Poly-α,β-aspartyl-Arg-Gly-Asp-Phe: A novel polymeric nanomedicine

Supporting Information

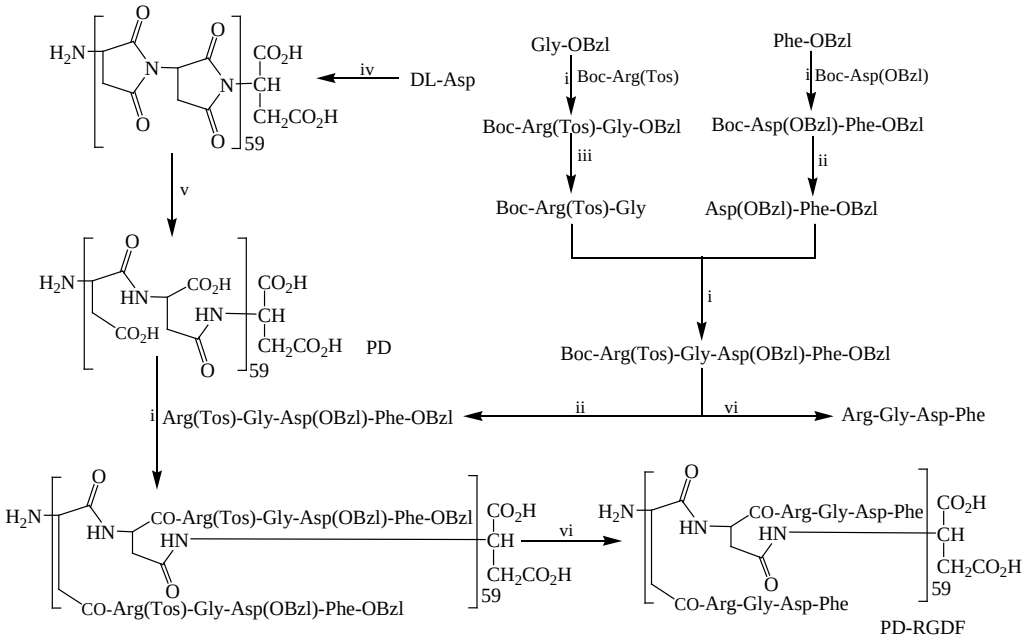
General

D,L-Asp and amino acids were purchased from Sigma Chemical Co. Protective L-Arg, Gly, L-Asp and L-Phe for preparing protective Arg-Gly-Asp-Phe were purchased from Sigma Chemical Co. GPIIb/IIIa (analytically pure) was purchased from Sigma-Aldrich Co. (LLC). Sodium citrate (analytically pure) was purchased from Beijing Chemical Factory. The column chromatography was carried out with Qingdao silica gel H. All chemical reactions were monitored with TLC (Qingdao silica gel, GF254, 0.25 mm layer thickness). The purity of PD-RGDF was examined with analytical HPLC (7 mg/mL, ultrahydrogel 120 column, 7.8×30 mm, 25 ºC; eluent, 0.1M aqueous $NaNO_3$; flow rate, 0.5 mL/min). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Advance 500 spectrometers. Amino acid analysis was carried out on a Sykam S433 (Germany). For platelet counts and platelet aggregation, a Chrono-Log 490-D Optical Aggregometer (Havertown,PA, USA) was used. Male SD rats were purchased from Animal Center of Peking University. The described assessments were performed based on a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures that the welfare of the rats was maintained in accordance with the requirements of the animal welfare act and in accordance with the guide for care and use of laboratory animals. The statistical analysis of all the biological data was carried out by use of an ANOVA test with $p <$

0.05 as significant cutoff.

Synthetic route of PD-RGDF

The preparation of PD-RGDF was performed according to the route depicted in Scheme 1.



Scheme 1 Synthetic route of poly-α,β-aspartyl-Arg-Gly-Asp-Phe (PD-RGDF). i) EDC, HOBt and NMM; ii) Hydrogen chloride in ethyl acetate (4 M); iii) Aqueous NaOH (2 M), 0 ºC; iv) H_3PO_4 (85%), 180 ºC, 0.1 MPa; v) Aqueous NaOH (2 M), 80 ºC; vi) CF_3CO_2H, CF_3SO_3H.

