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Targeted multifunctional redox-sensitive micelles co-delivery of DNA and doxorubicin for treatment of breast cancer

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1. Materials and methods

1. Blood compatibility assay support information

1.1. Hemolysis experiment

The hemolysis assay was measured according to the method reported by O'Leary and Guess[1]. Red blood cell (RBC) suspension (50 μL, 16% in PBS, v/v) was added to 1 mL of PBS solutions each containing different concentrations of the PPPT copolymer or PEI (25 kDa). PBS and distilled water were, respectively, used as the negative and positive controls[2]. All samples were incubated for 4 h. Then the RBC suspensions were centrifuged at 1000 × g for 5 min and the absorbance values of the released hemoglobin were tested at 540 nm with a microplate reader (MRX, Dynex Technologies). The experiment was performed three times. The hemolysis was calculated as the formula: [3]

$$Haemolysis\ (\%) = \frac{As - An}{Ap - An} X100\%$$

where As, An and Ap are the absorbance of the sample, negative and positive controls, respectively. All data are presented as the mean (±SD) of three measurements.

1.2. Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT)

Fresh, anticoagulated whole blood was centrifuged at 1000 ×g for 10 min and the resulting supernatant (platelet-poor plasma) was collected. The platelet-poor plasma (360 μL) was mixed with the PPPt copolymer (40 μL in PBS). Normal saline-treated platelet-poor plasma was used as a negative control (n=3)[4]. After adding the corresponding reagents, the APTT and PT of the samples were measured with an automatic coagulation analyser (STAR Evolution, Diagnostica Stago, Assiernes, France).

1.3. RBC aggregation and morphology

The citrated whole blood was centrifuged at 1000 × g for 5 min. After removing plasma and the buffy coat layer, the RBCs were washed with PBS. Morphology of RBCs was studied by incubating 10 μL of different concentrations of aqueous PPPT copolymer solutions with 90 μL of RBCs for 15 min at 37 °C. The same volume of RBCs incubated with PBS or 0.01 mg/mL PEI-25k was used as

the control. After that, the RBCs were obtained by centrifuge and fixed by 4% paraformaldehyde overnight at 25 °C and then dehydrated with 70, 85, 95 and 100% (v/v) ethanol for 10 min, respectively, and then dried in air. Then the dried RBCs were coated with gold and observed with a scanning electron microscope (SEM, Philips XL-30).

1.4 Serum stability of PPPT/DNA complexes

PPPT/DNA complexes (1 mg/mL) was incubated with PBS supplemented with 10% FBS at 37 ºC. At designated time intervals, the size of complexes was monitored by using a Zeta-Sizer Nano ZS (Malvern Instruments Ltd., UK).

2. Results and discussion

2.1. Blood compatibility assay

The instability of the carriers in the blood was considered as the most serious limitation in the therapeutics. The blood compatibility of PPPT was assessed by hemolysis analysis, PT and APTT assay and RBCs observation. Hemolysis study of PPPT was assessed by UV-visible spectrophotometry at 540 nm of hemoglobin release from erythrocytes after incubating PPPT micelles with RBCs. Hemolysis levels less than 20% are considered acceptable for nanoparticle formulations[5, 6]. Fig. S1A shows the percentage hemolysis of the blood in contact with different PPPT concentration of 0.001 mg/mL to 1 mg/mL, and PEI-25K was set as the control. It was found that PPPT exhibited good blood compatibility. Even after 4 h incubation with concentrated PPPT up to 200 μg/mL, the sample showed low-hemolytic effect, with the extent of hemolysis lower than the permissible level of 20%[5, 6]. However, PEI-25K showed obvious hemolysis, where 0.2 mg/mL PEI resulted in more than 60% hemolysis. This result indicated that PPPT was more suitable for drug/gene delivery through intravenous injection.

The effect of gene carriers on blood coagulation is one of the most important functions

regarding blood tissue[7]. PT and APTT study represents extrinsic and intrinsic pathways of coagulation[8, 9]. PT and APTT study was performed to evaluate the interaction of the micelles with coagulation factors. The acceptable range for PT and APTT is 11-14 s and 27-40 s, respectively[10]. The effects of the PPPT on APTT and PT are shown in Fig. S1B. Compared to the PBS control, PPPT did not significantly change the APTT and PT of the blood under the concentration of 0.5 mg mL^{-1}. The results indicated that PPPT under experimental concentrations do not cause activation of coagulation pathways and are biocompatible with respect to coagulation pathways.

To explore the effect of PPPT on RBCs the morphological changes of RBCs were observed by SEM (Fig. S1C). It was found that the aggregation or morphological of the RBCs did not change within the range from 0.001 to 0.2 mg/mL of the PPPT compared to PBS control. However, the RBCs aggregated in the presence of 0.5 mg/mL and 1 mg/mL of PPPT. The aggregated RBCs fully changed their shape, which enabled them to aggregate and eventually form large agglomerates. However, some of the RBCs changed from their normally biconcave shape to a hedgehog-like or to a spherical shape with pointed spicules. When the concentration reached 1 mg/mL, few normal RBCs were observed. The interaction of RBCs with foreign biomedical polymers is mainly driven by the electrostatic attraction between the negative RBC surface charge and the positive charge of polycations, and/or by the hydrophobic interaction between the hydrophobic groups of amphiphilic polymers and the lipid bilayer of the RBC membrane, and/or hydrogen bonds and van der Waals' forces[11]. Therefore, the RBC aggregation and morphological changes are attributed here to the electrostatic attraction between the RBC surface and the polycationic PEI on the backbone of the PPPT copolymer. According to previous study, the interactions of polymeric biomaterials and RBCs are mediated by the electrostatic and the hydrophobic interactions[12]. The introduction of PEG segments improved the blood compatibility of PPPT and made the resultant codelivery carrier safer to the blood. This result was in accordance with that of hemolysis analysis. PPPT displayed much better blood compatibility compared with PEI-25K. Furthermore, the used concentration of PPPT in this work was lower than 0.2 mg/mL, suggesting more suitable for drug/gene delivery through intravenous injection.

