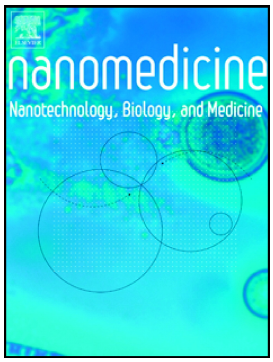


The Diosgenin Prodrug Nanoparticles with pH-Responsive as a Drug Delivery System Uniquely Prevents Thrombosis without Increased Bleeding Risk

Zeliang Wei, Guang Xin, Haibo Wang, Huajie Zheng, Chengjie Ji, Jun Gu, Limei Ma, Chaoyi Qin, Zhihua Xing, Hai Niu, Wen Huang

PII: S1549-9634(18)30004-2
DOI: https://doi.org/10.1016/j.nano.2017.12.019
Reference: NANO 1727

To appear in:

Received date: 5 June 2017
Revised date: 8 December 2017
Accepted date: 22 December 2017

Please cite this article as: Zeliang Wei, Guang Xin, Haibo Wang, Huajie Zheng, Chengjie Ji, Jun Gu, Limei Ma, Chaoyi Qin, Zhihua Xing, Hai Niu, Wen Huang , The Diosgenin Prodrug Nanoparticles with pH-Responsive as a Drug Delivery System Uniquely Prevents Thrombosis without Increased Bleeding Risk. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Nano(2018), https://doi.org/10.1016/j.nano.2017.12.019

Word count for Abstract: 150

Word count for manuscript: 4843

Number of References: 54

Number of figures: 7

Number of tables: 1

Number of Supplementary online-only files: 1

Title: The Diosgenin Prodrug Nanoparticles with pH-Responsive as a Drug Delivery System Uniquely Prevents Thrombosis without Increased Bleeding Risk

Zeliang Wei, PhD[1,#], Guang Xin, PhD[1,#], Haibo Wang, PhD[2,#], Huajie Zheng, MS[1,3], Chengjie Ji, PhD[4], Jun Gu, MD[5], Limei Ma, PhD[1], Chaoyi Qin, MD[5], Zhihua Xing, BS[1], Hai Niu, PhD[6,*], Wen Huang, PhD[1,*]

[1] Laboratory of Ethnopharmacology, West China Hospital, West China Medical School, Sichuan University Keyuan Road 4 No.1, Gaopeng Avenue, Gaoxin District, Chengdu, Sichuan, 610041, China.

[2] Textile Institute, College of Light Industry, Textile and Food Engineering, Sichuan University, Chengdu, 610065, China

[3] Department of Integrated Traditional Chinese and Western Medicine, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan 610041, China

[4] Clinical Laboratory, Hospital of University of Electronic Science and Technology of China and Sichuan Provincial People's Hospital, Chengdu, Sichuan 610072, China

[5] Department of Cardiovascular Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

[6] College of Mathematics, Sichuan University, Chengdu 610064, China

[#] Zeliang Wei, Guang Xin and Haibo Wang contributed equally to this work.

* **First corresponding author: Wen Huang**

Second corresponding author: Hai Niu

E-mail: huangwen@scu.edu.cn (Wen Huang); niuhai@scu.edu.cn (Hai Niu).

Keywords: pH-responsive, prodrug, self-assemble, thrombus therapy, diosgenin

Author Contributions

W. H., and H. N. designed experiments and analysed data, interpreted results and edited the paper; Z. L. W., G. X., and H. B. W. performed all experiments, analysed data, interpreted results and wrote the manuscript; H. J. Z., and C. J. J. took care of animals; L. M. M., and J. G. established venous and arterial thrombosis rats; C. Y. Q., and Z. H. X. performed statistical analysis.

Competing financial interests: The authors declare no competing financial interests.

[1] This work was partly supported by the China National '12.5' Foundation (Grant No. 2011BAJ07B04), National Natural Science Foundation of China (Grant No. 20972105), the Natural Science Foundation of China (Grant No. 8167371) and Natural Science Foundation of China (Grant No. 51503130).

Abstract

Thrombosis is the leading cause of death in patients with cardiovascular disease in the world. Current antithrombotic agent aspirin has serious side effects such as higher bleeding risk and serious gastrointestinal ulcers. Diosgenin reported in clinical research could prevent thrombosis without side effects. However, poor bioavailability and low knowledge on its molecular targets limit its clinical application. A novel prodrug with antithrombotic effect was prepared based on conjugating diosgenin derivatives to PEG with Schiff-base bond. The prodrug with long blood circulation time and satisfying safety could self-assemble into micelles in water. The prodrug micelles with pH-responsibility could targetedly release diosgenin in position of thrombus *in vivo*. The results indicate that the prodrug micelles without bleeding risk and histological damages prevent thrombosis by inhibiting platelet activation and apoptosis. Our studies demonstrate that the prodrug micelles could obviously enhance the efficacy in the prevention of arterial thrombus and venous thrombus than aspirin.

Introduction

Thrombotic disorders, including deep vein thrombosis, stroke, heart attack, and other circulatory diseases, have become the leading cause of premature death in the world.[1-3] However, lots of antithrombotic agents including aspirin and heparin approved by the Food and Drug Administration (FDA) have serious side effects, such as the bleeding risk, gastrointestinal ulcers and incidence of resistance.[4] Therefore, development of novel antithrombotic drug with minimal side effect is urgently needed.

In many clinical research reports, diosgenin exhibits antiplatelet and antithrombotic activities *in vivo*. It has attracted increasing attention because of its safety.[5,6] It was reported that no hormone and it's derivative

was detected in serum after mice treated with steroidal saponins from *Dioscorea zingiberensis* by oral administration.[7] And no significant toxicity on liver was observed in the mice treated with the diosgenin for a long time. In previous studies, we found that the diosgenin and its derivatives could prolong Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) or Thrombin Time (TT) in rats, also increase the clotting time, bleeding time and protection rate of pulmonary embolism in mice.[7-9] The studies indicated that diosgenin inhibited thrombosis by both improving the anticoagulation function and inhibiting platelet aggregation. However, the application of diosgenin and its derivatives were limited due to its strong hydrophobicity and low knowledge on its molecular targets.[10] It has been reported that drug delivery systems usually have a long circulation time and could control the release rate of the drug, which enhance the drug utilization and reduce side effect.[11,12] Thus, it is very necessary to develop a drug delivery system for improving bioavailability, which will enhance the efficiency of antithrombotic therapy. The nanocarriers have also been reported to improve the treatment of cerebral thrombosis.[13-16] Among the nanocarriers, polymeric prodrugs have emerged as one of the most promising platforms for drug delivery due to their advantages of increasing drug solubility, prolonging circulation time and improving pharmacokinetic properties.[17-22]

Nowadays, the polymeric prodrug with stimuli-sensitive properties such as responding to pH, redox, temperature, ultrasound, and light has been developed.[23] Thrombosis, which is mediated by activations of platelets, coagulation factors and the localized clotting of the blood, can occur in arterial or venous circulation and has a major medical barrier.[24] Numerous studies have reported that platelet activation and apoptosis are the major factors leading to thrombosis.[25,26] Kazuhiro Sako found the peripheral blood of blood clots provides less oxygen, resulting in lower pH of microenvironment of the thrombus.[27-29] Harris RJ and

Hirofumi Nakai reported that the local cerebral blood flow (LCBF) threshold for pH reduction was relatively stable.[30] The lower pH in location of thrombosis provided an opportunity to develop a more selective drug delivery system through pH response for overcoming medical barrier.

