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Ultra pH-Responsive and Tumor-Penetrating Nanopatform for Targeted siRNA Delivery with Robust Anti-Cancer Efficacy

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Abstract

RNA interference (RNAi) gene silencing technologies have shown significant potential for treating various diseases including cancer. However, clinical success in cancer therapy remains elusive, mainly due to suboptimal *in vivo* delivery of RNAi therapeutics such as small interference RNA (siRNA) to tumors. Herein, we developed a library of polymers that respond to a narrow pH change (ultra-pH-responsive), and demonstrated the utility of these materials in targeted and deep tumor-penetrating nanoparticle (NP) for *in vivo* RNAi. The new NP platform is mainly composed of the following key components: i) internalizing RGD (iRGD) to enhance tumor targeting and tissue penetration; ii) polyethylene glycol (PEG) chains to prolong blood circulation; and iii) sharp pH-responsive hydrophobic polymer to improve endosome escape. Through systematic studies of structure-function relationship, the optimized RNAi NPs (< 70 nm) showed efficient gene silencing and significant inhibition of tumor growth with negligible toxicities *in vivo*.

Graphical Abstract

A new tumor-penetrating and ultra pH-responsive nanopatform was developed for targeted siRNA delivery. This nano-sized nanopatform could efficiently use tumor-penetrating and ultra pH-responsive abilities to deliver therapeutic siRNA to cytoplasm, leading to a significant inhibition of tumor growth. This new nanopatform shows great promise as an siRNA delivery vehicle for cancer therapy.

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Keywords

Tumor penetration; Ultra pH-responsive; Nanoparticle; siRNA Delivery; Cancer therapy

RNAi technology has gained broad interest among academic and industry investigators for its potential to treat a myriad of diseases.^[1a, 1b] One major hurdle in clinical translation of RNAi therapeutics (*e.g.*, siRNA) may be attributed to the lack of effective and non-toxic delivery vehicles to transport siRNA into diseased tissues and cells.^[2] Specifically for cancer therapy, the barriers to effective *in vivo* siRNA delivery mainly include targeting to tumor, penetrating tumor tissue and cell membrane, escaping the endosome and releasing siRNAs in the cytoplasm.^[2] In the past decade, a variety of NP platforms made with cationic lipids,^[3] polymers^[4] or lipid/polymer hybrids^[5] have been demonstrated to significantly improve siRNA transfection *in vitro*. Nevertheless, their *in vivo* therapeutic efficacy is still limited by unsatisfactory blood circulation, poor tissue penetration, and endosomal entrapment.

Aiming for effective *in vivo* delivery of siRNA to tumor cells, it is highly necessary to understand the obstacles from tumor tissues. The vasculature of solid tumors is fenestrate, through which NPs can extravasate and accumulate in tumors via the enhanced permeability and retention (EPR) effect.^[6] However, efficiently crossing the vascular wall and penetrating deep into the tumor parenchyma against the elevated interstitial pressure remains an intractable problem.^[7] In addition, the subsequent endosomal entrapment is another key bottleneck for the widespread use of siRNA therapeutics. Therefore, an ideal platform for safe and effective siRNA delivery to tumors should possess at least the following features: i) long blood circulation for high EPR effect and tumor accumulation; ii) capability for suitable tumor tissue penetration and tumor cell-targeting; iii) efficient endosomal escape for cytosolic delivery of the active siRNA; iv) biocompatibility of material; and v) robust formulation processes that are amenable to scale-up using standard unit operations. To the best of our knowledge, a technology platform capable of satisfying all of these criteria has not been developed. Herein, we developed a novel long-circulating, tumor-penetrating, and ultra pH-responsive NP platform for effective *in vivo* siRNA delivery. This platform is made of a new PEGylated polymer, which shows an ultra pH-responsive characteristic with a pK_a close to the endosomal pH (6.0-6.5)^[8], and a tumor-penetrating peptide iRGD (Schemes 1A and 1B). After encapsulating siRNA, the resulting delivery system shows four unique features (Scheme 1C): i) the surface-encoded iRGD peptide endows the NPs with tumor-targeting and tumor-penetrating abilities; ii) the hydrophilic PEG shells prolong the blood circulation; iii) a small population of cationic lipid-like grafts randomly dispersed in the

hydrophobic poly(2-(diisopropylamino) ethylmethacrylate) (PDPA) segment can entrap siRNA in the hydrophobic cores of the NPs; iv) the rapid protonation of the ultra pH-responsive PDPA segment induces the endosomal swelling via the “proton sponge” effect, which synergizes with the insertion of the cationic lipid-like grafts into endosomal membrane to induce membrane destabilization^[9] and efficient endosomal escape.

