Virus-Mimicking Chimaeric Polymersomes Boost Targeted Cancer siRNA Therapy In Vivo

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Small interfering RNA (siRNA) offers a highly selective and effective pharmaceutical for various life-threatening diseases, including cancers. The clinical translation of siRNA is, however, challenged by its short plasma life, poor cell uptake, and cumbersome intracellular trafficking. Here, cNGQGEQc peptide-functionalized reversibly crosslinked chimaeric polymersomes (cNGQ/RCCPs) is shown to mediate high-efficiency targeted delivery of Polo-like kinase1 specific siRNA (siPLK1) to orthotopic human lung cancer in nude mice. Strikingly, siRNA is completely and tightly loaded into the aqueous lumen of the polymersomes at an unprecedentedly low N/P ratio of 0.45. cNGQ/RCCPs loaded with firefly luciferase specific siRNA (siGL3) or siPLK1 are efficiently taken up by $\alpha_3\beta_1$-integrin-overexpressing A549 lung cancer cells and quickly release the payloads to the cytoplasm, inducing highly potent and sequence-specific gene silencing in vitro. The in vivo studies using nude mice bearing orthotopic A549 human lung tumors reveal that siPLK1-loaded cNGQ/RCCPs boost long circulation, superb tumor accumulation and selectivity, effective suppression of tumor growth, and significantly improved survival time. These virus-mimicking chimaeric polymersomes provide a robust and potent platform for targeted cancer siRNA therapy.

Small interfering RNA (siRNA), a trigger of RNA interference (RNAi), offers a new and effective therapeutic modality for various human diseases, including viral infection, obesity, inflammation, and cancers.[1] However, in spite of its marvelous potential and tremendous advance, few siRNA formulations have been translated to the clinical trials.[2] The therapeutic efficacy of siRNA is depleted by its particularly short plasma life in vivo, inferior cellular uptake, and cumbersome intracellular trafficking pathway to the site of action.[3] Virus has shown to efficiently deliver nucleic acids including siRNA in vitro and in vivo.[4] The low selectivity and potential safety issues, nevertheless, restrict the clinical applications of viral systems. In contrast, nonviral vectors such as cationic liposomes,[5] cationic polymers,[6] polymeric micelles,[7] and polymersomes[8] have the clear merits of low immunogenicity and better safety. Unfortunately, though many synthetic siRNA systems have been reported to promote decent transfection in culture, only a few show pronounced silencing efficacy in vivo.[9] It should further be noted that most nonviral systems have excessive positive charge,[10] which is required for effective siRNA condensation but will also induce significant systemic toxicity and lead to low cell selectivity. In recent years, a couple of systems such as micelleplexes and cationic lipid-assisted PEG-PLA nanoparticles have been reported to effectively shield the positive charge and greatly improve systemic siRNA delivery.[11]

Here, we report a virus-mimicking vehicle based on cNGQGEQc peptide-functionalized reversibly crosslinked chimaeric polymersomes (cNGQ/RCCPs) for highly efficient and targeted delivery of siPLK1 to orthotopic human lung tumors in nude mice (**Figure 1**). Strikingly, siRNA is completely and tightly loaded into the aqueous lumen of polymersomes even at a high siRNA/polymer ratio of 80/100 (w/w), which corresponds to a notably low N/P ratio of 0.45. The outer surface of polymersomes consists of poly(ethylene gycol) (PEG) only

