Amphiphilic poly(α)glutamate polymeric micelles for systemic administration of siRNA to tumors

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Abstract

RNAi therapeutics carried a great promise to the area of personalized medicine: the ability to target “undruggable” oncogenic pathways. Nevertheless, their efficient tumor targeting via systemic administration had not been resolved yet. Amphiphilic alkylated poly(α)glutamate amine (APA) can serve as a cationic carrier to the negatively-charged oligonucleotides. APA polymers complexed with siRNA to form round-shaped, homogenous and reproducible nano-sized polyplexes bearing ~50 nm size and slightly negative charge. In addition, APA:siRNA polyplexes were shown to be potent gene regulators in vitro. In light of these preferred physico-chemical characteristics, their performance as systemically-administered siRNA nanocarriers was investigated. Intravenously-injected APA:siRNA polyplexes accumulated selectively in tumors and did not accumulate in the lungs, heart, liver or spleen. Nevertheless, the polyplexes failed to induce specific mRNA degradation, hence neither reduction in tumor volume nor prolonged mice survival was seen.

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In an era of personalized and targeted medicine,1 the ability to knockdown specific gene of interest brings unlimited potential to treat “undruggable” diseases. Exogenous double-stranded RNA (dsRNA) that is involved in RNA interference (RNAi),2 the innate cell machinery for regulated gene expression, causes downregulation of specific genes by targeting messenger RNA (mRNA) for cleavage or translational suppression. The approach of introducing artificial short interfering RNA (siRNA) into the cell was embraced by researchers in the field of cancer therapeutics in an attempt to specifically target and downregulate oncogenes.3 Unfortunately, siRNA molecules face many challenges when systemically administered to the body. Their negative charges and high molecular weights limit tissue penetration and cellular internalization, chemically unmodified siRNAs cause immune stimulation, undergo extensive degradation by RNases, exhibit rapid renal clearance and limited distribution and accumulation in tumor tissue.4 To overcome the obstacles and maximize the therapeutic potential of naked siRNA, enormous efforts were put in the development of nanocarriers that will bestow siRNA improved pharmacokinetic profile and anti-tumor efficacy.5 The most common non-viral nanocarriers for siRNA delivery are cationic lipids (lipoplexes) and cationic polymers (polyplexes). Several of the lipid-based

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systems have reached the clinical stage.\(^6,7\) Nevertheless, lipid-based delivery systems are limited to low administration doses due to carrier’s toxicity\(^8-10\) and suffer from preferential accumulation in the liver and spleen due to mononuclear phagocytic system (MPS) uptake, high blood flow and fenestrated vasculature.\(^11,12\) Utilizing the distribution pattern of lipid nanoparticles (LPN), they are mostly targeted towards liver diseases, kidney diseases or applied for local administration.\(^13,14\) Two polymer-based formulations have reached clinical evaluation. Unfortunately, cyclodextrin polymer (CDP)-based nanoparticles demonstrated dose-limiting toxicities (DLT) attributed to repeated exposure to the carrier\(^15,16\) in addition to liver and kidney toxicities that were observed in monkeys, possibly due to carrier’s components aggregation propensity.\(^17\) Dynamic Poly-Conjugates (DPC\(^\text{TM}\)) reached Phase 2 clinical trial for the treatment of Hepatitis B and Alpha-1 antitrypsin deficiency, both liver-related diseases.\(^19\) An ideal nanocarrier for siRNA delivery would retain advantageous properties of lipoplexes, such as narrow polydispersity and high transfection capacity, alongside low toxicity and higher loading capacity obtained by polyplexes.\(^19\) Here we present our attempt to develop a hybrid nanocarrier based on our previously published polymeric vehicle consisting of aminated poly(\(\gamma\)-glutamate) and low batch-to-batch reproducibility. Hence, microfluidics was previously used for the assembly of amphiphilic polymer was mixed with a solution of low toxicity and higher loading capacity obtained by polyplexes.\(^20-21\) The buffering capacity of the proton sponges leads to proton influx into endosomes, which results in membrane rupture and the release of their content to the cytoplasm.\(^29\) However, positively-charged polyplexes are known to form large aggregates and heterogeneous solutions in physiological fluids.\(^30\) Thus, to further improve the colloidal stability of our system, 40% of the functional groups were modified with alkyl groups and amphiphilic alkylated PGAamine (APA) was obtained. Hydrophobic modifications were found to have beneficial effects on cationic delivery systems including serum stability, membrane binding, improved dissociation in the cytoplasm, and decreased cytotoxicity.\(^31-33\) Nevertheless, manual mixing of bulk polymer and siRNA solutions resulted in poor uniformity and low batch-to-batch reproducibility. Hence, microfluidics methods were applied to achieve a homogenous, low-polydispersed and reproducible polyplexes when solution of amphiphilic polymer was mixed with a solution of siRNA.\(^34-36\) Microfluidics was previously used for the assembly of cationic polyethyleneimine (PEI) and plasmid DNA, and demonstrated advantageous manufacturing of smaller, moderately positive charged and narrow polydisperse nanoparticles compared with the bulked-mixed nanoparticles.\(^37\) Particles size was tuned by nitrogen/phosphate (N/P) ratio and alterations in concentration to obtain ~50 nm particles. Particles at this size are known to accumulate in the tumor site due to the enhanced permeability and retention (EPR) effect—the enlarged endothelial gaps and the impaired lymphatic drainage characterizing tumor vasculature. Indeed, in this report we show preferred accumulation of amphiphilic cationic nanoparticles in orthotopic mammary tumors following intravenous (IV) administration. Nevertheless, depletion of target gene was not observed in resected tumors, hence neither significant anti-tumor effect nor prolonged mice survival was obtained.

