Supplementary Material

Systemic siRNA Delivery With a Dual pH-Responsive and Tumor-targeted Nanovector for Inhibiting Tumor Growth and Spontaneous Metastasis in Orthotopic Murine Model of Breast Carcinoma

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1. Materials

Branched polyethyleneimine with the MW of 1,800 (PEI1.8k) and 3-(3,4-dihydroxyphenyl) propionic acid (DHPA) were purchased from Alfa Aesar Ltd. (Tianjin, China). α-methoxy ω-amino poly(ethylene glycol) amine (mPEG-NH$_2$, MW 2kDa) was purchased from JenKem Technology Co. Ltd. (Beijing, China). 2-(bromomethyl) phenylboronic acid (2-Br-PBA) was purchased from Aladdin Industrial Inc. (Shanghai, China). N,N-Diisopropylethylamine (DIEA), Alizarin Red S (ARS) was bought from J&K Chemical Ltd. (Beijing, China). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was obtained from Sigma-Aldrich Inc. (Shanghai, China). Fluorescein isothiocyanate labeled sambubus nigra lectin (FITC-SNA), fluorescein isothiocyanate labeled wheat germ agglutinin (FITC-WGA) and fluorescein isothiocyanate labeled maackla amurensis lectin (FITC-MAA) were supplied from Vector Laboratories (Burlingame, CA, USA). Sialidase was purchased from New England Biolabs, Inc. (Hitchin, UK).

2. Synthesis and characterization of the polymers

2.1. Synthesis of mPEG-catechol (mPEG-Cat)

mPEG-Cat was synthesized by coupling between mPEG-NH$_2$ (MW 2 kDa) and DHPA (Figure S1A). DHPA (182.2mg, 1.0mmol) dissolved in 5ml DMF was activated with EDC (383.4mg, 2mmol) and NHS (230.2mg, 2mmol) for 2 h, and then added dropwise to mPEG-NH$_2$ (1g, 0.5mmol) in 5 ml DMF with DIEA (262 µl, 1.5mmol). The reaction solution was stirred for 24 hrs at room temperature. Then, the mixture was transferred into a dialysis tube (MWCO 1,000) and dialyzed against deionized water for 48 hrs, followed by lyophilized to obtain white product. The conjugation of DHPA to mPEG was confirmed by $^1$H-NMR spectroscopy in CDCl$_3$. $^1$H NMR (600MHz, CDCl$_3$, δ): 6.75-6.80 (d, 1H, aromatic), 6.70 (s, 1H, aromatic), 6.55-6.60 (d, 1H, aromatic), 2.80-2.85 (t, 2H, adjacent to aromatic), 2.40-2.45
(t, 2H, -NHCO-CH2-), 3.50-3.70 (m, -O-CH2CH2O-), 3.37(s, 3H, OCH3). The absorbance of catechol group at 280 nm was measured with a UV-Vis spectrophotometer (Implen GmbH., Germany), and the coupling yield was calculated by determining the catechol group concentration from a standard curve of dopamine hydrochloride in a concentration range of 50–250 mM.

As depicted in **Figure S1B**, the structure of mPEG-Cat was confirmed by the characteristic peaks at 6.5–7.0 ppm (m, 3H, aromatic) of DHPA and 3.55–3.67 ppm (m, -O-CH2CH2O-) of PEG backbone, respectively.

### 2.2. Synthesis of PEI-PBA

PEI-PBA was prepared according to the reference with some modifications[1]. Briefly, 2-(bromomethyl) phenylboronic acid (1.289 g, 6 mmol) and PEI1.8k (0.900 g, 0.5 mmol) were dissolved in methanol and stirred at 70°C for 24 hrs. After reaction, the mixed solution was dropped into 30 mL cold ether to acquire a pale-yellow precipitate. Then the crude product was purified by dialysis (MWCO 1,000 Da) against distilled water for 48 h. The polymers were characterized by 1H NMR spectroscopy (Varian Mercury-600 MHz spectrometer, Varian Medical Systems, Inc., USA). As presented in **Figure S2B**, the peaks at 7.8–6.8 ppm (m, 3H, aromatic) of PBA and 3.0–2.0 ppm (m, 4H, -NCH2CH2N) of PEI were evidence for the successful conjugation. Herein, the modification degree of PBA was based on the integration ratio of resonances at 7.8–6.8 ppm (the benzene proton signals of PBA) and 3.0–2.0 ppm (the ethylene proton signals of PEI) in the 1H NMR spectra. PBA were grafted on PEI1.8k at various substitution degree (DS) (data not shown), and the optimal DS was selected as 20.1%.