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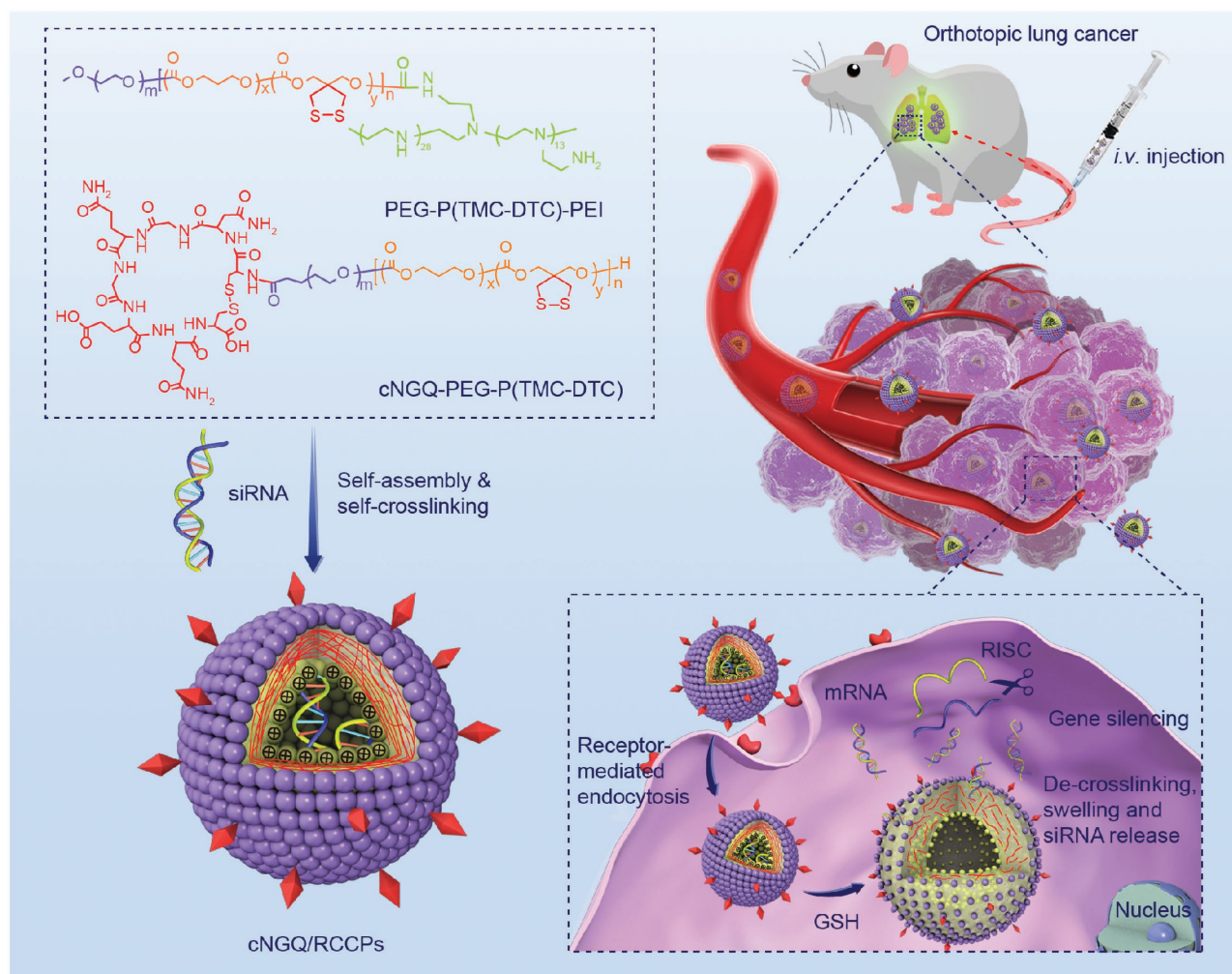
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Figure 1. Efficient and targeted siRNA delivery to orthotopic lung tumors in nude mice by a cNGQ peptide-directed reversibly crosslinked chimaeric polymersomes (cNGQ/RCCPs). cNGQ/RCCPs are co-self-assembled from biodegradable PEG-P(TMC-DTC)-PEI asymmetric triblock copolymer and cNGQ-PEG-P(TMC-DTC) diblock copolymer. cNGQ/RCCPs can efficiently load siRNA into their lumen and protect siRNA from degradation. siPLK1-loaded cNGQ/RCCPs boost a long circulation time, high tumor accumulation and selectivity, efficient uptake by $\alpha_3\beta_1$-overexpressing A549 lung cancer cells, and fast cytoplasmic release of siPLK1, resulting in effective treatment of nude mice bearing orthotopic A549 human lung tumors.

warranting excellent biocompatibility and low nonspecific protein adsorption. The further functionalization of polymersome surface with cNGQ peptide enables active targeting to $\alpha_3\beta_1$-integrin-overexpressing tumor cells. The disulfide crosslinking of the membrane leads to not only excellent stability in circulation but also fast cytoplasmic cargo release, as also reported for disulfide-crosslinked nanomedicines carrying chemotherapeutic drugs.[12] It should further be noted that these polymersomes following loading with siRNA are self-crosslinked, in which no any catalyst is needed and no any byproduct is generated. The in vivo studies in nude mice bearing orthotopic A549 human lung tumors reveal that siPLK1-loaded cNGQ/RCCPs boost a long circulation, superb tumor accumulation and selectivity, effective suppression of tumor growth and metastasis, and significantly improved survival time. The remarkable silencing effect and easy fabrication of siRNA-loaded cNGQ/RCCPs renders these multifunctional chimaeric polymersomes a highly appealing platform for targeted cancer siRNA therapy.