The amphiphilic polymer, methoxyl-polyethylene glycol-*b*-poly (2-(diisopropylamino) ethylmethacrylate-*co*-glycidyl methacrylate) (Meo-PEG-*b*-P(DPA-*co*-GMA)) was first synthesized (Scheme S1 and Table S1), which was further grafted by tetraethylenepentamine (TEPA) and 1,2-epoxyhexadecane to obtain Meo-PEG-*b*-P(DPA-*co*-GMA-TEPA-C14) (Scheme 1A). We varied the length of PDPA segment to adjust siRNA encapsulation efficiency (EE%). As the PDPA length increases, the EE% and size of the resulting NPs increase (Table S3), possibly because the increased PDPA length leads to an increase in the size of the hydrophobic core. Specifically, the EE% reaches almost 100% for the polymer with 80 (PDPA₈₀) or 100 (PDPA₁₀₀) DPA repeat units. Notably, using a mixture of Meo-PEG-*b*-P(DPA-*co*-GMA-TEPA-C14) (90 mol%) and tumor-penetrating polymer (iRGD-PEG-*b*-PDPA, 10 mol%, Scheme 1A) to prepare NPs does not cause obvious change in the EE% or particle size (Table S4).

The polymer, PDPA₈₀ (pK_a 6.24, Table S3), was chosen for pH response evaluation by incorporating a near-infrared dye, Cy 5.5, into its PDPA segment (Scheme S2). Due to the quenching of the aggregated fluorophores inside the hydrophobic cores of the NPs,^[8] there is no fluorescence signal at a pH above pK_a of PDPA₈₀ (Figure 1A). In contrast, at a pH below pK_a , the protonated PDPA segment induces the disassembly of the NPs and a dramatic increase in the fluorescence signal. Measurement of the fluorescence intensity upon pH change reveals that the pH difference from 10 to 90% fluorescence activation ($pH_{10-90\%}$) is 0.34 (Figure 1B and Table S3),^[8] which is much smaller than that of small molecule dyes (about 2 pH units),^[10] indicating the ultra-fast pH response of PDPA₈₀. This characteristic is confirmed by transmission electron microscope (TEM). The spherical siRNA-loaded NPs with could be visualized at a pH of 6.5 (Figure 1C), with an average size of 69.7 nm determined by dynamic light scattering (DLS, Table S3). If altering pH to 6.0, there are no observable NPs after 20 min incubation (Figure 1D). With this morphological change, the NPs offer super-fast release of DY547-labelled GL3 siRNA (DY547-siRNA) (Figure 1E). Around 90% loaded siRNA has been released within 4 h at a pH of 6.0. Within the same time frame, less than 30% of the loaded siRNA is released at a pH of 7.4.

Luciferase-expressing HeLa (Luc-HeLa) cells were used to evaluate the gene silencing efficacy. GL3 siRNA was employed to suppress luciferase expression. All the siRNA-loaded NPs show a reduction in luciferase expression at a 10 nM siRNA dose (Figure 2A), with the differential silencing efficacy depending upon the polymer structure. In comparison, the NPs with iRGD peptide (denoted iRGD-NPs) offer much better gene silencing efficacy. In particular, the iRGD-NPs₈₀ prepared from PDPA₈₀ show the best gene silencing efficacy, *i.e.*, > 90% knockdown in luciferase expression without obvious cytotoxicity (Figure S4).

After acquiring the nanoplatform with optimal silencing efficacy (iRGD-NPs₈₀), flow cytometry was employed to evaluate its *in vitro* tumor-targeting ability. With the specific

recognition between integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$, Figure S5) on Luc-HeLa cells and iRGD, the uptake of DY547-siRNA-loaded iRGD-NPs₈₀ is 3-fold higher than that of iRGD-absent NPs₈₀ (Figures 2B and S6), demonstrating the excellent tumor-targeting ability of iRGD-NPs₈₀. Endosomal escape ability was assessed by staining the endosomes with lysotracker green. As shown in Figure 2C, a majority of the internalized siRNA-loaded NPs enter the cytoplasm after 4 h incubation, indicating the effective endosomal escape of the iRGD-NPs₈₀. In comparison, for the iRGD-NPs prepared from polymer without lipid-like grafts or pH response (Scheme S4), the endosome escape ability is relatively weaker (Figure S8), thus leading to a much lower silencing efficacy (Figure S9).