### Methods

Preparation of \(\gamma\)-aminohexane\(_{40}\%)\)-diaminoethane\(_{60}\%)\) L-polyglutamate (APA). PGA was prepared by N-Carboxyanhydride (NCA) polymerization as reported before.\(^20\) APA was synthesized from the PGA backbone by conjugation of ethylenediamine and hexylamine using carbonyldimidazole. Detailed description of the synthesis and the rest of the methods appear in the supporting information.

\(^1\)H Nuclear Magnetic Resonance (NMR), Multi Angle Static Light Scattering (MALS), and Electrophoretic mobility shift assay (EMSA), were performed as previously described.\(^20\)

Zeta potential and Hydrodynamic radius determination were performed using a ZetaSizer Nano ZS instrument (\(\lambda = 633 \text{ nm} \)); Malvern Instruments Ltd., Malvern, Worcestershire, UK).

Critical micellar concentration (CMC) was determined by measuring the emission of Nile red in polyplex/polymer solution at 640 nm.

Transmission Electron Microscopy (TEM) and Cryo-TEM images were taken using JEM 1200EX TEM (JEOL Ltd., Tokyo, Japan) and Tecnai 12 TWIN TEM (FEL, Oregon, USA), respectively.

Direct Stochastic Optical Reconstruction Microscopy (STORM) images were acquired using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging.

Confocal microscopy images were taken following 30 min-48 h incubation of MDA-MB-231 cells treated with 100 nM of APA:CY5-Rac1 siRNA (Cy5-siRac1).

In vitro silencing of Plk1 gene by Plk1 siRNA (siPlk1)-carrier polyplex was evaluated with pCsiCHECK reporter assay (Promega Madison, Wisconsin, USA). Cells transfected with pCsiCHECK plasmid were treated with 50, 100 and 250 nM of APA:siPlk1 polyplexes. Following 72 h, cells were lysed and measured for luminescence.

Western blot analysis for protein expression was performed on cells treated with 250 nM of APA:siPlk1 polyplexes for 48 h.

Cell growth inhibition was assessed counting the harvested cells following 72 h incubation with 50, 100 and 250 nM of APA:siPlk1 polyplexes. Following 72 h, cells were lysed and measured for luminescence.

Heparin displacement assay was performed as previously described.\(^20,21\)

APA:siRNA polyplexes’ integrity in serum was assessed by release of siRNA from the complex as measured by EMSA.

Red blood cell lysis was performed as previously described.\(^21\)

PK, accumulation and silencing activity of polyplexes in A549 subcutaneous tumor bearing nu/nu mice were determined using stem-loop qPCR method and EZ-RNA II total RNA
isolation kit respectively. Following single injection of polyplexes, blood samples were taken at 0-24 h. Tumors were collected at 24 h and analyzed.

Biodistribution of labeled polyplexes in mCherry-labeled MDA-MB-231 orthotopic tumor-bearing nu/nu mice was performed using CRI maestro, at 24 h following single APA:Cy5-siRac1 IV injection.

Accumulation and silencing activity of APA:siRac1 polyplexes in orthotopically-inoculated MDA-MB-231 tumor-bearing nu/nu mice. Mice were IV injected with polyplexes solution for 3 sequential days. After 24 h, organs were collected and siRNA levels were determined using stem-loop qPCR.

Accumulation and silencing activity of APA:siRac1 polyplexes in orthotopically-inoculated MDA-MB-231 tumors-bearing nu/nu mice. Mice were treated with polyplex solution for 9 subsequent days then monitored twice a week.