cNGQ/RCCPs are co-self-assembled from poly(ethylene glycol)-b-poly(trimethylene carbonate-co-dithiolane trimethylene carbonate)-b-polyethylenimine (PEG-P(TMC-DTC)-PEI) asymmetric triblock copolymer with a number-average molecular weight (M_n) of 5.0–24.2–1.8 kg mol^{-1} and cNGQ peptide-functionalized PEG-P(TMC-DTC) (cNGQ-PEG-P(TMC-DTC), M_n = 6.5–24.5 kg mol^{-1}) diblock copolymer. PEG-P(TMC-DTC)-PEI was obtained by ring-opening copolymerization of trimethylene carbonate (TMC) with dithiolane-functionalized trimethylene carbonate (DTC) using MeO-PEG-OH as a macroinitiator, as reported previously,[13] followed by end-conjugation with 1.8 kDa branched PEI (Scheme S1 and Figure S1, Supporting Information). cNGQ-PEG-P(TMC-DTC) was prepared by copolymerization of TMC and DTC using maleimido-PEG-OH as an initiator followed by coupling with cNGQGEQc.[14] The pendent dithiolane rings in P(TMC-DTC) are analogous to the lipoyl ring in lipoic acid, which can reversibly stabilize nanoparticles via forming reduction-sensitive disulfide crosslinks.[12d]
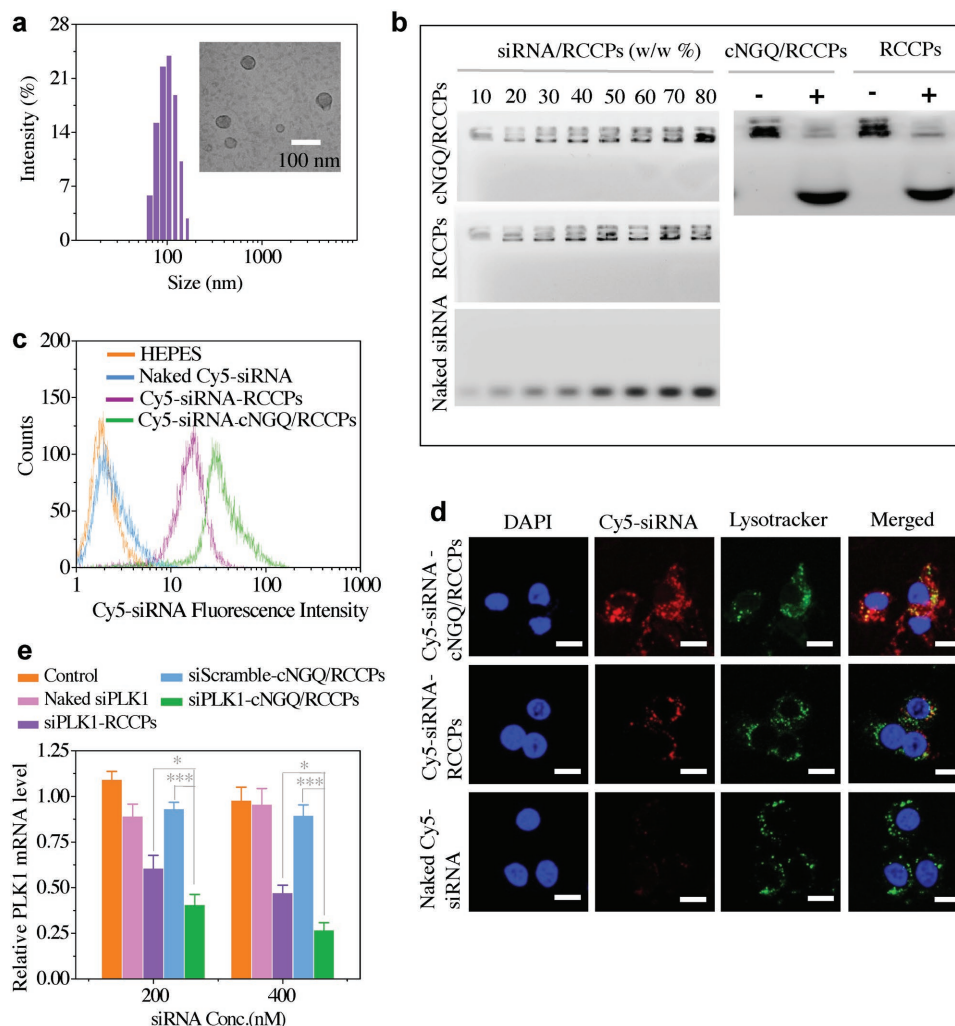
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Figure 2. a) Size distribution of siRNA-cNGQ/RCCPs (10 wt% siRNA) determined by DLS and TEM. b) Gel retardation assays of siRNA-cNGQ/RCCPs with (+) or without (−) 10×10^{-3} M GSH treatment. The polymersome concentration was fixed at 0.5 mg mL^{-1}. Naked siRNA was used as a control. c) Flow cytometry of A549-luc cells following 4 h incubation with Cy5-siRNA-cNGQ/RCCPs (siRNA dosage: 200×10^{-9} M). d) CLSM images of A549-luc cells following transfection with Cy5-siRNA-cNGQ/RCCPs (siRNA dosage: 200×10^{-9} M). The media were removed at 4 h and replenished with fresh culture media. The cells were further cultured for another 4 h. For each panel, the images from left to right were cell nuclei stained by 4′,6-diamidino-2-phenylindole (DAPI, blue), lysosome stained by lysotracker green (green), Cy5-siRNA (red), and overlays of the three images. The bar represents 20 µm. e) Gene-silencing ability of siPLK1-cNGQ/RCCPs in A549-luc cells following 48 h incubation (200 or 400×10^{-9} M siRNA). Data are presented as mean ± SD ($n = 4$, one-way Anova and Tukey multiple comparisons tests, $*p < 0.05$, $***p < 0.001$).