In the present research, we designed a novel prodrug nanocarriers based on PEG and diosgenin derivatives for inhibiting thrombosis. The compound was conjugated to the PEG with Schiff-base bond for forming the prodrug. The prodrug was expected to self-assemble into nanomicelles in aqueous solution. We found the prodrug micelles could be cleaved under the acidic condition in the location of thrombosis, and released diosgenin rapidly to inhibit thrombosis. At the same time, it was observed that the prodrug micelles improved the blood drug concentration. What is more,we found that the diosgenin prodrug micelles without bleeding risk prevented thrombosis by inhibiting activation and apoptosis of platelet, and its efficiency is better than that from clinical antithrombotic agent as aspirin.

Methods

Materials

Dichloromethane, anhydrous ethyl ether, methanol, tetrahydrofuran (THF), N,N′-Dicyclohexylcarbodiimide (DCC, ≥98.0%), 4-dimethylaminopyridine (DMAP, ≥99.0%) and 4-Formylbenzoic acid (CBA, 98.0%) were purchased from Aladdin Chemical Co., Ltd. (Shanghai, China). Themethyl poly(ethylene glycol) (mPEG–OH, Mw 1900), Diosgenin (≥99.0%) , dimethylsulfoxide and (DMSO) 3‑[4,5‑Dimethylthiazol‑2‑yl]‑2,5‑diphenyltetrazolium (MTT), were all ordered from Sigma-Aldrich (Shanghai, China).

Synthesis of prodrugs

Methoxy-PEG-4-Formylbenzoic acid (MPEG-CBA) was synthesized as previously described,[31] and the product was purified by recrystallization repeatedly for consequent reaction. Diosgenin amino caproic acid ester was synthesized by our research group.[32] Then diosgenin amino caproic acid ester (2.7 g) and MPEG-CBA (13.00 g) were added to tetrahydrofuran (THF, 350 mL) solution with stirring at 40 °C for 12 h. Subsequently, the mixture was evaporated under vacuum, and product was dissolved in methanol, purified by precipitating in anhydrous ethyl ether. After filtration to remove the impurities, the product was recovered by precipitation in isopropanol. The pure prodrugs were dried in the vacuum drying set at 40 °C for a future experiment.

Cells and animals

The human hepatocyte LO2 cell line and human kidney HK-2 cell line were obtained from Laboratory of Transplantation Immunity, Sichuan University. Both kinds of cells were cultured in DMEM (HyClone, USA) supplemented with 10% FBS (HyClone, USA), 100 U mL^{-1} penicillins and 100 μg mL^{-1} streptomycin sulfate. Cells were incubated at 37°C in a humidified atmosphere and 5% CO_2 concentration. Male Sprague-Dawley rats (eight weeks old, 200±25 g; Chengdu, China) and male Balb/C mice (four weeks old, 20 ±5 g; Chengdu, China) were used in this study. The animals were housed separately and acclimatized in a temperature controlled (25 ± 2 °C) and illumination-controlled (12 h light/dark cycle) room for at least 1 week prior to experiments. They were fed standard animal food and water. The experimental protocol was approved by the Animal Ethics Committee of Sichuan University for Animal Experimentation.

Platelets adhesion assay

The experiments were performed as previously described.[33] Briefly, washed platelets were exposed to U0126, diosgenin and prodrug micelles (50 μM) at 37 °C for 3 h, and then they were incubated with phalloidin (200 nM) (Sigma-Aldrich) for 30 min at 37 °C in the dark. Collagen was coated on coverslips forming the lower surface by a custom-made flow chamber (Chamlide CF; Live Cell Instruments). Platelets were perfused over the coverslip at 150 s^{-1} by a syringe pump (Harvard Apparatus). After the termination of perfusion, platelets were washed with phosphate-buffered saline (PBS) and fixed with cold 4% paraformaldehyde for 15min to remove non-adherent cells and fix adherent platelets. The images were recorded from at least 10 independent fields of per flow experiment by a fluorescence microscope Olympus BX51 microscope and photographed using a DP-70 digital camera (Olympus).

Platelets aggregation assay in vitro

The experiments were performed as previously described.[34] The platelet (pre-incubated with various concentrations (2.5, 5, 25, 50 μM) diosgenin or prodrug micelles for 3 h at 37 °C) aggregation assay was performed at 37 °C by a LBY-NJ4 aggregometer (Pulisheng, Beijing, China). Aggregation was initiated by the addition of ADP solution (final concentration, 20 μM), thrombin (0.1 U/mL) and arachidonic acid (AA: 900 μM) *in vitro*. Aggregation was recorded for 5 min, and data were expressed as the change in light transmission.

Platelets aggregation assay in vivo

The experiments were performed as previously described.[34] The platelet (rats pre-treated with 100mg/kg/d diosgenin by oral administration or 40mg/kg/d prodrug micelles by tail intravenous injection for 5d) aggregation assay was performed at 37 °C by a LBY-NJ4 aggregometer (Pulisheng, Beijing, China).

Aggregation was initiated by the addition of ADP solution (final concentration, 20 μM), thrombin (0.1 U/mL) and arachidonic acid (AA: 900 μM) *in vitro*. Aggregation was recorded for 5 min, and data were expressed as the change in light transmission.

P-selectin and integrin αIIbβ3 assay

The experiments were performed as previously described.[35] After treatment *in vivo* (pre-treated with 100 mg/kg/d diosgenin by oral administration or 40 mg/kg/d prodrug micelles by tail intravenous injection for 5 d) or *in vitro* (pre-incubated with diosgenin or prodrug micelles (50 μM) for 18h at room temperature), platelets were incubated with PE-CD62P (P-Selectin antibody, Sigma, USA) or PAC-1-FITC (Integrin αIIbβ3 antibody, Sigma, USA) for 30 min in the dark, and the reaction was stopped by adding ice-cold PBS. The surface expression of P-selectin and integrin-αIIbβ3 on the platelets was measured by flow cytometer (Becton - Dickinson, San Jose, CA, USA).

$FeCl_3$-induced arterial thrombosis

One hour after the last administration (100 mg/kg/d aspirin and diosgenin by oral administration or 40mg/kg/d prodrug micelles by tail intravenous injection for 5d), rats were anesthetized with intraperitoneal (i.p.) pentobarbital sodium (40 mg/kg) and placed on a surgical table. An arterial thrombus was induced by $FeCl_3$ application onto the surface of the right carotid artery, as described by Zheng *et al.* with slight modification.[36] In brief, following anesthetization, the right carotid artery was dissected, and a 10 × 10 mm strip of filter paper soaked in 10 μL $FeCl_3$ solution (10% in water) was applied onto the external surface of the right carotid artery for 30 min. Afterward the paper was removed and the vessel was left in situ for 60 min, to enable thrombus formation. At the end of the 60 min period, the right carotid artery was harvested for

measurement of the length and weight of the thrombus.