Preparing polysuccimide

For preparing polysuccimide 20 g (0.15 mol) of DL- Asp and 14 mL (0.09 mol) phosphoric acid (85%) was heated at 180 °C in vacuum (0.1 MPa) for 3.5 h. The resulting tawny powders were filtered and successively washed with water (5 × 70 mL), 1% hydrochloric acid (3 × 70 mL) and water (10 × 70 mL) to provide 14.4 g (99%) of polysuccimide. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 5.294 (CH),

3.233-2.737 (CH_2) . ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 173.96, 172.69, 169.70, 47.82, 33.10.

Preparing PD

A solution of 3 g (30 mmol) of polysuccimide in an aqueous solution of 1.4 g of NaOH in 20 mL of water was stirred at 0 ºC for 1 h. The pH of the solution was adjusted to 1 with 35% hydrochloric acid. The solution was poured into 300 mL methanol, the formed precipitates were collected by filtration and dried at 40 °C under reduced pressure to provide 3.3 g (91%) of PD. ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 177.87, 173.4, 172.7, 172.0, 171.8, 51.76, 51.60, 39.12, 37.61.

Preparing PD-RGDF

At 0 ºC to a solution of 83 mg (0.72 mmol) of PD in 20 mL anhydrous DMF 97 mg (0.72 mmol) of HOBt and 360 mg (1.87 mmol) of HCl·EDC were added, the solution was stirred at 0 ºC for 30 min, then 621mg (0.72 mmol) of HCl·Arg(Tos)-Gly-Asp-(OBzl)-Phe-OBzl was added, the pH of the reaction mixture was kept ~9 with N-methylmorpholine and stirred at room temperature for 24 h. The reaction mixture was successively washed with ether (3×30 mL), 5% aqueous $KHSO_4$ (3×30 mL) and water (3×30 mL). At 37 °C the reaction mixture was evaporated under reduced pressure and the yellowish powders of poly-α,β-Arg(Tos)-Gly-Asp(OBzl)-Phe-OBzl

were obtained. The solution of poly-α,β-Arg(Tos)-Gly-Asp(OBzl)-Phe-OBzl in 5.4 mL CF_3CO_2H and 1.8 mL CF_3SO_3H was stirred at 0 °C for 90 min. Upon removal of CF_3CO_2H and CF_3SO_3H the residue was triturated with ether (5×30 mL). The powders were dissolved in 5 mL distilled water, centrifuged for 30 min and the supernatant was lyophilized to provide 174 mg (43%) colorless powders of PD-RGDF. ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 177.33, 171.92, 170.50, 170.07, 156.79, 138.48, 137.48, 129.79, 129.43, 128.56, 126.84, 60.52, 56.21, 53.66, 53.09, 51.25, 42.43, 40.58, 38.83, 37.63, 27.96, 24.32. $[\alpha]_D^{24}$ (C 0.01, H_2O) = -21.56. HPLC purity was 98.4% and retention time was 11.5 min.

Recording transmission electron microscopy (TEM) images

The shape and size of the nanoparticles of PD-RGDF was imaged on TEM (JSM-6360 LV, JEOL, Tokyo, Japan). A 10^{-6} nM solution of PD-RGDF in ultrapure water was dripped onto a formvar-coated copper grid and then a drop of anhydrous ethanol was added to promote removing water. The grid was first allowed to thoroughly dry in air and then at 37 °C for 24 h. The samples were viewed under the TEM. The shape and size distribution of the nanospecies were determined from counting over 100 species in randomly selected regions on the TEM copper grid. All the determinations were carried out in triplicate grids. TEM was operated at 120 kV, electron beam accelerating voltage. Images were recorded on an imaging plate (Gatan Bioscan Camera Model 1792) with 20 eV energy windows at 6000-400,000× and

were digitally enlarged.

Recording scanning electron microscopy (SEM) image

A 10^{-6} nM solution of PD-RGDF in ultrapure water was dripped onto a glass plate, and a drop of anhydrous ethanol was added to promote removing water. The nano-particles of PD-RGDF were characterized for their shape and size on SEM (JEM-1230, JEOL, Tokyo, Japan) at 50 kV. The specimens were coated with 20 nm gold-palladium using JEOL JFC-1600 Auto Fine Coater. The coater was operated at 15 kV, 30 mA, 200 mTorr (argon) for 60 s. The shape and size distribution of the nano-particles were determined from counting over 100 particles in randomly selected regions on the SEM alloy. All the determinations were carried out in triplicate grids. Images were recorded on an imaging plate (Gatan Bioscan Camera Model 1792) with 20 eV energy windows at 100-10,000× and were digitally enlarged.