cNGQ peptide has shown a high and specific affinity to $\alpha_3\beta_1$ integrins that overexpress in A549 human lung cancer cells.[15]

siRNA-loaded cNGQ/RCCPs (siRNA-cNGQ/RCCPs) were readily prepared from 80 wt% PEG-P(TMC-DTC)-PEI and 20 wt% cNGQ-PEG-P(TMC-DTC) via solvent exchange method. We have shown previously that polymersomes with a cNGQ surface density of about 20% possess excellent targetability toward $\alpha_3\beta_1$-overexpressing A549 lung tumors in vivo.[14] The dynamic light scattering (DLS) showed that cNGQ/RCCPs following loading with 10 wt% siRNA had a small size of ≈109 nm and low polydispersity index (PDI) of 0.13 (**Figure 2**a). The transmission electron microscopy (TEM) image showed a distinctive spherical vesicular structure. The static light scattering (SLS) measurements of blank cNGQ/RCCPs revealed a radius of gyration (R_g) of 48.9 nm (Figure S2, Supporting Information).

The ratio of R_g to hydrodynamic radius ($R_h = 101$ nm, Table S1, Supporting Information) determined by DLS was calculated to be ≈0.97, confirming that cNGQ/RCCPs have a vesicular structure.[16] The size of siRNA-cNGQ/RCCPs increased from 109 to 175 nm with increasing siRNA/polymer ratios from 10/100 to 50/100 (w/w). The zeta potential measurements showed that all siRNA-cNGQ/RCCPs had a close to neutral surface charge (Table S1, Supporting Information), supporting that only PEG is present in outer surface and siRNA is tightly loaded into the aqueous lumen. In the following, siRNA-cNGQ/RCCPs with siRNA/polymer ratio at 20/100 (w/w) (126 nm, PDI 0.15), which corresponded to an N/P ratio of 1.8, was used for further in vitro and in vivo studies. It is interesting to note that most synthetic carriers including systems based on PEI and PEG require a high N/P ratio (ranging from 10 to 40) to achieve good siRNA

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complexation,[6a,17] which would nevertheless also induce systemic toxicity.[10a,18] The agarose gel retardation assays revealed that siRNA was completely and tightly loaded into cNGQ/RCCPs even at a high siRNA/polymer ratio up to 80/100 (w/w) (Figure 2b), and remained stable against 10% fetal bovine serum (FBS) confirming that siRNA-cNGQ/RCCPs have excellent stability (Figure S3a, Supporting Information). However, when treated with 10×10^{-3} M glutathione (GSH) for 20 h, most siRNA was released due to de-crosslinking and swelling of the polymersome (Figure S3b, Supporting Information).

The cellular uptake and intracellular release behavior of Cy5-siRNA-loaded cNGQ/RCCPs were studied in $\alpha_3\beta_1$-integrin-overexpressing A549 lung cancer cells using flow cytometry and confocal laser scanning microscopy (CLSM). Notably, flow cytometric analysis revealed that cNGQ functionalization significantly enhanced the cellular uptake of RCCPs into A549 cells (Figure 2c). CLSM images displayed that Cy5-siRNA-cNGQ/RCCPs could effectively escape from endo/lysosomes. Moreover, the cells treated with Cy5-siRNA-cNGQ/RCCPs exhibited much stronger Cy5 fluorescence than those with the nontargeting Cy5-siRNA-RCCPs and naked Cy5-siRNA controls (Figure 2d). To investigate the specific silencing effect of Polo-like kinase1 specific siRNA (siPLK1) loaded inside

polymersomes, we performed quantitative real-time polymerase chain reaction (PCR) study on A549-luc lung cancer cells after 48 h transfection. Figure 2e shows that both siPLK1-cNGQ/RCCPs and siPLK1-RCCPs substantially downregulated PLK1 mRNA level in A549-luc cells, whereas siScramble-cNGQ/RCCPs and naked siPLK1 brought about little reduction in PLK1 mRNA level, corroborating the sequence-specific gene silencing activity of siPLK1.[19] Moreover, siPLK1-cNGQ/RCCPs displayed significantly better silencing efficacy than siPLK1-RCCPs (73.2% vs 52.7% at 400×10^{-9} M siPLK1). The superior silencing ability of siPLK1-cNGQ/RCCPs in A549-luc cells is probably due to their stable encapsulation and protection of siRNA, efficient cellular internalization by receptor-mediated endocytosis mechanism, rapid endosomal escape, and fast cytoplasmic release of intact siRNA.