Middle cerebral artery occlusion experiments

The experiments were performed as previously described.[37] After treated with 100 mg/kg/d diosgenin by oral administration or 40 mg/kg/d prodrug micelles by tail intravenous injection for 5 d, a 200 μm silicon-coated nylon suture was introduced into the external carotid artery and advanced up the internal carotid artery to block the origin of the middle cerebral artery (MCA), under isoflurane anesthesia. After 90 min, the filament was removed and the MCA reperfused. Animals recovered in heated incubators for 30 min and body temperature kept at 35-36 °C. Sham surgeries included identical preparatory and surgical steps as MCAO except for filament blocking of the MCA. Examining the measurements of brain infarct size by staining with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) to quantify infarct size using computer-assisted image analysis, and neurologic deficit score by animal behavioral study at 24 h after stroke onset.

Statistical analysis

All statistical analyses were conducted using the statistical software package SPSS19.0 (SPSS, Inc., Armonk, NY, USA). Data were presented as the means ± standard deviation (SD), and the statistical significance of the differences was determined using a two-sample t-test or a one-way ANOVA. The statistically significant difference was considered for $P<0.05$.

Date availability

Uncropped methods including preparation and characteristic of prodrug micelles, cytotoxicity assay *in vitro*, hematological parameters, preparation of rat platelets, bleeding time assay, $FeCl_3$-induced venous thrombosis, western blot, biodistribution and pharmacokinetics in *in vivo* and biosafety evaluation of prodrug micelles *in*

vivo, were described in the online supporting information.

Results

Preparation and characterization of prodrug

To obtain the pH-responsive prodrug delivery platform, a methoxyl-poly(ethylene glycol) derivative precursor with a 4-Formylbenzoic acid (MPEG-CBA) was synthesized firstly (Figure 1, A). And then the MPEG-CBA reacted with 6-aminohexanoic acid diosgenylester to yield a pH-responsive prodrug with a Schiff base linkage. The structure characteristics of prodrug was determined by the ^{1}H nuclear magnetic resonance (NMR), as depicted in the Supporting Information (Figure S1). The peak **a** was terminal methoxyl group (-OCH_3〉of MPEG，and the strong peak **p** was methyl (-CH_3) of diosgenin. As expected, the peak area of **a** was twice as the peak area of **p** in ^{1}H-NMR spectrum. The results revealed that the prodrug has been synthesized successfully.

The synthesized polymeric prodrug is composed of diosgenin derivative (hydrophobic part) and PEG (hydrophilic moiety). Therefore, the polymeric prodrug has the property which could self-assemble into micelles with diosgenin derivative as the core and PEG as the corona in aqueous solution (Figure 1, B). Next, we determined the shape and size of prodrug micelles by transmission electron microscopy (TEM) and dynamic light scattering (DLS) measurements. As shown in Figure 2, A and B, the prodrug exclusively formed homogenous spherical shapes with the average hydrodynamic diameter of 70.6 ± 5.4 nm. The results indicated that the prodrug micelles were suitable to be used as drug delivery system and achieved drug delivery in mice without the need of additional carriers.

Moreover, the stability of prodrug micelles was evaluated by monitoring the change of micellar size at

different temperatures under a simulative physiological condition. It was found that the prodrug micelles were quite stable in aqueous solution, and the size of micelles changed slightly in different temperatures during the incubation (Figure 2, D). It may be noted that the diameter of prodrug micelles dramatically increased in PBS solution in time course with the pH varying from 7.4 to 6.5 (Figure 2, C). The pH-responsibility of prodrug micelles may be attributed to the cleavage of Schiff-base bond, which is easily hydrolyzed in relatively weak acidic condition (pH 6.5~6.8), and stable in the neutral environment. [31,39,40]

Effects of prodrug micelles on cell viability

Cell culture system is a traditional method to rapidly assess the potential safety and cell viability for new drugs and compounds *in vitro*.[41] In the study, we employed LO2 cells and HK-2 cells to examine cell viability by MTT assay after treatment with diosgenin and prodrug micelles. The optimal concentration of the prodrug micelles using in our study was chosen based on the IC50 value and more than 50% of viable cell number after prodrug micelles treatment. The results showed that no effect on LO2 and HK-2 cells viability was observed after treated with prodrug micelles with concentrations varying from 2.5 to 50 μM (Figure 3, A and B). It is found that the cytotoxicity of diosgenin was higher than prodrug micelles at concentration of 50 μM. After the drugs treated 24h with varying concentrations, no significant change on cell proliferation and cell morphology were observed under a microscope (Figure S2 and S3). Meanwhile, the effect of prodrug micelles on platelet properties was determined. The results showed that after treatment with prodrug micelles, the properties of platelet including platelet count, platelet distribution width, mean platelet volume, thrombocytocrit, platelet ratio, and immature platelet fraction of mice did not change compared with control (Table 1).

Effects of prodrug micelles on adhesion, activation and aggregation of platelet

It is reported that thrombosis depends on the adhesion, aggregation and activation of platelets.[43] Therefore, the effect of prodrug micelles on adhesion of platelets was assessed. The results illustrated that the prodrug micelles and diosgenin inhibited platelet adhesion and activation on collagen-coated surfaces (Figure 4, A). Importantly, the prodrug micelles could more effectively reduce the platelet adhesive function compared with diosgenin *in vitro*.

It was well known that αIIbβ3 and P-selectin expressions are two key indicators of platelet activation.[47] The data showed that diosgenin and prodrug micelles significantly suppressed αIIbβ3 and P-selectin expressions of ADP-induced platelet activation *in vitro* (Figure 4, E-H) and *in vivo* (Figure S4 and S5). Furthermore, the group treated with prodrug micelles had a significantly better outcome in terms of platelet activation than the group treated with diosgenin.

The effect of prodrug micelles on platelet aggregation induced by ADP was also investigated *in vitro*. The results showed that after rats treated with prodrug micelles at concentrations of 12.5, 25, 50μM, platelet aggregation were 52.17%, 46.35% or 33.33%, respectively, which was lower than the group treated with ADP (65.26%) *in vitro*. Meanwhile, we found that after rats treated with diosgenin at concentrations of 12.5, 25, 50 μM, platelet aggregation were 57.97%, 48.65% and 45.45%, respectively. It may be noted that inhibitory effect of prodrug micelles on the platelet aggregation was superior to that of diosgenin *in vitro* (Figure 4, B and C). As shown in Figure 4, D, the prodrug micelles also exhibited more obviously efficiency in inhibiting platelet aggregation than diosgenin *in vivo*.

Effects of prodrug micelles on coagulation, blood coagulation factor activity and apoptosis

It is well known that thrombosis is mediated by activation of platelets and coagulation factors.[50] The results showed that the group treatment with prodrug micelles significantly prolonged APTT values compared with the control group (Figure 5, C), while the group treatment with prodrug micelles had no changes in PT and TT values. It may be noted that after treatment with diosgenin, the change of APTT value is similar as that from prodrug micelles. Normal APTT times required the presence of the following intrinsic coagulation factors: VIII, IX, XI and so on. In our study, we found that the prodrug micelles and diosgenin could only regulate coagulation factor VIII activity (Figure S6).

Numerous studies have reported that when thrombosis occurs, the platelets show the apoptosis accompanying by distinctive apoptotic features of phosphatidylserine (PS) exposure, integrin expression and caspase-3 activation in final state.[25,26] Our results showed that prodrug micelles could dose-dependently decreased cleaved caspase-3 up to 50 μM (Figure 5, A), suggesting the prodrug inhibited thrombosis by reducing platelets apoptosis to protect platelet.