### 2.3. The formation of PEG-CPB-PEI (PCPP)

PEG-CPB-PEI was prepared by mPEG-Cat and PEI-PBA at 1:2 molar ratios of catechol to PBA. Herein, the borate esters act as a “joint” to spontaneously integrate mPEG-Cat and
PEI-PBA into one entity via the conjugation of PBA and Cat in neutral aqueous medium without any catalysts. A typical procedure was used as follows: mPEG-Cat and PEI-PBA were dissolved in HEPES buffer (pH 7.4) and stirred overnight under nitrogen. The product was dialyzed (MWCO 5,000 Da) against distilled water for 48h. Then, the PEG-CPB-PEI polymer was lyophilized and stored at -20 ℃ for further use. For convenience, PEG-CPB-PEI was further abbreviated as PCPP in the article.

3. The binding capacity of PEI-PBA with cis-diols

To verify the binding capacity of PEI-PBA with cis-diols in our system, the apparent pKₐ of PEI-PBA was first determined by supervising the decrease of absorbance at 268 nm upon increasing pH according to Wang et al.[2]. The UV absorption variations occurred due to the change of hybridization state of boron, which transformed from the trigonal sp² geometry into the tetrahedral sp³ geometry. As shown in Figure S4A, the pKₐ value was determined to be 7.18. Because most boronic acids are weak lewis acids with a pKₐ of ca. 8–9, it usually need to be performed in alkaline solution to form stable borate esters (the pH of medium should be equal or greater than the pKₐ)[3]. However, this induced the inconvenient operation and a hazard of disassembly of borate esters in physical conditions. In our design, the strategy of borate formation at pH 7.4 is based on the introduction of an amine in ortho-position to the boronic acid, which could form an intramolecular B…N interacton (Wulff-type boronic acids)[4]. As the chemical structure of PEI-PBA illustrated in Figure S2A, the introduction of an ortho aminomethyl group to boronic acid decreased pKₐ of boronic acid (Figure S4A), which facilitated higher association constants between PBA and cis-diols at neutral pH.

Then Alizarin Red S (ARS) was used to investigated the binding affinities of different diols for PBA[2]. ARS is a catechol-containing dye, which could display dramatic color and fluorescent changes upon binding to boronic acid. A PEI-PBA solution (15 mM) was mixed with the ARS solution (0.75mM) in HEPES buffer at pH 7.4. Then, the fluorescence intensity
of PBA-ARS complex was measured after adding different concentrations of \textit{cis}-diols at pH 7.4 using a fluorescence spectrometer (Fluorolog-Tau-3, ISA Co., Ltd., USA) ($\lambda_{\text{ex}}$ 468 nm, $\lambda_{\text{em}}$ 572 nm). As illustrated in Figure S4B, the binding of PBA with catechol, ribose or glucose induces the fluorescence quenching of PBA-ARS complex due to competitive association between diols\cite{5}. Furthermore, the binding capacity of PBA-glucose was lower than that of PBA-ribose or PBA-catechol, indicating that the glucose level during blood circulation might have little impact on the stability of the siRNA delivery system after intravenous administration.

4. Cytotoxicity assay

The cell toxicity of PCPP copolymer was examined by MTT assays. 4T1 cells were seeded into a 96-well plate. After 24 h culture, cells were incubated with the polymers of serial concentrations from 10 to 500 $\mu$g mL$^{-1}$ for 24 or 48 h. Then the medium was removed and 200 $\mu$L fresh RPMI 1640 containing 20 $\mu$L MTT solution (5 mg/mL in PBS buffer) was added. After further incubation for another 4 h, the medium was discarded and 150 $\mu$L DMSO was added to dissolve the formazan crystals. The absorbance was detected at 570 nm using a Synergy H1m Monochromator-Based Multi-Mode Microplate Reader (BioTek., USA). The cell viability was calculated as Equation (S1). The in vitro cytotoxicity of nanoparticles containing 100 nM siN.C. at different N/P ratios was also studied as described above.

\begin{equation}
\text{Equation (S1): cell viability (\%)} = \frac{OD_{570(\text{sample})}}{OD_{570(\text{control})}} \times 100
\end{equation}

\textbf{Figure S5} showed that the cell viability was above 80\% when the concentration of polymer was ranging from 10 to 300 $\mu$g mL$^{-1}$, indicating negligible cytotoxicity of the PCPP copolymer during this range. Furthermore, no obvious cytotoxicities of PCPP$_{\text{siN.C.}}$ nanoparticles were obtained at different N/P-ratios.