To confirm the in vitro silencing efficacy of siRNA-cNGQ/RCCPs, transfection experiments were performed using siGL3 in A549-luc cells.[20] The results showed that luciferase expression was significantly downregulated by siGL3-cNGQ/RCCPs that silenced ≈48% and 62% of luciferase expression at 200 and 400×10^{-9} M siRNA, respectively (**Figure 3**a). In comparison, siGL3-RCCPs (nontargeting control) showed much lower silencing efficacy. As expected, siScramble-cNGQ/RCCPs
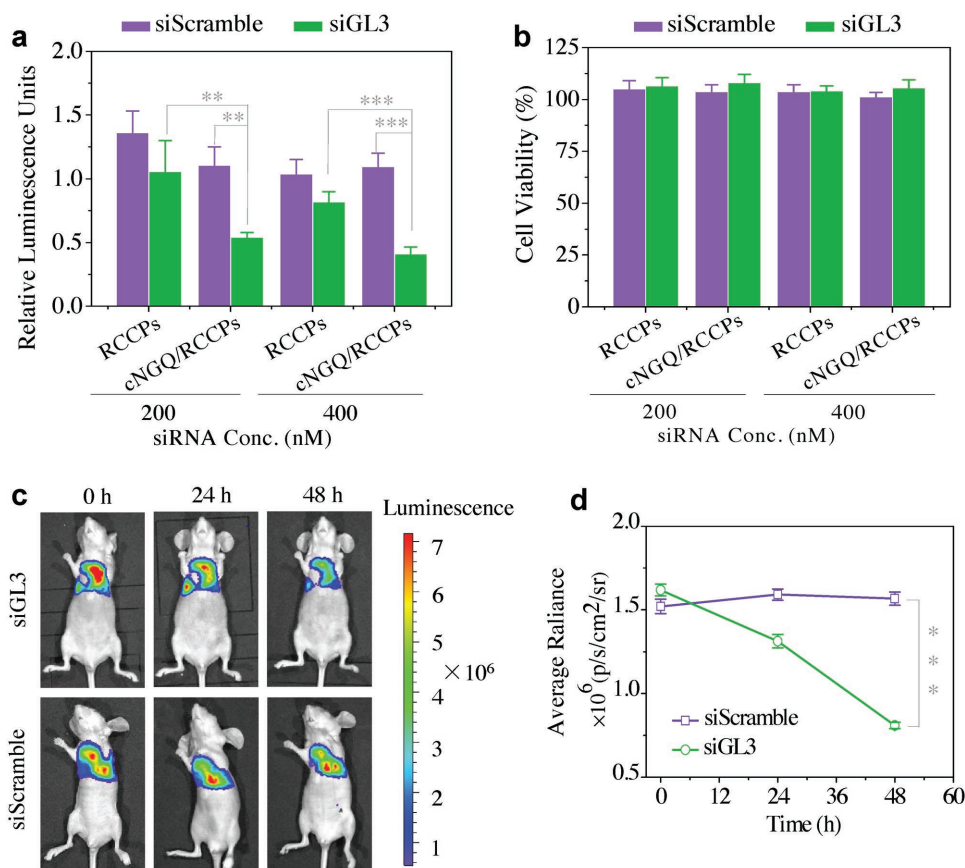

Figure 3. a) In vitro luciferase gene knockdown efficacy of siGL3-cNGQ/RCCPs, siGL3-RCCPs, and siScramble-cNGQ/RCCPs in A549-luc cells. The transfection was carried out for 48 h at a dose of 200 or 400×10^{-9} M siGL3. b) Viability of A549-luc lung cells following 48 h incubation with siRNA-cNGQ/RCCPs. c) Luciferase expression of lung in the mice before, 24 or 48 h postinjection of siGL3-cNGQ/RCCPs or siScramble-cNGQ/RCCPs (2 mg siRNA equiv. kg^{-1}). d) Bioluminescence intensity of the lung tumor. Data are presented as mean ± SD ($n = 3$, one-way Anova and Tukey multiple comparisons tests, **$p < 0.01$, ***$p < 0.001$).

resulted in no reduction in luminescence. Importantly, CCK-8 cell viability assays showed that both siGL3-cNGQ/RCCPs and siGL3-RCCPs were non-cytotoxic (Figure 3b).

To unveil their in vivo silencing performance, we established an orthotopic-A549-luc-human-lung-tumor model in nude mice. Figure 3c demonstrates that siGL3-cNGQ/RCCPs treatment led to obvious reduction of tumor bioluminescence. The quantitative analyses revealed that siGL3-cNGQ/RCCPs resulted in 76% and 53% reduction of tumor bioluminescence at 24 and 48 h post systemic injection, respectively (Figure 3d), indicating that siGL3-cNGQ/RCCPs induce efficient luciferase gene silencing in the lung tumor. In contrast, no reduction in the bioluminescence was observed for mice treated with siScramble-cNGQ/RCCPs. These results were consistent with luciferase gene knockdown in orthotopic HCC1299-Luc lung tumors by siGL3 functional polyester NPs.[21]

To study their in vivo pharmacokinetics, we monitored the plasma level of Cy5-siRNA following a single intraveous (i.v.) injection of polymersomal siRNA in tumor-free mice (20 μg Cy5-siRNA per mouse). Notably, both Cy5-siRNA-cNGQ/RCCPs and Cy5-siRNA-RCCPs demonstrated a long blood circulation time with an elimination half-life ($t_{1/2,\beta}$) of ≈1.8 h, which was significantly longer than naked Cy5-siRNA