Atomic force microscopy (AFM)

***In vitro* anti-platelet aggregation assay**

An H-10 cell counter was used to determine the platelet count and a Two-Channel Chronolog Aggregometer was used to evaluate platelet aggregation. After collection, the pig blood was centrifuged at 116 *g* for 10 min and the platelet rich plasma (PRP)

was removed. The remaining blood was centrifuged for an additional 10 min at 200 *g* to prepare platelet poor plasma (PPP). The final platelet count of the citrated plasma samples was adjusted to 2×10^8 platelets/mL with autologous PPP. To an optical aggregometry testing tuber, 0.5 mL of the adjusted plasma sample and 5 μL of NS or 5 μL solution of PD-RGDF in NS (final concentration: 20, 10, 2 and 0.2 nM) was added. After adjustment of the baseline, 5 μL solution of PAF in NS (final concentration 1 μM) or 5μL solution of ADP in NS (final concentration 10 μM) or 5μL solution of AA in NS (final concentration 0.15 mg/mL), or 5 μL solution of TH in NS (final concentration 1 U/ml) was added and the aggregation was measured at 37 ºC for 5 min. The effects of RGDF (concentrations ranging from 112 μM to 112 nM) on the platelet aggregation induced by PAF or ADP or AA or TH were observed. All the anti-platelet aggregation tests were carried out in sixplicate tubers. The maximal rate of platelet aggregation (A_m%) was represented by the peak height of aggregation curve. The inhibition rate was calculated by inhibition% = [(A_m%$_{NS}$) - (A_m%$_{sample}$)]/ (A_m%$_{NS}$), wherein A_m%$_{NS}$ = 55.83 ± 5.56%. The concentration versus inhibition rate curve is plotted to determine the IC_{50} values of PD-RGDF or RGDF against the four aggregators induced platelet aggregation via GWBASIC.EXE program.

***In vivo* antithrombotic assay**

Aspirin (167 μmol/kg) or RGDF (5 μmol/kg), or PD (1 nmmol/kg) or PD (1 nmmol/kg) plus RGDF (1 nmol/kg) or PD-RGDF (10, 1 and 0.1 nmol/kg) were dissolved in NS before administration and kept in an ice bath. Male SD rats weighing

230-250 g were fed with NS, or the solution of aspirin or RGDF or PD or PD plus RGDF or PD-RGDF in NS. The rats were anesthetized with urethane (140 mg/kg, i.p.) and the right carotid artery and left jugular vein were separated. A weighed 6 cm thread was inserted into the middle of a polyethylene tube. The polyethylene tube was filled with heparin sodium (50 IU/mL) in NS and one end was inserted into the left jugular vein, the other end of the polyethylene tube was inserted into the right carotid artery. Blood was allowed to flow from the right carotid artery to the left jugular vein through the polyethylene tube for 15 min. The thread was removed to obtain the fresh thrombus for weighing.

GPIIb/IIIa expression assay

GPIIb/IIIa levels were measured on citrated rat blood samples by enzyme immunoassay according to the manufacturer's instructions (rat platelet membrane glycoprotein IIb/IIIa ELISA kit, Shanghai Yuanye Biotechnology Co., China). The blood was collected in 3.8% aqueous solution of sodium citrate (1/9, v/v) and immediately centrifuged at 116 *g* for 10 min to collect PRP. In 15.5 mL NS 1 mg PD-RGDF was dissolved to prepare 1 μM solution. To 96 μL PRP 2 μL PD-RGDF (1 μM) or 2 μL NS or 2 μL RGDF (10 μM) or 2 μL RGDF (1 mM) was added and incubated at 37 °C for 5 min, to which 2 μL solution of AA in NS (7.5 mg/mL) was added and incubated for 3 min. This is the test sample, i.e. PD-RGDF treated PRP sample or the black control sample, i.e. NS treated PRP sample. To the control well and the test well of the 96-well plate coated with the enzyme, 10 μL NS treated PRP sample and 10 μL