5. In vitro gene silencing effects of PCPP$_{\text{siLuc}}$ nanoparticles for targeting study
The silencing capacity on luciferase reporter gene in 4T1-Luc cells was investigated after treatment with different formulations. As controls, the naked siLuc, PEI1.8k_siluc, PEI-PBA_siluc and Lipofectamine 2000_siluc complexes were used. The reporter assay results were showed in Figure S6. The siLuc dose was first investigated. As we can see in Figure S6A, when the 4T1-Luc cells were treated with PCPP_siluc at 50 nM, 25.6 ± 2.0% reduction of the luciferase expression was obtained. When the siLuc concentration was improved to 100 nM, 53.3 ± 3.6% reduction was observed. However, as the siLuc concentration increased further from 100 nM to 200 nM, the luciferase expression reduced without statistically significant difference (P > 0.05). Hence, 100 nM was served as the fixed concentration in future research.

The result of gene silencing effects with different formulations was shown in Figure S6B. As compared with PEI1.8k_siluc, PCPP_siluc displayed much higher gene silencing ability, indicating the enhanced effect is attribute to the interaction of PBA with cells. Interestingly, the gene silencing efficiency in the PCPP_siluc group was almost at the same level as that of PEI-PBA_siluc, which suggested that the detachable PEG modification did not inhibit cellular uptake of nanoparticles by target cells. Compared to the permanent PEGylation[6], the detachable PEGylation of gene carriers might achieve prolonged circulation, advanced targeting capacity and enhanced cellular uptake at the same time.

6. The expression of sialylated antigens on cell surface after treatment with sialidase

In order to verify the hydrolysis effect of sialidase, flow cytometry analysis was performed to detect the binding of FITC-SNA, MAA or WGA to SA on 4T1 cell surfaces. Cells were incubated with sialidase (50 mU mL⁻¹) for overnight prior to addition of PCPP/Cy3-siRNA. Sialidase is an enzyme that can cleave SA epitopes from cells. Then the cells were incubated with FITC-SNA, MAA or WGA (SNA 1μg mL⁻¹, MAA 5μg mL⁻¹, WGA 5μg mL⁻¹) for 30 min, followed by flow cytometry analysis. SNA, MAA and WGA are the
lectins, widely used to determinate the sialylation in cancer cells[7, 8]. These three lectins each bind different structural subclasses of sialylated epitopes. MAA could specifically recognize α2,3-linked sialic acid[9]. SNA primarily binds to α2,6-linked SA on the terminal galactose and to a less degree α2,3-linkage[10]. WGA has a broader selectivity range with binding in any linkage (α2,3-, α2,6- or α2,8-) to N-acetyleneuraminic acid and N-acetyl-D-galactosamine besides N-acetyl-D-glucosamine and its β-1,4 linked oligosaccharides[11, 12].

As represented in Figure S7, compared with the control groups (PBS treated groups), sharp decreased fluorescent signals ($P < 0.01$) could be determined at the cell surface after sialidase treatment. This meant declined expression of SA after cells were incubated with sialidase overnight.

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Figure S1. Synthesis of mPEG-Cat (A). $^1$H NMR spectra of mPEG-Cat in CDCl$_3$ (B)
Figure S2. Synthesis of PEI-PBA (A). $^1$H NMR spectra of PEI-PBA in D$_2$O (B)
**Figure S3.** Synthesis of PCPP

**Figure S4.** The pKₐ determination of PEI-PBA by the absorbance change at 268 nm. PBA at 1.0 mM in 0.10 M phosphate buffer (A). The fluorescence of ARS/PEI-PBA in HEPES buffer at pH 7.4 was measured in the presence of catechol, ribose and glucose at various concentrations. I₀ and I represent the fluorescence intensity without and with cis-diol, respectively (B).
Figure S5. Cytotoxicity of PCPP with different concentrations (A) and PCPPsiN.C. nanoparticles at various N/P-ratios (B) on 4T1 cells for 24 or 48h (n=6).

Figure S6. Luciferase gene silencing study of treatment with PCPPsiLuc at various doses (A) and different nanoparticles at the siLuc dose of 100 nM (B). (n = 3, *** P < 0.001).
Figure S7. After treatment with sialidase, the expression of α-2,3-sialylated antigens, α-2,6-sialylated antigens or a broader linkage of sialylated antigens on 4T1 cell surface was quantified by flow cytometry using FITC-MAA (a), FITC-SNA (b) or FITC-WGA(c).

Figure S8. The UV spectra of mPEG-Cat (A) and PEI-PBA (B).

Scheme S1. Equilibrium between phenylboronic acids and cis-diol containing compounds in an aqueous solution.