Effects of prodrug micelles on pharmacokinetics and biodistribution in vivo

After rats treated with prodrug micelles and free diosgenin, the plasma concentration of diosgenin was detected by HPLC. As presented in Figure 5, B, the group treatment with prodrug micelles had a 8.06-fold increase in the maximal plasma concentration (C max) and a 3.02-fold increase in the area under the curve (AUC (0-t)) of diosgenin compared with group treatment with free diosgenin. Moreover, the group treatment with prodrug micelles exhibited a 1.25-fold higher drug half-life ($t_{½}$) compared with group treatment with free diosgenin. The results revealed that prodrug micelles improved the drug retention in blood and increased the bioavailability *in vivo*.

To observe the distribution of the prodrug micelles and diosgenin in the position of thrombus, a fluorescent dye (Cy5) was used as a signal-label. As a control, diosgenin was fluorescent-labeled with NBD-Cl by chemical reaction. As shown in Figure S7, the group treated with prodrug micelles had strong fluorescence intensity, but no significant fluorescence intensity was observed of the group treated with diosgenin in the fluorescent images of transverse and longitudinal thrombus freezing section. The results indicated that the prodrug micelles were more easily aggregated in position of thrombus, due to that the prodrug micelles have a long circulation time in blood and release drug rapidly at the acidic condition of thrombus site. Thereby, the high accumulation of prodrug micelles in position of thrombus could provide an essential prerequisite for effectively inhibiting thrombosis.

Effects of prodrug micelles on arterial thrombosis, venous thrombosis and cerebral thrombosis in vivo

Detecting bleeding time is a classic index of a study of antithrombotic effect *in vivo*. As shown in Figure 6, G, the average tail bleeding time of the control group, diosgenin group or aspirin group was 344s, 577s or 666s, respectively, while that of the group treated with prodrug micelles was 703s. It may be noted that the rats treated with prodrug micelles had more less bleeding risk than those treated with aspirin (Figure 5, D),a clinical antithrombotic agent.[51,52] The results revealed that prodrug micelles could more significantly prolong bleeding time compared with diosgenin.

Nextly, we evaluated the effect of drugs on arterial and venous thrombosis induced by $FeCl_3$ *in vivo*. The length and weight of average arterial and venous thrombus in aspirin group were significantly decreased (Figure 6, A-F). The diosgenin group could also reduce either arterial and venous thrombus length or weight (Figure 6, A-F). The results suggested that prodrug micelles could significantly inhibit arterial and venous

thrombosis compared with diosgenin and aspirin.

In addition, the effect of prodrug micelles on cerebral thrombosis was also evaluated by the model of middle cerebral artery occlusion (MCAO). The prodrug micelles and diosgenin could obviously reduce infarct volumes from 37.73% to 21.64% and diosgenin could reduce infarct volumes from 37.73% to 30.45% as shown in Figure 6, H and I. At the same time, neurologic deficit score of group treatment with prodrug micelles was also significantly improved compared with group treatment with diosgenin (Figure 6, J). Collectively, these results indicated that prodrug micelles could more effectively prevent ischemic cerebral thrombosis compared with diosgenin.

Effects of prodrug micelles on biosafety in vivo

In the acute toxicity study, no significant difference between the groups treated with the prodrug micelles and diosgenin in the levels of hematological parameters (ASL, ALT) in the serum of mice compared with the control (Table S1). In addition, no obvious alterations in organ morphological characteristics were observed in the mice treated with the prodrug micelles and diosgenin, as compared with the control group. Histopathological studies of the mice treated with high concentrations diosgenin and prodrug micelles were analyzed by hematoxylin–eosin (H&E) staining to determine the acute toxicity of prodrug micelles on all major organ tissue (heart, liver, spleen, lung, kidney and brain). These results verified that the tissue samples did not exhibit obvious histological damages after the mice treated with prodrug micelles, compared with the control group (Figure 7), further confirmed that the prodrug micelles had good biocompatibility and biosafety.

Discussion

Thromboembolic diseases including stroke, cerebral thrombosis, deep vein thrombosis and pulmonary embolism mainly have high incidence due to mortality and morbidity in the current society.[1-3] However, most commercial antithrombotic drugs result in many side effects, such as the bleeding risk, gastrointestinal ulcers and incidence of resistance.[4] Therefore, it is necessary to develop novel antithrombotic drugs with high potential therapeutic effect and minimal side effects.

In recent years, our group mainly focuses on modifying and synthesizing novel diosgenin derivatives with activities of anti-tumor, antithrombus and anti-hyperlipidemia.[9,32,36] Diosgenin, as an active ingredient of Chinese medicine group attracting attention recently, has anti-platelet aggregation, thrombosis and other cardiovascular pharmacological effects.[7-9] Nevertheless, the application of diosgenin was restricted because of its poor bioavailability and low knowledge on its molecular targets. It has been reported that nanoparticles with smaller size could promote their constant circulation in blood to enhance the efficacy.[38] To our knowledge, no information about nano-drug delivery systems loading diosgenin with antithrombotic effect is available.

In this study, we had successfully designed and reconstructed the prodrug micelles, which could self-assemble into micelles in aqueous solutions with the mean diameter of 70.6 ± 5.4 nm (Figure 2). As it was known, compared with normal tissue, the microenvironment at thrombus site is weakly acidic condition. We found that the prodrug micelles with pH-responsibility, could keep stable in the blood circulation, and release drug at thrombus site with acidic condition. Furthermore, our results revealed its safety by using LO2 cells and HK-2 cells *in vitro*, because liver and kidney are major organs of bioactivation, metabolism and

elimination.[42] Meanwhile, the effect of prodrug micelles on platelet properties was also not observed.

Thrombosis begins with the adhesion of platelets to a vessel wall, followed by the aggregation of additional platelets.[44,45] And the processes of thrombosis are classified as involving platelets, relating to fibrin formation or blood coagulation.[46] The mechanisms of thrombosis included platelet adhesion, subsequent aggregation of platelets at the site of vascular injury, and blood coagulation. It is known that platelet is a key component of thrombosis, and platelet also directly participates in a lot of functions necessary for clotting including triggering coagulation cascade, recognizing vascular lesions, and activating other platelets.[48] It has been demonstrated that inhibiting platelet activation is effective in reducing the mortality and morbidity of thrombus.[49] Our results show that the prodrug micelles could inhibit the adhesion, aggregation and activation of platelets *in vitro* and *in vivo*, and the efficacy of prodrug micelles are significantly better than diosgenin (Figure 4).

Coagulation factors, which catalyze the conversion of fibrinogen to fibrin, and then assembles into large stringy networks, are important in the blood coagulation process. These networks can trap blood cells to form a red thrombus.[53] Additionally, APTT is used to determine the intrinsic clotting index, whereas PT and TT were related to extrinsic clotting pathway in the clinical study.[54] In our study, the coagulation data showed that diosgenin and prodrug micelles could significantly prolong APTT value, while PT and TT were not affected. The results suggest that prodrug micelles do not take part in the extrinsic coagulation but rather through the intrinsic pathway to inhibit thrombosis. Furthermore, we found that only the coagulation factor VIII activitiy, which plays an important role in the intrinsic pathway, was affected in the prodrug micelles group and diosgenin group.[8] Therefore, our results imply that the prodrug micelles and diosgenin could efficiently prolong APTT values by regulating factor FVIII activities, and the efficacy of prodrug micelles is

significantly superior to diosgenin. The mechanisms of inhibiting thrombosis of diosgenin related to factor FVIII activities need to continue to explore in the future research.