PD-RGDF treated PRP sample were added, respectively, and then 40 μL sample diluents as well as 100 μL HRP- conjugate reagent were added. The plate was at 37 °C incubated for 60 min. Upon the removal of the mixed liquids, the wells were washed with washing solution (from the kit, 5×350 μL), to each of which 50 μL chromogen solution A and 50 μL chromogen solution B (from the kit) were successively added, gently mixed and kept at 37 °C in dark for 15 min for coloration. To each well 50 μL stop solution (from the kit) was added to stop the coloration. The OD value of each well was measured at 450 nm and the GPIIb/IIIa level was calculated according to the standard samples (from the kit). The samples should be kept at 2-8 °C in their pouch with the desiccant provided.

SEM image of PD-RGDF treated platelets

Rat blood was collected in an aqueous solution of 3.8% sodium citrate (1:9, v/v) and immediately centrifuged at 160 g for 15 min to collect platelet-rich plasma (PRP). To 500 μL of PRP 10 μL arachidonic acid (Sigma) (AA, final concentration,15 μM) and 10 μL NS or 10 μL PD-RGDF (final concentration, 10^{-6} nM) was successively added, and incubated at 37 ºC for 5 min. The system was incubated for 5min at 37 ºC and centrifuged at 1917 g for 15 min allowing the platelets to precipitate. The precipitated pellet was immersed in 3% glutaraldehyde solution (Sinopharm Chemical Reagent Beijing Co., Ltd) for 1 hour. Glutaraldehyde was separated by centrifugation, and the supernatant was discarded. Precipitants was subsequently were washed in phosphate buffer 3 times and postfixed in 1% osmium tetroxide (Sigma) for 1 hour. Sample was

centrifuged again and treated with 50% ethanol for 10 min and 2 times. Sample was dehydrated in graded series of ethanol (70%, 80%, 90% and 100%) and continued for 10-min incubation. Sample was dried using critical point dryer (CPD300, Leica Ltd., German). The dried samples were mounted on an aluminum stub, coated with platinum using auto fine coater (JFC-1600, JEOL, Japan), and observed on SEM (S-4800, Hitachi Ltd., Japan).

Size and PDI of the particles during 8 days and Mean size with SD

To represent the stability of the nanoparticles 10^{-6} nM solutions of PD and PD-RGDF in ultrapure water were prepared, the 8-day particle sizes were tested for size and polydispersity index (PDI) by dynamic light scattering on a Malvern's Zeta Sizer (Nano-ZS90) with the DTS (Nano) Program, and the data are shown in Table 1 and Table 2.

Table 1 Eight-day particle size and PDI of PD-RGDF in ultrapure water (10^{-6} nM)

Day	1	2	3	4	5	6	7	8
Size, nm	60.1	94.1	153.1	166.5	166.9	171.8	169.4	170.4
PDI	0.814	0.817	0.647	0.716	0.798	0.844	0.801	0.871

Table 2 Eight-day particle size and PDI of PD in ultrapure water (10^{-6} nM)

Day	1	2	3	4	5	6	7	8
Size, nm	70.4	81.4	122.3	131.6	140.1	142.5	145.0	144.8
PDI	0.655	0.630	0.611	0.594	0.506	0.637	0.553	0.631

The solutions of PD and PD- RGDF in ultrapure water (10^{-6} nM) were prepared and stored at room temperature for 12 days to measure the particle size and PDI, and

the results are shown in Fig. 1 and Fig. 2. As seen, on 12th day the particle size of PD and PD-RGDF are 89.4 ± 56.6 nm and 101.6 ± 85.4 nm, respectively, while the PDI of PD and PD- RGDF are 0.288 and 0.221, respectively.