We next examined whether the iRGD-NPs₈₀ can be used to downregulate survivin expression, an inhibitor of apoptosis protein that is over-expressed in most cancers.^[11] PC3 cells, a prostate cancer cell line showing targeted uptake of iRGD-NPs (Figures S5 and S6), were used as a model cell line. The western blot analysis in Figure 2D indicates the survivin siRNA-loaded iRGD-NPs₈₀ significantly suppress survivin expression (> 80% knockdown) at a 10 nM siRNA dose. At a 50 nM siRNA dose, survivin expression is nearly absent (< 3%, Figure 2E). The similar result can be also found in the immunofluorescence staining analysis (Figures 2F and 2G). Very weak red fluorescence corresponding to the residual survivin can be observed in the cells treated with iRGD-NPs₈₀ at a 10 nM siRNA dose (Figure 2G). With such suppressed survivin expression, the proliferation rate of PC3 cells is very slow. There is only 2.5-fold increase in cell number after 8 days incubation (Figure 2H).

After validating the efficient gene silencing of iRGD-NPs₈₀, we then assessed their *in vivo* tumor-targeting ability. Pharmacokinetics was first examined by intravenous injection of DY647-siRNA-loaded NPs. As shown in Figure 3A, the blood half-life ($t_{1/2}$) of iRGD-NPs₈₀ is around 3.56 h, which is far longer than that of naked siRNA ($t_{1/2}$ < 30min). This prolonged blood circulation is mainly due to the protection of PEG outer layer and small particle size.^[12] The *in vivo* tumor-targeting ability was evaluated by intravenously injecting DY677-siRNA-loaded NPs into PC3 xenograft tumor-bearing mice. As shown in Figure 3B, with the iRGD-mediated tumor-targeting, the iRGD-NPs₈₀ show a much higher tumor accumulation than that of NPs₈₀ at 24 h post-injection. The tumors and main organs were harvested (Figure S11) and the biodistribution is shown in Figure 3C. Naked siRNA has a characteristic biodistribution, *i.e.*, high accumulation in kidney but extremely low accumulation in tumor. With the specific recognition between iRGD and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ over-expressed on tumor cells and angiogenic tumor vasculature,^[8, 13] the tumor accumulation of the iRGD-NPs₈₀ is around 3-fold higher than that of NPs₈₀.

To evaluate the tumor-penetrating ability of the iRGD-NPs₈₀, the tumors were collected at 4 h post-injection of the DY677-siRNA-loaded NPs and then sectioned for immunofluorescence staining. As shown in Figure 3D, there is nearly no naked siRNA in the tumor section. For the NPs₈₀ (Figure 3E), the number of NPs in tumor section is very low. Additionally, most of these NPs are positioned in the tumor vessels, and only a small number reach the extravascular tumor parenchyma. In contrast, highly concentrated iRGD-NPs₈₀ with bright red fluorescence could be visualized in the tumor section (Figure 3F). Remarkably, a majority of these NPs can cross tumor vessels and reach the extravascular

tumor parenchyma, strongly demonstrating the deep tumor-penetrating characteristic of iRGD-NP_{s80}.

Finally, we evaluated the *in vivo* inhibition of survivin expression and anti-cancer efficacy. The survivin siRNA-loaded NPs were intravenously injected into the PC3 xenograft tumor-bearing mice (650 µg/kg siRNA dose, n = 3) for three consecutive days. The siRNA-loaded NPs indeed suppress survivin expression in tumor (Figure 4A). In particular, the administration of iRGD-NP_{s80} induces > 60% knockdown in survivin expression (Figure S12), which is around 3-fold greater than that of NP_{s80}. Notably, the administration of NPs shows negligible *in vivo* side effects (Figure S13). To confirm whether the NP-mediated survivin silencing has an anti-cancer effect, the survivin siRNA-loaded NPs were intravenously injected to the mice once every two days at a 650 µg/kg siRNA dose (n = 5). After five consecutive injections (Figure 4B), the tumor growth is inhibited compared to the mice treated with PBS or GL3 siRNA-loaded NPs (Control NPs). Particularly, with the excellent tumor-targeting and penetrating abilities, the iRGD-NP_{s80} can significantly suppress tumor growth, and there is only around 2-fold increase in tumor size at day 24 (Figure 4C).