When thrombosis occurs, the platelets exhibit distinctive apoptotic features, including phosphatidylserine (PS) exposure, integrin expression and caspase-3 activation in final state.[25,26] We found that prodrug micelles could inhibit thrombosis by reducing platelets apoptosis in final state to protect platelet. Meanwhile, it was also observed that prodrug micelles could improve the drug retention in blood, increase the bioavailability. The results of drug distribution indicated that prodrug micelles could targetedly concentrate in the position of thrombus *in vivo*, due to its nano scaled size and pH-response feature (Figure 5 and S7). Our results *in vivo* show that prodrug micelles without bleeding risk and obvious histological damages are more efficacious on inhibiting arterial and venous thrombosis than diosgenin and aspirin (Figure 6 and 7). It has been reported that diosgenin has poor bioavailability after treatment of oral administration.[10] We compared plasma drug concentration from rats treated with diosgenin by intravenous administration to rats treated with prodrug micelles by intravenous administration, but a few diosgenin could be detected in the rats treated with diosgenin by intravenous administration due to poor bioavailability and strong hydrophobicity of diosgenin. Therefore, we designed prodrug micelles of diosgenin to prolong blood circulation time and increase molecular target. When rats were treated with 40 mg/kg diosgenin by oral administration for 5d, we found that diosgenin did not have antithrombotic effect in $FeCl_3$-induced venous thrombosis model. Meanwhile, prodrug micelles was observed to have antithrombotic effect in $FeCl_3$-induced venous thrombosis model after rats were treated with 40 mg/kg prodrug micelles by tail intravenous injection for 5d. To explain the experimental phenomena, the pharmacokinetics of rats treated with 100 mg/kg diosgenin and 40 mg/kg prodrug micelles were studied. In conclusion, prodrug micelles have a long circulation time, high plasma

concentration, good safety and higher bioavailability, leading to have a better therapeutic effect than clinical antithrombotic agent as aspirin. Also, the prodrug micelles without bleeding risk prevent thrombosis by inhibiting platelet activation and apoptosis

To our knowledge, this study is firstly reported that a novel antithrombotic agent with pH-response feature, which could targetedly release drug to thrombus to inhibit arterial thrombosis and venous thrombosis. The passive targeted drug delivery to the position of thrombus by pH-responsive way provide a new insight for developing a novel selective carrier system for thrombus-targeted therapy.

Supplementary data

References

1. M. G. Beckman, W. C. Hooper, S. E. Critchley and T. L. Ortel, American Journal of Preventive Medicine, 2010, 38, S495–S501.

2. O. Osinbowale, L. Ali and Y. W. Chi, Postgraduate Medicine, 2010, 122, 54-65.

3. D. H. Lau, M. Alasady, A. G. Brooks and P. Sanders, Expert Review of Cardiovascular Therapy, 2010, 8, 941-948.

4. E. M. V. Soest, V. E. Valkhoff, G. Mazzaglia, R. Schade, M. Molokhia, J. L. Goldstein, S. Hernandez-Diaz, G. Trifiro, J. Dieleman and E. J. Kuipers, Gut, 2011, 60, 1650-1659. 7

5. S. G. Sparg, M. E. Light and J. van Staden, Journal of ethnopharmacology, 2004, 94, 219-243.

6. M. A. Hai-Ying, Q. L. Zhou and B. X. Wang, Chinese Journal of Hospital Pharmacy, 2002.

7. Y. Qin, X. Wu, W. Huang, G. Gong, D. Li, Y. He and Y. Zhao, Journal of ethnopharmacology, 2009, 126, 543-550.

8. R. Zhang, B. Huang, D. Du, X. Guo, G. Xin, Z. Xing, Y. Liang, Y. Chen, Q. Chen, Y. He and W. Huang, Steroids, 2013, 78, 1064-1070.

9. G. Gong, Y. Qin and W. Huang, Phytomedicine: international journal of phytotherapy and phytopharmacology, 2011, 18, 458-463.

10. M. Okawara, F. Hashimoto, H. Todo, K. Sugibayashi and Y. Tokudome, International Journal of Pharmaceutics, 2014, 472, 257–261.

11. J. An, X. Dai, Z. Wu, Y. Zhao, Z. Lu, Q. Guo, X. Zhang and C. Li, Biomacromolecules, 2015, 16, 2444-2454.

12. S. Namfa, K. Wichan, B. Prapaporn, P. Narubodee and W. Ruedeekorn, European Journal of Pharmaceutical Sciences Official Journal of the European Federation for Pharmaceutical Sciences, 2013, 50, 459-466.

13. H. Han, H. Wang, Y. Chen, Z. Li, Y. Wang, Q. Jin and J. Ji, Nanoscale, 2016, 8, 283-291.

14. Z. Min, R. Shaobo, L. Shi, S. Tingting, Q. Dan, Z. Haifeng, X. Zhigang, G. Huile, J. Xiabin and S. Zaicheng, Acs Nano, 2015, 9, 11455-11461.

15. J. D. A. K. Twibanire and T. B. Grindley, Polymers, 2014, 6, 179-213.

16. R. Alyautdin, I. Khalin, M. I. Nafeeza, M. H. Haron and D. Kuznetsov, International Journal of

Nanomedicine, 2014, 9, 795-811.

17. W. Lin, D. K. Nagesha, S. Selvarasah, M. R. Dokmeci and R. L. Carrier, Journal of Nanobiotechnology, 2008, 6, 11-11.

18. J. Qian, S. Ruan, X. Cao, X. Cun, J. Chen, S. Shen, X. Jiang, Q. He, J. Zhu and H. Gao, Journal of colloid and interface science, 2014, 436, 227-233.

19. C. Li, L. Dai, K. Liu, L. Deng, T. Pei and J. Lei, RSC Adv., 2015, 5, 74828-74834.

20. N. Bertrand, J. Wu, X. Xu, N. Kamaly and O. C. Farokhzad, Advanced drug delivery reviews, 2014, 66, 2-25.

21. E. Vlashi, L. E. Kelderhouse, J. E. Sturgis and P. S. Low, Acs Nano, 2013, 7, 8573-8582.

22. F. Danhier, O. Feron and V. Preat, Journal of controlled release: official journal of the Controlled Release Society, 2010, 148, 135-146.

23. X. Guo, X. Wei, Y. Jing and S. Zhou, Advanced Materials, 2015, 27.

24. N. Mackman, Nature, 2008, 451, 914-918.

25. O. N. Bae, K. M. Lim, J. Y. Noh, S. M. Chung, S. H. Kim and J. H. Chung, Toxicol Appl Pharmacol, 2009, 239, 144-153.

26. V. Leytin, D. J. Allen, S. Mykhaylov, E. Lyubimov and J. Freedman, Journal of thrombosis and haemostasis : JTH, 2006, 4, 2656-2663.