2.2. Serum stability of PPPT/DNA complexes

To further confirm the stability of PPPT/DNA during circulation, we have introduced experiments of serum stability, the relevant data were shown in Fig. S2. The particle sizes of HPAA/MMP-9 and HPAA-MTX/MMP-9 maintained the initial size over the experimental time in presence of 10% fetal bovine serum (FBS), this result indicated that PPPT/DNA exhibited excellent stability in FBS.

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Table S1. Average zeta potential and size of the nanostructures for PPPT+GSH (20 mM) at 0, 1, 3, 6, 12 and 24h by DLS.

Time (h)	Size (nm)	Zeta potential(mV)
0	109.5±0.12	50.1±0.80
1	106.8±0.29	49.2±0.44
3	104.1±0.13	48.6±0.38
6	98.5±0.22	42.9±0.21
12	91.45±0.27	38.1±0.63
24	91.05±0.89	37.6±2.25

Figure Captions:

Fig.S1. (A) Effect of PPPT and PEI-25k with different concentrations on the hemolysis. (B) PT and APTT assay of PPPT polymer micelles. (C). Morphology and aggregation of the RBCs in the presence of different concentrations of PPPT copolymer as observed with SEM.

Fig.S2. Stability of PPPT/DNA in PBS (pH 7.4) containing 10% fetal bovine serum (FBS) measured by dynamic light scattering.

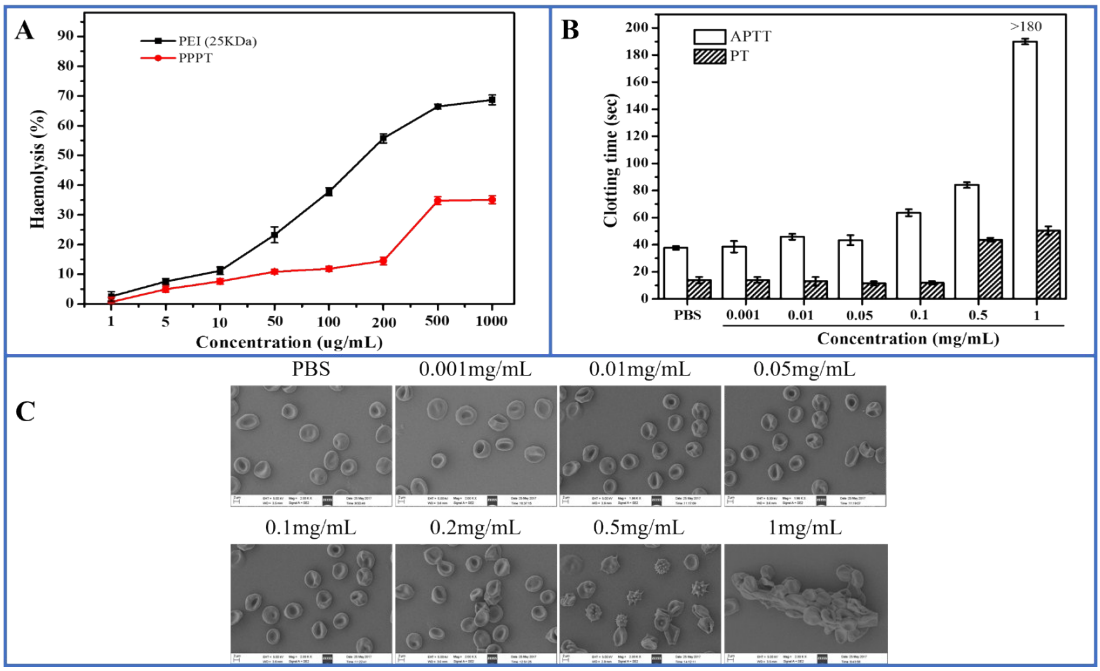
A
PEI (25KDa)
PPPT
Haemolysis (%)
Concentration (ug/mL)
B
APTT
PT
>180
Clotting time (sec)
PBS
0.001
0.01
0.05
0.1
0.5
1
Concentration (mg/mL)
C
PBS
0.001mg/mL
0.01mg/mL
0.05mg/mL
0.1mg/mL
0.2mg/mL
0.5mg/mL
1mg/mL

Fig.S1.

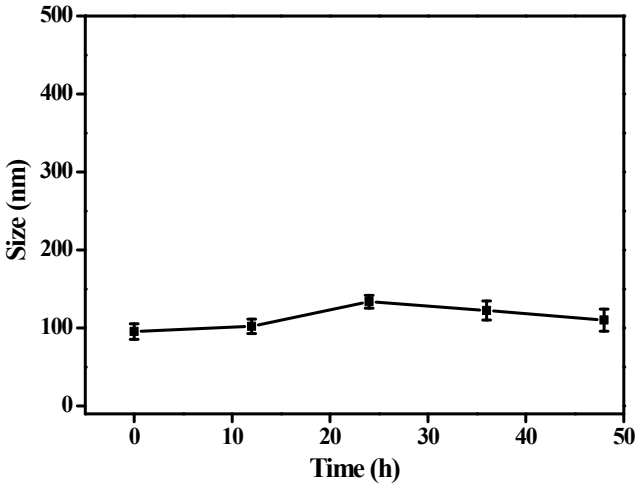

Fig.S2.