group (Figure 4B). PAMN-treated HUVEC cells show much higher fluorescence intensity (99.77) than PBS-treatment groups meaning the specific interaction between the drug carrier and the target. The mean fluorescence intensity (149.53) of HUVEC cells incubated with mNGR peptide was higher than PAMN-treated group which may attribute to its less steric hindrance effect. Targeting peptide competitive inhibition experiment showed the similar results (Supplementary Figure 4). Therefore, the drug carrier PAMN can implement APN-mediated active targeting.

In vitro cytotoxicity assay

The general cytotoxicity and specific cytotoxicity effects of free CDDP and PAMCN against HUVEC cells after 24 and 48 h incubations were detected by MTT assay. The results showed that CDDP and PAMCN inhibited cell growth in a dose-dependent manner (Supplementary Figure 3). As shown in Table 2 and Figure 5, in general toxicity, the IC\textsubscript{50} value of PAMCN was much lower than that of free CDDP which may be due to the rapid diffuse of free CDDP into cells. While in the specific toxicity assay, the drug was removed after 10 min of incubation, more significant cytotoxic effects were observed in PAMCN group comparing with the free CDDP group which indicated the specific bind effect of PAMCN on HUVEC cells. The data above suggested that the PAMCN drug delivery system could effectively target HUVEC cells and enhance drug efficacy.

Table 2. In vitro cytotoxicity of CDDP or PAMCN against HUVEC cells.

| Exposure time (h) | CDDP     | PAMCN    |
|------------------|----------|----------|
| General toxicity |          |          |
| 24 h             | 23.86 ± 3.64 | 90.83 ± 33.00 |
| 48 h             | 11.59 ± 2.38 | 28.88 ± 1.830 |
| Specific toxicity|          |          |
| 24 h             | 1311.89 ± 111.18 | 365.93 ± 33.00 |
| 48 h             | 240.32 ± 56.80 | 62.43 ± 23.40 |

In vitro matrigel angiogenesis inhibition effects

After 10 min incubation with PBS, free CDDP or PAMCN, the cells were washed with PBS for three times and fresh culture medium was added to incubate for another 12 h. In the same incubation period, tube formation amounts of PAMCN were significantly decreased \((p < 0.001)\) than free CDDP, thus, the drug delivery system PAMCN generates higher tube formation inhibition ability. The tube formation amounts of free CDDP group were still significantly lower than that of the PBS group (Figure 6), which accounted for CDDP inhibition. Neovascularization plays crucial role in tumor
Figure 6. *In vitro* matrigel angiogenesis inhibition assay. $5 \times 10^4$/well of HUVEC cells were seeded in 24-well plate for 4 h and then the cells were treated with (A) PBS, (B) CDDP (200\(\mu\)g/ml) and (C) PAMCN (equal to CDDP concentration of 200\(\mu\)g/ml) for 10 min then incubated with fresh medium for another 12 h and observed under 10-fold objective lens. (D) Mean endothelial cell tube formation.*$p<0.05$, **$p<0.01$.

Figure 7. Survival rate and body weight change rate of normal KM mice that received CDDP, PBS, PAMCN and PAMN. (A) Survival rate (%), (B) body weight change rate (%). Body weight change rate (%) was calculated by the equation: body weight at measurement day after injection/body weight before injection \times 100\%. Each sample was injected into normal mice (female, 8 weeks, via tail veins). ***$p<0.001$, CDDP group comparing with the other three groups.
effectively target HUVEC cells and enhance CDDP efficacy. A specific cytotoxicity assay demonstrated that PAMCN could significantly reduce acute toxicity comparing with free CDDP. The acute toxicity results indicated that PAMN as drug carrier for cisplatin reduced its integrated toxicities compared with free CDDP. Although anticancer drug cisplatin has been extensively used in clinical, it manifests the drawback of cytotoxicity such as bone marrow suppression [34], acute nephrotoxicity [35] and hepatotoxicity [36]. The nephrotoxicity and its altered biochemical characters, we developed a selective tumor vasculature drug targeting carrier PAMN, characterized by significant difference in serum BUN, CRE, ALT and AST levels. No significant difference between PAMCN-treated group and the control groups (PBS, PBS) (p > 0.05) were detected, indicating that PAMCN displayed lower toxicity. Although anticancer drug cisplatin has been extensively used in clinical, it manifests the drawback of cytotoxicity such as bone marrow suppression [34], acute nephrotoxicity [35] and hepatotoxicity [36]. The in vivo acute toxicity results indicated that PAMN as drug carrier for cisplatin reduced its integrated toxicities comparing with free CDDP.