27. E. A. Frost, C. U. Arancibia and K. Shulman, Journal of Neurosurgery, 1979, 50, 768-772.

28. K. Sako, K. Kobatake, Y. L. Yamamoto and M. Diksic, Stroke, 1985, 16, 828-834.

29. H. Nakai, Y. L. Yamamoto, M. Diksic, K. J. Worsley and E. Takara, Stroke; a journal of cerebral circulation, 1988, 19, 764.

30. R. J. Harris, P. G. Richards, L. Symon, A. H. A. Habib and J. Rosenstein, Journal of Cerebral Blood Flow & Metabolism Official Journal of the International Society of Cerebral Blood Flow & Metabolism, 1987, 7, 599-604.

31. N. Song, M. Ding, Z. Pan, J. Li, L. Zhou, H. Tan and Q. Fu, Biomacromolecules, 2013, 14, 4407-4419.

32. B. Huang, D. Du, R. Zhang, X. Wu, Z. Xing, Y. He and W. Huang, Bioorganic & Medicinal Chemistry Letters, 2012, 22, 7330-7334.

33. Q. Zhou, L. Jiang, C. Xu, D. Luo, C. Zeng, P. Liu, M. Yue, Y. Liu, X. Hu and H. Hu, Thrombosis research, 2014, 133, 57-65.

34. Z. Tang, Y. Wang, Y. Xiao, Z. Ming and S. Peng, Thrombosis Research, 2003, 110, 127-133.

35. S. Dayal, K. M. Wilson, D. G. Motto, M. F. Jr, A. K. Chauhan and S. R. Lentz, Circulation, 2013, 127, 1308-1316.

36. H. Zheng, Z. Wei, G. Xin, C. Ji, W. Li, Q. Xia, N. Hai and H. Wen, Bioorganic & Medicinal Chemistry Letters, 2016, 26, 3364-3369.

37. M. Lin, S. Wei, G. Wan, Y. Ding, Y. Zhuang and H. Qi, Acta Pharmaceutica Sinica B, 2015, 5, 277-284.

38. İ. Özcan, F. Segurasánchez, K. Bouchemal, M. Sezak, Ö. Özer, T. Güneri and G. Ponchel, International Journal of Nanomedicine, 2009, 5, 1103–1111.

39. J. Gu, W. Cheng, J. Liu, S. Lo, D. Smith, X. Qu and Z. Yang, Biomacromolecules, 2008, 9, 255-262.

40. C. Ding, J. Gu, X. Qu and Z. Yang, Bioconjugate Chemistry, 2009, 20, 1163-1170.

41. M. J. D. Clift, C. Endes, D. Vanhecke, P. Wick, P. Gehr, R. P. F. Schins, A. Petrifink and B. Rothenrutishauser, Toxicological Sciences An Official Journal of the Society of Toxicology, 2014, 137, 55-64.

42. L. Zhang, X. Mu, J. Fu and Z. Zhou, Toxicology in Vitro, 2007, 21, 734-740.

43. S. M. King, R. A. Mcnamee, A. K. Houng, R. Patel, M. Brands and G. L. Reed, Circulation, 2009, 120, 785-791.

44. Y. G. †, M. L. †, L. Piao, X. Li, F. Yang, J. Zhang, B. Xiao, Q. Zhang, W. L. Song and H. Yin, British Journal of Pharmacology, 2015, 172, 5647-5660.

45. K. Sugiyama, M. Iyori, A. Sawaguchi, S. Akashi, J. R. Tame, S. Y. Park and S. Yoshida, Journal of Biological Chemistry, 2014, 289, 16303-16312.

46. E. Morgenstern, A. Ruf and H. Patscheke, Blood Coagulation & Fibrinolysis An International Journal in Haemostasis & Thrombosis, 1990, 1, 543-546.

47. A. A. Dimitriou, P. Stathopoulos, J. V. Mitsios, M. Sakarellosdaitsiotis, J. Goudevenos, V. Tsikaris and A. D. Tselepis, Platelets, 2009, 20, 539-547.

48. J. K. W. Chesnutt and H. C. Han, Computers in Biology & Medicine, 2015, 56, 182–191.

49. P. A. D. A. C. Group, European Journal of Vascular & Endovascular Surgery the Official Journal of the European Society for Vascular Surgery, 2003, 26, 1-16.

50. T. Renné, B. Nieswandt and D. Gailani, Blood Cells Molecules & Diseases, 2006, 36, 148-151.

51. G. Xin, Z. l. Wei, C. J. Ji, H. j. Zheng, J. Gu, L. M. Ma, W. F. Huang, S. L. Morris-Natschke, J. L. Yeh, R. Zhang, C. Y. Qin, L. Wen, Z.H. Xing, Y. Cao, Q. Xia, Y. R. Lu, K. Li, H. Niu, K.H. Lee, and W. Huang. Free Radical Biology & Medicine. 2017, 108: 247-257.

52. G. Xin, Z. l. Wei, C. J. Ji, H. j. Zheng, J. Gu, L. M. Ma, W. F. Huang, S. L. Morris-Natschke, J. L. Yeh, R. Zhang, C. Y. Qin, L. Wen, Z.H. Xing, Y. Cao, Q. Xia, Y. R. Lu, K. Li, H. Niu, K.H. Lee, and W. Huang. Scientific Reports. Publication 2016 Nov 2;6:36222. Doi: 10.1038/srep36222.

53. N. Ma, X. W. Liu, Y. J. Yang, J. Y. Li, I. Mohamed, G. R. Liu and J. Y. Zhang, Plos One, 2015, 10.

54. A. P. S. Azevedo, J. C. Farias, G. C. Costa, S. C. P. Ferreira, W. C. Aragão-Filho, P. R. A. Sousa, M. T. Pinheiro, M. C. G. Maciel, L. A. Silva and A. S. Lopes, Journal of ethnopharmacology, 2007, 111, 155-159.

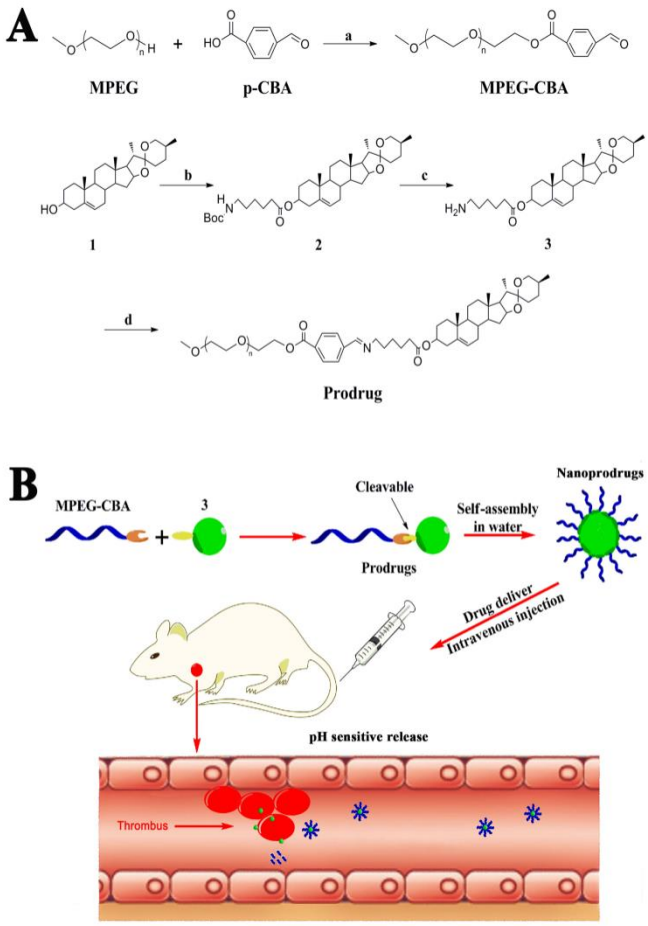


Figure 1 (**A**) Reaction scheme of prodrug initiated by diosgenin and MPEG: i) DCC, DMAP, CH_2Cl_2, 60h, 90%; ii) EDC, DMAP, CH_2Cl_2, 24h, 85%; iii) CF_3COOH, CH_2Cl_2, rt, 86%; iv) THF, 40℃,12h. (**B**) Schematic illustration showed the structure and self-assembly in aqueous solution of prodrug for *in vivo* drug deliver treating thrombus.