($t_{1/2,\beta}$ = 0.14 h) (**Figure 4**a) and reported formulations like siRNA/cationic poly(DMAEMA-co-BMA) polyplexes (0.31 h)[22] and PEGylated siRNA mixed micelleplexes (0.30 h).[23] The half-life of PEGylated polycation/siRNA complexes was reported to be less than 5 min due to rapid decomplexation in serum and systemic removal in the kidney.[24] We further studied the tumor accumulation of Cy5-siRNA-cNGQ/RCCPs following i.v. injection into nude mice bearing orthotopic A549-luc tumors using in vivo near-infrared fluorescence imaging. The images showed strong Cy5-siRNA fluorescence in the tumor of the lung at 2 h post-injection of Cy5-siRNA-cNGQ/RCCPs and kept high until 24 h (Figure 4b). In comparison, Cy5-siRNA-RCCPs exhibited significantly less tumor accumulation, signifying the important role of active targeting in achieving high tumor accumulation and retention. Remarkably, ex vivo imaging results display that the mice treated with Cy5-siRNA-cNGQ/RCCPs showed significantly stronger Cy5-siRNA fluorescence in the lung than in the other major organs (Figure 4c). In contrast, nontargeting Cy5-siRNA-RCCPs exhibited weak Cy5-siRNA fluorescence in the lung while strong fluorescence in the liver and kidney. The biodistribution of Cy5-siRNA quantified using fluorometry measurements demonstrated that accumulation in the lung reached 4.02% of injected dose per gram of tissue (% ID g^{-1}),
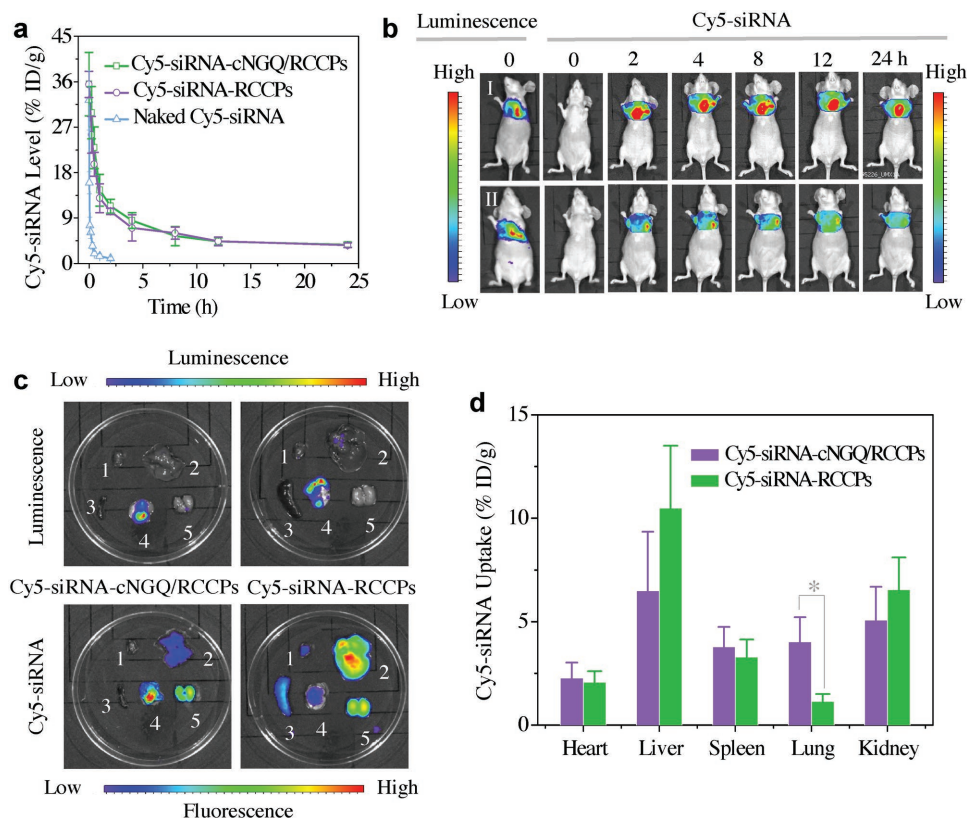

Figure 4. a) In vivo pharmacokinetics of Cy5-siRNA-cNGQ/RCCPs, Cy5-siRNA-RCCPs, and naked Cy5-siRNA in mice. Cy5-siRNA levels were determined by fluorometry and expressed as injected dose per gram of blood (% ID g^{-1}). Data are presented as mean ± SD ($n = 3$). b) The in vivo fluorescence images of nude mice bearing orthotopic A549-luc lung tumors at different time points following i.v. injection of Cy5-siRNA-cNGQ/RCCPs (I) and Cy5-siRNA-RCCPs (II). c) Bioluminescence and Cy5-siRNA fluorescence images of major organs of the nude mice bearing orthotopic A549-luc tumors 4 h after i.v. injection of Cy5-siRNA-cNGQ/RCCPs or Cy5-siRNA-RCCPs (1 mg Cy5-siRNA equiv. kg^{-1}): 1) heart; 2) liver; 3) spleen; 4) lung; 5) kidney. d) Quantification of Cy5-siRNA accumulated in different organs. Cy5-siRNA levels were determined by fluorometry and expressed as injected dose per gram of tissue (% ID g^{-1}). Data are presented as mean ± SD ($n = 3$, one-way Anova and Tukey multiple comparisons tests, *$p < 0.05$).

