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Electroneutral composite polymersomes self-assembled by amphiphilic polyphosphazenes for effective miR-200c *in vivo* delivery to inhibit drug resistant lung cancer

Yan Peng [b, 1], Xiumei Zhu [b, 1], Liyan Qiu [a, c, *]

[a] Ministry of Education (MOE) Key Laboratory of Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, 38 Zheda Road, Hangzhou 310027, China
[b] College of Pharmaceutical Sciences, Zhejiang University, 866 Yu-Hang-Tang Road, Hangzhou 310058, China
[c] Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China

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ABSTRACT

MiR-200c has been confirmed to display remarkable effects on proliferation inhibition and apoptosis induction of certain cancer cells, but the main challenge for its successful translation into the clinic remains its effective delivery to the action site *in vivo*. In this study, a novel composite polyphosphazene vesicle system composed of amphiphilic $[NP(PEG)_{0.3}(EAB)_{1.7}]_n$ (PEEP) and weakly cationic $[NP(PEG)_{0.5}(DPA)_{1.5}]_n$ (PEDP) was prepared via a very simple dialysis method. The loading of miR-200c was accomplished with high efficiency by taking advantage of the combination effect of physical encapsulation and electrostatic interaction between vectors and miR-200c. The resultant miR-200c-loaded PEEP-PEDP polymersome (Nano-ED-200c) displayed suitable particle size, electric neutrality, excellent Ribonuclease stability and hemocompatibility. We also evaluated its subsequent miR-200c function in paclitaxel resistance human lung cancer (A549/T) cells in culture and tumor xenografts in nude mice. The results showed that Nano-ED-200c could achieve a higher miR-200c level and the enhanced antitumor efficacy with 68% tumor inhibition ratio at a very low dose of 1.0 mg/kg than PEEP nanoparticle, PEDP nanoparticle, even than Lipo2000. All these evidences indicated that this miR-200c delivery via polyphosphazene vesicles could act as a potential new therapeutic option for paclitaxel resistant human lung cancer.

1. Introduction

Lung cancer is the most commonly diagnosed cancer in the world and approximately 85% cases are classified into the non-small-cell lung carcinoma (NSCLC) subtype. In the last decade, molecular therapies including EGFR-tyrosine kinase inhibitors (TKIs), which target epidermal growth factor receptor (EGFR) mutation, have dramatically changed clinical outcome and been recommended as the first choice for the treatment of adults with locally advanced or metastatic NSCLC [1–3]. However, similar to conventional chemo-therapeutics, these therapeutic agents also showed a propensity to fail due to the development of resistance [4]. Therefore, identifying new drugs or new therapy strategies is critical to any future success in NSCLC therapies, especially for those resistant to conventional drugs.

MicroRNA (miRNA) therapy against cancers appears as a novel field, in which miRNA activity becomes the major target of the intervention [5–7]. By replacing miRNA to restore a loss-of-function or antagonizing silence to repress aberrant miRNA expression, miRNA therapy has played such key roles in different kinds of cancers with significant therapeutic benefits [8–11]. Recently, miR-200c has been revealed to impact cell differentiation, proliferation and apoptosis in several types of tumors [12,13]. The inhibitory effect of miR-200c on proliferation and cell cycle in

* Corresponding author. Ministry of Education (MOE) Key Laboratory of Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, 38 Zheda Road, Hangzhou 310027, China.
E-mail address: lyqiu@zju.edu.cn (L. Qiu).
[1] These two authors equally contributed to this work.

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A549 cells has been proved by directly suppressing the expression level of KRAS protein which is involved in a variety of cellular processes such as differentiation, proliferation and survival [14]. A study found that the expression of miR-200c was significantly lower in A549, H1299 and SPC-A-1sci NSCLC cell [15]. Another study confirmed that lower miR-200c expression levels were associated with a poor grade of differentiation ($P = 0.04$) in tumor specimens obtained from 69 patients with consecutively resected NSCLC [16]. Moreover, it was reported that increasing miR-200c expression would reduce the protein levels of B-cell lymphoma-2 (BCL2) and X-linked inhibitor of apoptosis protein (XIAP) and further cause apoptosis of cancer cells by binding and inhibiting Caspase-3, -7, -9 in SGC7901/VCR and A549/CDDP cells [17]. Therefore, replacement of miR-200c as a therapeutic strategy seems to hold great promise for NSCLC treatment.

Indeed, despite miR-200c displays remarkable effects on cancer inhibition, the main challenge for its successful translation into the clinic remains *in vivo* delivery to the action site, which will be the focus of future study and therapeutic development to harness the full potential of miRNAs. In a word, a successful gene therapy is predominantly dependent on an efficient delivery vector. Besides viral vectors, cationic polymers and lipids have been commonly investigated for gene delivery in the form of condensed complex with genetic substances via electrostatic interaction. Generally, the higher positive charges the vectors bear, the better the gene transfection efficiency is. But, contradictorily, high positive charges always induce the systemic toxicity related to complement activation and inflammatory response [18,19]. As for miR-200c, quite few studies considering its situation of *in vivo* delivery have been reported.