**In vivo acute toxicity**

In order to evaluate the acute systemic toxicity of PAMCN in vivo, we examined the survival rate and body weight changes in normal KM mice. According to our observation, the survival rate for the control groups and PAMCN treatment groups is both 100% while for the free CDDP treatment group is around 50% at the end of the experiment (Figure 7A). The in vivo toxicity differences were not only recognized in survival rate but also in body weight test. When free CDDP was administered, the loss of body weight was about 17% (p < 0.001) at the end of the experiment while PAMCN had no obvious influence on the body weight change (Figure 7B). Mice treated with PAMCN showed no significant decrease (p > 0.05) in WBC, RBC comparing with PBS or PAMN control groups (Table 3), however, the free CDDP-treated group with significant changes (p < 0.01). Renal function (BUN and CRE) and hepatic function (ALT and AST) were evaluated and the data were listed in Table 3. Serum analysis indicated that only free CDDP group caused a significant reduction in renal function and hepatic function, as characterized by significant difference in serum BUN, CRE, ALT and AST levels. No significant difference between PAMCN-treated group and the control groups (PAMN, PBS) (p > 0.05) were detected, indicating that PAMCN displayed lower toxicity. Although anticancer drug cisplatin has been extensively used in clinical, it manifests the drawback of cytotoxicity such as bone marrow suppression [34], acute nephrotoxicity [35] and hepatotoxicity [36]. The in vivo acute toxicity results indicated that PAMN as drug carrier for cisplatin reduced its integrated toxicities comparing with free CDDP.

**Conclusion**

In this article, on the basis of tumor vascular microenvironment and its altered biochemical characters, we developed a selective tumor vasculature drug targeting carrier PAMN, which could specifically bind to APN/CD13 that is highly expressed by the endothelium of angiogenic blood vessels. The carrier made the most use of the combination of passive and active targeting approach to the targeted cells. In vitro cell targeting showed drug carrier PAMN specifically bound to the APN/CD13 positive line HUVEC. What’s more, in vitro specific cytotoxicity assay demonstrated that PAMCN could effectively target HUVEC cells and enhance CDDP efficacy. Based on the findings of the present study, we believe that the in vivo safe PAMCN bears potential to become a fundamental drug delivery system in treating tumor.

**Declaration of interest**

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

This research work was supported by the National Natural Science Foundation of China (31170920), National 863 Project (2009AA03Z429) and large instruments Open Foundation of East China Normal University.

**Table 3. Biochemical parameters in PAMCN-treated animals.**

| Groups | RBC (10^12/l) | WBC (10^9/l) | AST (mmol/l) | ALT (mmol/l) | BUN (mmol/l) | CREAP (mmol/l) |
|--------|--------------|--------------|--------------|--------------|--------------|---------------|
| PBS    | 9.26 ± 0.34  | 19.98 ± 4.89 | 118.58 ± 30.872 | 49.10 ± 5.13  | 6.91 ± 1.02  | 15.62 ± 2.11  |
| PAMN   | 9.92 ± 0.49  | 20.38 ± 4.62 | 99.78 ± 28.434 | 40.36 ± 19.81 | 7.83 ± 0.76  | 14.14 ± 1.88  |
| CDDP   | 3.00 ± 0.64 *** | 9.13 ± 3.48 *** | 265.25 ± 4.72 *** | 131.97 ± 19.62 *** | 41.06 ± 8.38 *** | 41.73 ± 5.55 ** |
| PAMCN  | 8.62 ± 0.43  | 21.38 ± 7.68 | 90.98 ± 11.88 | 41.60 ± 21.32 | 7.83 ± 0.56  | 14.24 ± 1.25  |

CDDP group comparing with the other three groups (n = 5).

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Supplementary material available online
Supplementary Figures S1–S4.