In summary, we have successfully developed a new ultra pH-responsive and tumor-penetrating nanoplatform for targeted systemic siRNA delivery. The *in vitro* and *in vivo* results demonstrate that this polymeric NP has a long blood circulation, and can efficiently target tumor and penetrate tumor parenchyma, leading to efficient gene silencing and tumor growth inhibition. The polymeric nanoplatform reported herein may represent a robust siRNA delivery vehicle for the treatment of a myriad of important diseases including cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Scheme 1.

(A) Molecular structures of the ultra pH-responsive polymer, Meo-PEG-*b*-P(DPA-co-GMA-TEPA-C14), and the tumor-penetrating polymer, iRGD-PEG-*b*-PDPA. (B) Ultra pH-responsive and tumor-penetrating nanoplatform for siRNA loading and release. (C) Schematic illustration of the nanoplatform for targeted *in vivo* siRNA delivery and cancer therapy. The iRGD-conjugated NPs target tumor tissue via the specific recognition between the RGD motif and α_v integrin-expressing endothelial cells in tumor tissue. Subsequently, the iRGD peptide is cleaved by cell-surface protease to expose CRGDR sequence, which can bind to neuropilin-1 to induce penetration of tumor tissue and cells. After cellular uptake, the rapid protonation of the PDPA segment induces the endosomal swelling, which synergizes with the insertion of the cationic lipid-like grafts into endosomal membrane to induce fast endosomal escape and efficient gene silencing.

Figure 1. (A) Fluorescent image of the Cy5.5-labelled NPs of PDPA₈₀ at different pHs. (B) Normalized fluorescence intensity as a function of pH for the Cy .5.5-labelled NPs of PDPA₈₀. TEM images of the siRNA-loaded NPs of PDPA₈₀ at a pH of (C) 6.5 and (D) 6.0. (E) *In vitro* siRNA release from the NPs of PDPA₈₀ at 37 °C.

Figure 2. (A) Luciferase expression in Luc-HeLa cells transfected with siRNA-loaded NPs at a 10 nM siRNA dose. (B) Flow cytometry profile of Luc-HeLa cells incubated with the siRNA-loaded NPs₈₀ and iRGD-NPs₈₀ for 4 h. (C) Fluorescent image of Luc-HeLa cells incubated with the siRNA-loaded iRGD-NPs₈₀ for 4 h. (D, E) Western blot analysis of survivin expression in PC3 cells treated by survivin siRNA-loaded NPs₈₀ and iRGD-NPs₈₀. Fluorescent images of PC3 cells treated by survivin siRNA-loaded (F) NPs₈₀ and (G) iRGD-NPs₈₀ at a 10 nM siRNA dose. (H) Proliferation profile of PC3 cells incubated with survivin siRNA-loaded NPs₈₀ and iRGD-NPs₈₀ at a 10 nM siRNA dose. GL3 siRNA-loaded NPs₈₀ were used as a control.

Figure 3. (A) Pharmacokinetics of naked siRNA, and siRNA-loaded NPs. (B) Overlaid fluorescent image of PC3 xenograft tumor-bearing mice at 24 h post-injection of naked siRNA and siRNA-loaded NPs. (C) Biodistribution of the NPs in the PC3 xenograft tumor-bearing mice sacrificed at 24 h post-injection of naked siRNA, and siRNA-loaded NPs. Fluorescent images of the tumor sections of the PC3 xenograft tumor-bearing mice sacrificed at 4 h post-injection of (D) naked siRNA, and siRNA-loaded (E) NP_{80} and (F) iRGD- NP_{80} .

Figure 4.

(A) Western blot analysis of survivin expression in the PC3 tumor tissue after systemic treatment by control NPs and survivin siRNA-loaded NPs. (B) Relative tumor size of the PC3 xenograft tumor-bearing mice after treatment by PBS, control NPs, and survivin siRNA-loaded NPs. The intravenous injections are indicated by the arrows. (C) Representative image of the harvested PC3 tumor from each group at day 16. GL3 siRNA-loaded NPs_{80} were used as a control. * $P < 0.05$; ** $P < 0.01$