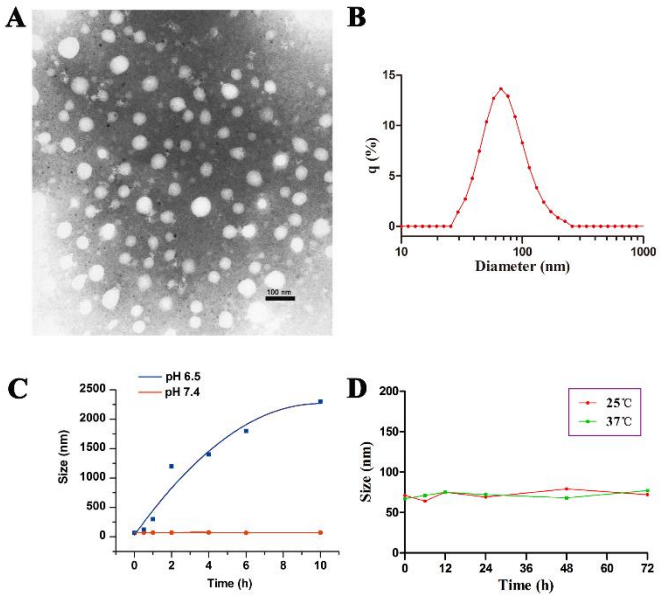


Figure 2. (**A**) TEM images of prodrug micelles. (**B**) The size distribution of prodrug micelles. (**C**) The size changes of prodrug micelles in response to pH with different times. (**D**) The size changes of prodrug micelles in different temperatures as a function of time.

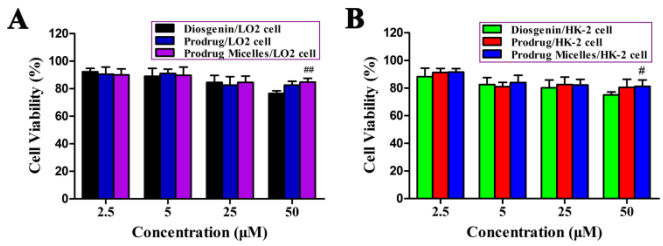


Figure 3. Toxicity of diosgenin and prodrug micelles to (**A**) LO2 cells and (**B**) HK-2 cells after incubation with various concentrations 24 h *in vitro*. $^{\#}P<0.05$ vs diosgenin, $^{\#\#}P<0.01$ vs diosgenin. Data are expressed as mean ± SD. n=3-4.

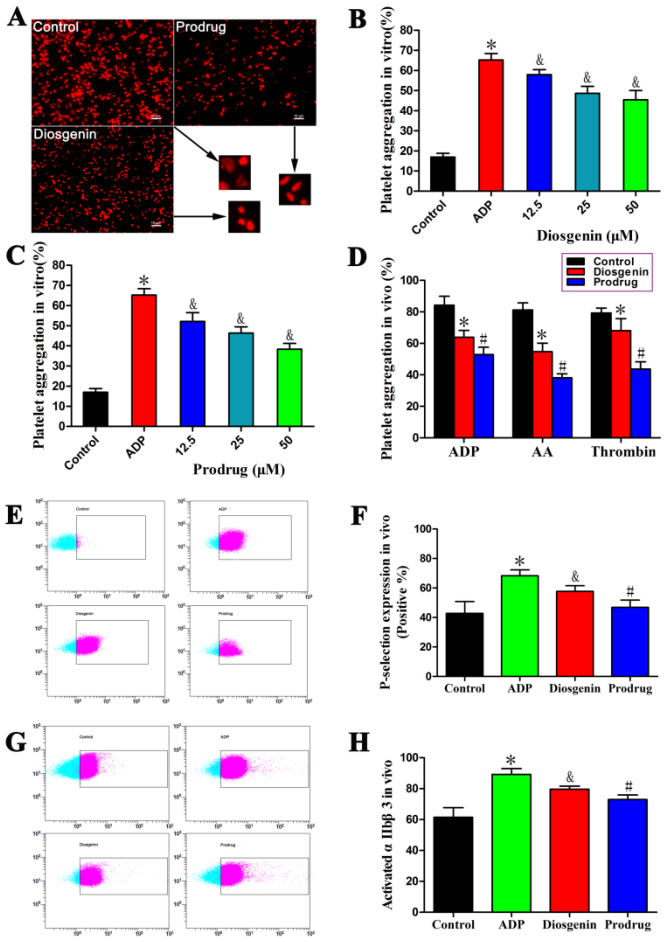


Figure 4. Experiments on platelets *in vitro* and *in vivo*. (**A**) Effect of prodrug micelles on platelets aggregation induced by ADP, AA, and thrombin *in vivo*. Effect of (**B**) diosgeninand (**C**) prodrug micelles with different concentrations on platelets aggregation induced by ADP *in vitro*.V(**D**) Effect of prodrug micelles on platelets adhesion on collagen surfaces.(**E**) P-selectin and (**G**) αIIbβ3 expression in ADP-activated platelets treated with prodrug micelles *in vitro*. Graph summarizing the effect of prodrug micelles on the (**F**) P-selectin and (**H**) αIIbβ3 expression. **P*<0.05 vs control. $^{\&}P$<0.05 vs ADP, $^{\#}P$<0.05 vs diosgenin. Data are expressed as mean ± SD. n=3-4.

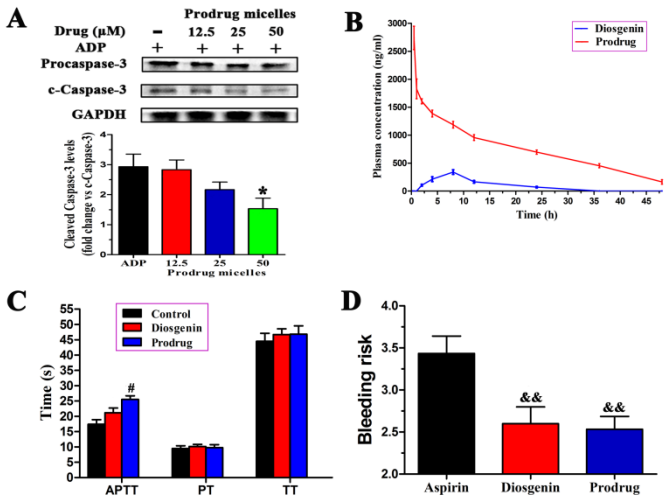


Figure 5. Western blots, pharmacokinetics and effects on blood coagulation. (**A**) Western blots of platelets treated with prodrug micelles incubated with caspase-3. (**B**) Plasma concentration of diosgenin at different times in rats after oral administration of free diosgenin (100 mg/kg) or intravenous administration of produg (40 mg/kg). (**C**) Effects of the drugs on APTT, PT and TT levels in rats. (**D**) Reduces the bleeding risk (quantitative value of bleeding risk = [(Bleeding time in the drug group - control group bleeding time) / control group bleeding time] / [(model group thrombosis weight - drug group thrombosis weight) / model group thrombosis weight]). *$P < 0.05$ vs ADP, $^{\#}P < 0.05$ vs control and diosgenin, $^{\&\&}P < 0.01$ vs aspirin. Data are expressed as mean ± SD. n=6-8.