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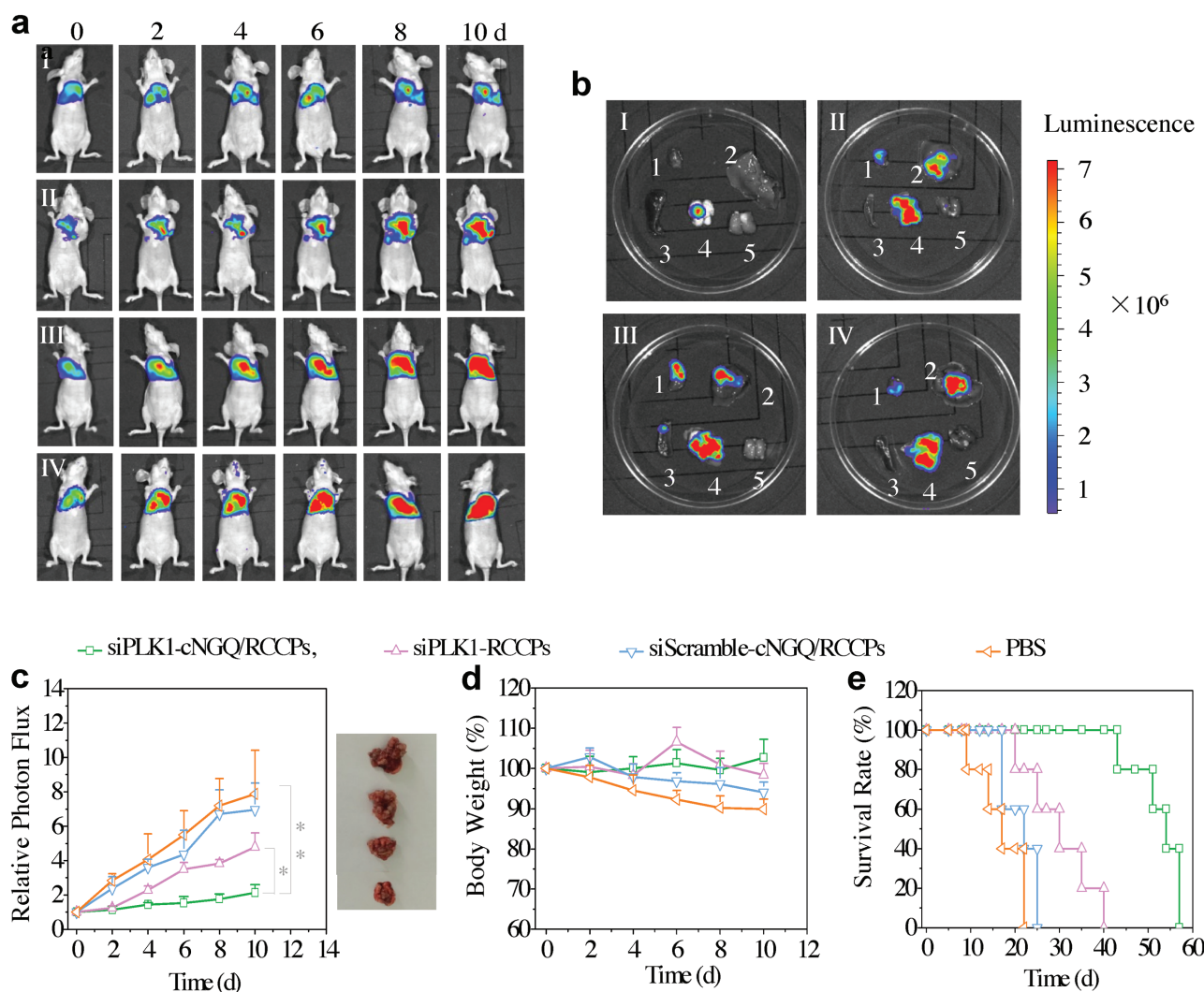
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Figure 5. a) Luminescence optical images of nude mice bearing orthotopic A549-luc tumors following treatment with siPLK1-cNGQ/RCCPs (I), siPLK1-RCCPs (II), siScramble-cNGQ/RCCPs (III), and PBS (IV), respectively. The mice were intravenously injected the dosage at 2 mg siRNA equiv. kg^{-1} on day 0, 2, 4, 6 and, 8. b) Ex vivo luminescence optical images of the major organs excised from the mice bearing the orthotopic A549-luc tumors following 10 d treatment: 1) heart; 2) liver; 3) spleen; 4) lung; 5) kidney. c) Quantified luminescence levels of mice using Lumina IVIS II system and images of the mouse lungs on day 10. Data are presented as mean ± SD ($n = 5$, one-way Anova and Tukey multiple comparisons tests, *$p < 0.05$, **$p < 0.01$). d) Body weight changes of mice. e) Mice survival rates. Statistical analysis: siPLK1-cNGQ/RCCPs versus siPLK1-RCCPs, siScramble-cNGQ/RCCPs or PBS, $p < 0.01$; siPLK1-RCCPs versus PBS, $p < 0.05$ (Kaplan–Meier analysis, log-rank test).