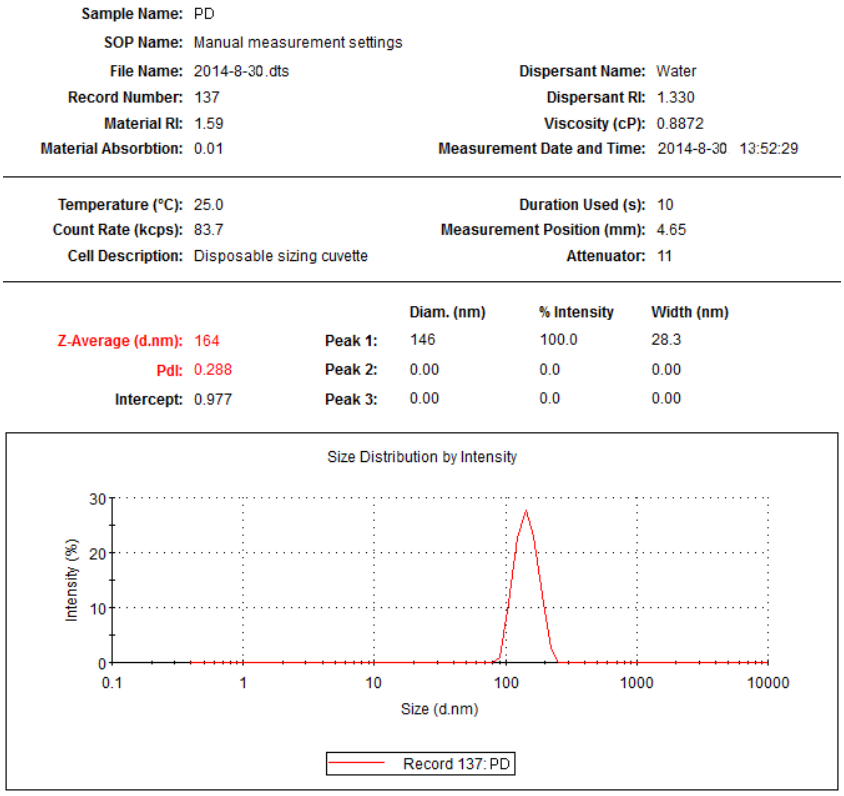


Fig. 1 The mean size, half-peak width and PDI of the nanoparticles of PD.

Sample Name:	PD-GFDF		
SOP Name:	Manual measurement settings		
File Name:	2014-8-30.dts	**Dispersant Name:**	Water
Record Number:	17	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.01	**Measurement Date and Time:**	2014-8-30 13:42:54

Temperature (°C):	25.0	**Duration Used (s):**	10
Count Rate (kcps):	85.7	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	11

			Diam. (nm)	**% Intensity**	**Width (nm)**
Z-Average (d.nm):	198	**Peak 1:**	187	100.0	42.7
PdI:	0.221	**Peak 2:**	0.00	0.0	0.00
Intercept:	0.974	**Peak 3:**	0.00	0.0	0.00

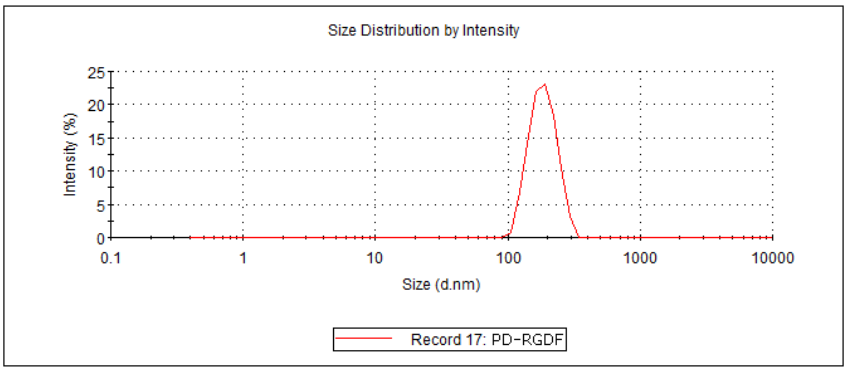


Fig. 2 The mean size, half-peak width and PDI of the nanoparticles of PD-RGDF.

Zeta potential of the nanoparticles of PD and PD-RGDF on 12th day

After 12-day storage at room temperature the solutions of PD and PD-RGDF in ultrapure water (10^{-6} nm) were received zeta potential measurements at the mode of automatic measurement on a BROOKHAVEN INSTRUMENTS CORPORATION (Zeta Plus Zeta Potential Analyzer). The measurements explored that the zeta potentials of PD-RGDF and PD were -29.15 mV and -39.12 mV, respectively.