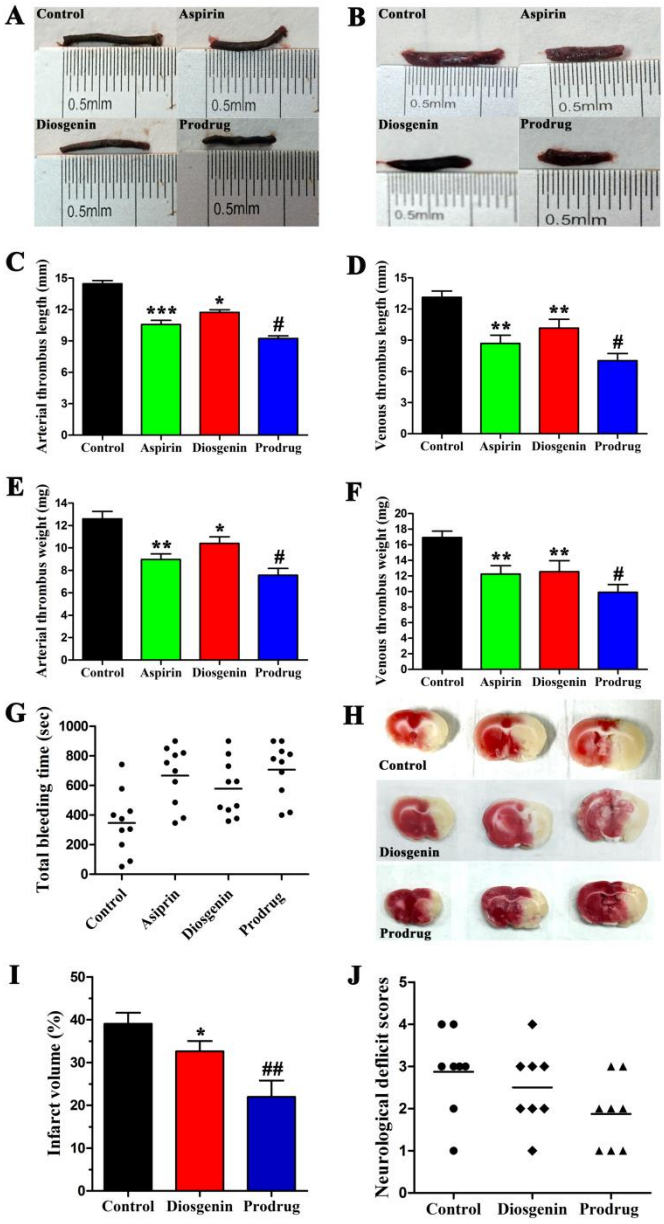


Figure 6. Effect of prodrug micelles on thrombus *in vivo*. Inhibiting effect of prodrug micelles on arterial thrombus (**A, C**) length and (**E**) weight. Inhibiting effect of prodrug micelles on venous thrombus (**B, D**) length and (**F**) weight. (**G**) Prodrug micelles increased bleeding time in the male Balb/c mice. (**H, I**) Brain tissue sections and graph summarizing of infarct size. (**J**) Prodrug micelles improve neurologic deficit score after stroke onset 24 h. **P*<0.05 vs control, ***P*<0.01 vs control, ****P*<0.001 vs control, $^{\#}$*P*<0.05 vs

diosgenin and aspirin, $^{\#\#}P<0.01$ vs diosgenin and control. Data are expressed as mean ± SD. n=6-8.

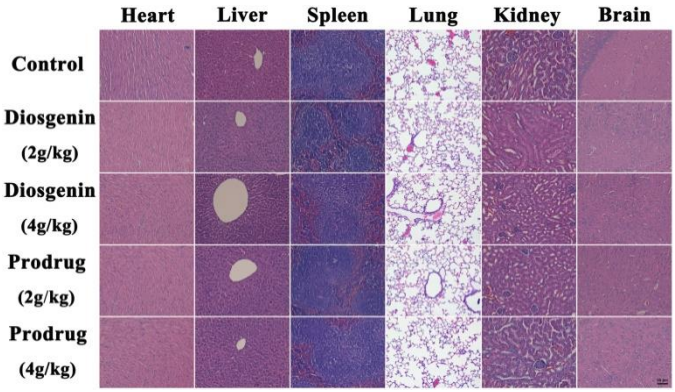


Figure 7. Histological examination of heart, liver, spleen, lung, kidney and brain after treated with diosgenin or prodrug micelles seven days by oral administration in mice.

Table 1.

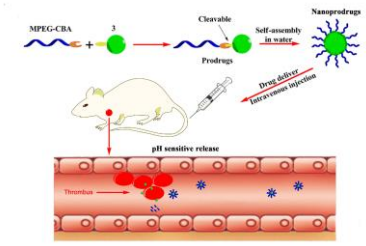
Effects of the Prodrug on PLT, PDW, MPV, PCT, P-LCR and IPF in rats.

Group	PLT (109/L)	PDW (fL)	MPV (fL)	PCT	P-LCR (%)	IPF (%)
Control	865 ± 108	7.90 ± 0.55	7.52 ± 0.51	0.38 ± 0.08	7.40 ± 0.48	0.50 ± 0.06
Prodrug	871 ± 102	7.94 ± 0.49	7.62 ± 0.48	0.40 ± 0.07	7.49 ± 0.55	0.55 ± 0.09

Data are expressed as mean ± SD; n=6-8.PLT: Platelet count, PDW: Platelet distribution width, MPV: Mean platelet volume PLT, PCT: Thrombocytocrit, P-LCR: Platelet ratio, IPF: Immature platelet fraction.

Graphical Abstract

The nanoparticle， a novel prodrug based on conjugating diosgenin to PEG with Schiff-base bond, could self-assemble into micelles in aqueous solutions. The prodrug micelles exhibited stability at different temperatures, and targeted release drugs by pH-response in the position of thrombus *in vivo*. The prodrug micelles had a long circulation time in blood and better antithrombotic activity than commercial antithrombotic agents as aspirin. Furthermore, the prodrug micelles were not observed to histological damages on tissues, and bleeding risk. Importantly, we found that the targeted delivery prodrug micelles inhibited platelet activation by suppressing apoptosis of platelet in final state. These results confirmed that the prodrug micelles was a promising new class of agent for targeted therapy multiple thrombus diseases.



Graphical Abstract