Polyphosphazene is a kind of unique polymers containing an inorganic backbone of alternating phosphorus and nitrogen atoms [20]. Compared to those popular biomaterials such as polycaprolactone and poly(lactic acid), the most advantage of polyphosphazenes for drug delivery is the wide regulation of two side groups attached on the polymer backbone, therefore, multifunctionalized polyphosphazenes with a variation of physical and chemical characteristics could be obtained at ease [21–24]. Recently, we have synthesized an amphiphilic polyphosphazene PEEP using monomethoxy poly(ethylene glycol) (mPEG) as hydrophilic chains and ethyl-*p*-aminobenzoate (EAB) as hydrophobic side groups at the mole ratio of 0.3–1.7. PEEP could self-assemble into spherical-like polymersomes in water, which provided a potential to encapsulate miR-200c in their central aqueous lumens. More interestingly, another amphiphilic polyphosphazene PEDP was introduced in the process of PEEP polymersome formation. Due to the existence of positive-charged group *N*,*N*-diisopropylethylenediamine (DPA) in PEDP, this composite polymersome was expected to achieve improved loading efficiency of miR-200c on the basis of the combination effect of physical encapsulation and electrostatic interaction between vectors and miR-200c. Furthermore, after miR-200c loading, the nanoparticles became nearly neutral and would evade systemic toxicity *in vivo* and acquire long circulation in blood. Once internalized into endosomes of cancer cells, the relatively low pH would trigger the protonation of PEDP to facilitate miR-200c escape from endosomes and subsequent action in the cytoplasm. Therefore, we obtained an innovative and effective vector trapping miR-200c alone instead of co-encapsulating drugs and siRNAs/miRNAs as a number of references reported [25,26]. Based on the synthesis of two polyphosphazenes and construction of miR-200c loaded composite polymersome, we evaluated its ability to suppress the growth of paclitaxel resistance A549 human lung cancer cells both *in vitro* and *in vivo*.

2. Results and discussion

2.1. Synthesis and characterization of PEEP and PEDP polymers

Two amphiphilic graft polyphosphazenes PEEP and PEDP containing EAB and DPA, respectively, were synthesized by grafting side groups onto poly(dichlorophosphazene) backbone. The synthesis route was shown in Scheme 1. Their chemical structures were confirmed by ^{1}H NMR and FTIR spectra. The characteristic bands of mPEG and EAB in PEEP and DPA in PEDP were observed in FTIR spectra (Fig. 1a): mPEG at 2910 cm^{-1} (−CH_2− stretching vibration), 1464 cm^{-1} (−CH_2− deformation vibration), 1090 cm^{-1} (C−O−C stretching vibration), 1700 cm^{-1} (C═O stretching vibration). EAB at 856 cm^{-1} (*p*-substituted benzene vibration). DPA at 2910 cm^{-1} (−CH− stretching vibration). Additionally, P═N stretching band of backbone existed at 1021 cm^{-1} and the P−N stretching vibration was at 920 cm^{-1}. The detailed peak analysis of polymers by ^{1}H NMR (Fig. 1b) was presented as follows. PEEP ($[NP(PEG)_x(EAB)_y]_n$): δ(ppm) = 3.6 (mPEG, 4H, −OCH_2CH_2), 3.4 (mPEG, 3H, −OCH_3), 7.2–7.6 (EAB, 4H, phenyl), 4.2 (EAB, 2H, −OCH_2−), 1.3 (EAB, 3H, −CH_3). PEDP ($[NP(PEG)_x(DPA)_y]_n$): δ(ppm) = 3.6 (mPEG, 4H, -OCH_2CH_2), 3.4 (mPEG, 3H, −OCH_3), 2.4–3.1 (DPA, 6H, −CH_2−, −CH−), 1.0 (DPA, 12H, −CH_3). The grafted molar ratio (x/y) of mPEG to EAB in PEEP was calculated as 0.3/1.7 by comparing the peak intensities of methyl ether proton of mPEG at 3.4 ppm to the methyl protons of EAB at 1.3 ppm. Similarly, the grafted molar ratio (x/y) of mPEG to DPA in PEDP was 0.5/1.5 by comparing the peak intensities of methyl ether proton of PEG at 3.4 ppm to the methyl protons of DPA at 1.0 ppm. The molecular weights of PEEP and PEDP were determined as 39117 (PDI 1.58) and 36202 (PDI 1.51) by GPC, respectively (Fig. S1).

The acid-base titration measurement was performed to determine the pK_b value of PEDP. As the NaOH solution was dropwise added, the pH of PEDP solution was gradually increased until a buffering region ranging from pH 5.5 to pH 6.5 was reached (Fig. 1c). Therefore, the pK_b value of PEDP was about 6.1.

2.2. Nanoparticles preparation and characterization

It has been validated that amphiphilic copolymers tend to form various ordered self-assembly by non-covalently interaction between macromolecules. Their sophisticated microstructures are dependent on the MW of the polymer, the fraction of each block and the effective interaction energy between monomers [27,28]. As shown in the TEM images (Fig. 2), by a common dialysis method, PEEP and PEDP polymers can self-assemble into nanoparticles, but a blank nanoparticle of PEEP (Nano-E) appeared as a polymersome with internal cavum and legible shell (Fig. 2a) while a blank nanoparticle of PEDP (Nano-D) was more like a micelle with solid hydrophobic core (Fig. 2b). Also, Nano-D carried much more positive charges than Nano-E as shown in Table S1.

Since DNA/RNA encapsulation efficiency of nanoparticles is one of the most important dependent variables, we prepared cy3-miRNA loaded Nano-E or Nano-D by dialysis method and made a comparison. It is well known that cationic polymers or lipids can fully complex with negatively charged nucleic acids through electrostatic force when their N/P ratio achieves a certain value. As showed in Fig. 3a, the free cy3-miRNA was decreased with the increase of weight of PEDP. When PEDP was 450 μg, there was no more free cy3-miRNA, suggesting that there was a direct electrostatic interaction between positive charged PEDP and negative charged miRNA. Although the zeta potential of Nano-D-NC was significantly lower than that of Nano-D after the negative charged miRNA was loaded (Table S1), it was rapidly increased along with the increasing weight of PEDP, which reached 27.6 ± 3.5 mV when