over three times higher than Cy5-siRNA-RCCPs (1.13% ID g^{-1}) (Figure 4d). These results verify that disulfide crosslinking of membrane and cNGQ functionalization can remarkably enhance tumor accumulation and retention of polymersomes.

To evaluate the therapeutic efficacy of polymersomal siRNA in vivo, we used siPLK1 as a therapeutic siRNA and mice bearing orthotopic A549-luc tumors as a tumor model. PLK1 is overexpressed in a broad range of tumors and plays a crucial role in cell mitosis. siPLK1 has been used for the treatment of Her2 positive breast cancer in vivo.[25] The tumor growth can easily be monitored by in vivo bioluminescence imaging. The results showed that siPLK1-cNGQ/RCCPs effectively inhibited tumor growth (**Figure** 5a). The nontargeting siPLK1-RCCPs could also partly suppress tumor proliferation. In contrast, rapid tumor growth was observed for siScramble-cNGQ/RCCPs and

phosphate buffered saline (PBS) control groups. The ex vivo images of the major organs excised from the mice bearing the orthotopic-A549-luc tumors at 10 d post-treatment showed that the lung of siPLK1-cNGQ/RCCPs group had much lower bioluminescence than that of siPLK1-RCCPs, siScramble-cNGQ/RCCPs and PBS groups (Figure 5b). It is remarkable to note that while clear sign of metastasis to liver was observed for mice treated with siPLK1-RCCPs, siScramble-cNGQ/RCCPs, and PBS, no metastasis was detected for those with siPLK1-cNGQ/RCCPs. The quantitative luminescence assays further corroborated effective suppression of A549-luc levels in the lungs by siPLK1-cNGQ/RCCPs (Figure 5c). The photograph of lungs collected from different treatment groups at day 10 confirmed a high therapeutic efficacy of siPLK1-cNGQ/RCCPs. Notably, no body weight loss was observed for mice treated

with siPLK1-cNGQ/RCCPs (Figure 5d), supporting that they can effectively inhibit tumor growth without causing adverse effects. In contrast, mice treated with siScramble-cNGQ/RCCPs and PBS revealed significant body weight loss likely due to malfunctions of lung and liver resulting from metastasis. Interestingly, survival curves showed that the treatment with siPLK1-cNGQ/RCCPs markedly increased the survived rate with a median survival time of 54 d, which was significantly longer than those with siPLK1-RCCPs (30 d) and siScramble-cNGQ/RCCPs (22 d) (Figure 5e). These results highlight that siPLK1-cNGQ/RCCPs mediate safe, highly efficient, and targeted siRNA delivery to orthotopic lung tumors in mice.

In summary, we have demonstrated that cNGQGEQc peptide-functionalized reversibly crosslinked chimaeric biodegradable polymersomes (siRNA-cNGQ/RCCPs) present high loading capacity toward siRNA, promote potent, targeted, and sequence-specific in vitro gene silencing in $\alpha_3\beta_1$-overexpressing A549 lung cancer cells, and mediate highly efficient and targeted delivery of siPLK1 to orthotopic-A549-human-lung-tumor xenografts in nude mice, which leads to effective suppression of tumor growth, inhibition of metastasis, and markedly improved survival time. This is the first report on development of chimaeric polymersomes for efficacious loading and targeted delivery of siRNA in vivo. siRNA-cNGQ/RCCPs have several unique features for cancer siRNA therapy: i) the chimaeric structure of polymersomes allows efficient loading of siRNA into their inner lumen and excellent protection of siRNA from degradation in vivo; ii) in contrast to common polycationic systems, they are stable in blood circulation and have a low systemic toxicity due to disulfide crosslinking of the polymersomal membrane and effective stealth by PEG on the outer surface; iii) they can be efficiently and selectively internalized by $\alpha_3\beta_1$-integrin-overexpressing A549-lung-tumor cells via receptor-mediated uptake and quickly release siRNA into the cytoplasm due to glutathione-triggered de-crosslinking of the polymersomes, resulting in efficacious and specific gene silencing; and iv) in addition to high transfection efficacy, they are safe and easy to fabricate, which renders them particularly interesting for clinical translation. These virus-mimicking chimaeric polymersomes have emerged as a simple, robust, multifunctional, and versatile platform for targeted cancer siRNA therapy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors declare no conflict of interest.

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