RACE procedure performed on RNA extracted from frozen tumors included RNA adaptor ligation and two steps of PCR amplification of the cleavage product which was finally detected by hybridization to a specific probe.38

All animal procedures were performed in compliance with Tel Aviv University guidelines approved by the Institutional Animal Care and Use Committee (IACUC). Mice body weight change was monitored twice a week. Mice were euthanized according to ethical protocol when showing signs of distress or with rapid weight loss (above 10% within a few days or 20% from the initial weight). For tumor-bearing mice, animals were also euthanized in case the tumor size exceeded 1000 mm3 or if the tumor was necrotic/ulcerative.

Results

Synthesis and characterization of APA polymer

Poly-α-glutamic acid (PGA) was synthesized as previously described.20 In order to conjugate ethylenediamine and hexylamine to the PGA backbone via the carboxylic acid moieties, carbonyldiimidazole (CDI) was used. The two conjugation processes were performed simultaneously, using 0.41 and 0.61 equivalents of the conjugation reagents respectively in order to obtain 40 and 60 conjugation percentages (Figure 1). 1H NMR of the APA was obtained (Figure 2, A inset) and compared to that of the PGA precursor (Figure 2, A inset), demonstrating the addition of 2 peaks of the ethylenediamine moiety at 3.42 (1.5H, s), 3.06 (2H, s) ppm and 3 peaks of the hexylamine moiety at 1.40 (0.5H, s), 1.20 (1.5H, s), and 0.78 (0.5H, s) ppm. The ratio of conjugation with each moiety was determined by 1H NMR. The Mw of the PGA precursor was measured by MALS to give 7018 g/mol, indicating a backbone of ~46 units. The Mw of the APA measured by MALS was 10,560 g/mol, confirming the 40 and 60 conjugation percentages that obtain theoretical Mw of 10,277 g/mol (considering acetate as counter ion) (Figure 2, B-C).

TEM and Cryo-TEM images of the APA polymer revealed rod-shaped particles bearing width of 5 nm and various lengths (Figure 2, D-E).

APA formed spherical polyplexes when complexed with siRNA, bearing ~50 nm diameter and slightly negative charge.

In order to evaluate the ability of APA polymer to bind siRNA, EMSA was performed (Figure 3, A). Migration retardation started already at 1 N/P, without complete neutralization of charge as indicated by the slower migration towards the positive electrode of 1, 1.5 and 2 (N/P) ratio polyplexes. Complete neutralization of charge was obtained at N/P ratio of 3. A screen of several polyplexes revealed high correlation between N/P ratio and concentration to the obtained size of polyplex (Supplementary Table 1). Since particles bearing diameter of 10-100 nm demonstrated better in vivo delivery efficiency to tumors than particles with 100-200 nm diameter,39 N/P ratio of 1.5 and a concentration of 1.5 mg/kg were selected for all further evaluations if not stated otherwise. In addition, since positively charged polyplexes are known for their high toxicity,40-42 these slightly negatively charged polyplexes were found beneficial in our study. APAsiPlk1/Ctrl siRNA (siCtrl) polyplexes were prepared using 5% glucose solution as excipient to obtain physiologically compatible solution that will further suit parenteral administration for in vivo applications. In order to maintain size reproducibility during complex preparation, a microfluidic chip was used for the controlled assembly of the polyplexes (Figure S1) that was siRNA type-dependent. Interestingly and in accordance with previous reports,37 all three complexes prepared by microfluidic chip obtained smaller sizes than the manually mixed ones. Our selected complex was further characterized for its size and charge. The hydrodynamic diameter of the complex was 43 ± 12 nm and it demonstrated narrow polydispersity of 0.127 (Figure 3, B, E). Particles at a
size range of 20-200 nm were shown before to selectively accumulate and retain in the tumor tissue due to the EPR effect. Critical micellar concentration (CMC) assay showed that APA and siRNA complex micelles are formed already at APA concentration of 0.015 mg/mL, which is equivalent to siRNA dose of 0.12 mg/kg. APA alone formed micelles only at 0.136 mg/mL (Figure S2). STORM images confirmed that the complexes are composed of both APA and siRNA as indicated by their high degree of colocalization (Figure 3, F). Zeta potential measurements revealed slightly negative charge of $-2.56 \pm 3.79$ mV, as expected by the migration of the complex towards the positive electrode in EMSA (Figure 3, C, E). The polyplex was further imaged by JEM 1200EX TEM and revealed spherical morphology with diameter of 50 nm (Figure 3, D, E).

Cryo-TEM images further confirmed a spherical structure bearing diameter of around 60 nm (Figure 3, E, F).

**Time course internalization of APA:Cy5-siRac1 polyplexes**

The uptake of APA:Cy5-siRac1 polyplexes by MDA-MB-231 cells was assessed by confocal microscopy (Figure 4). Cy5 punctuated structures appeared inside cells following 4 h of treatment. The fluorescent stains increased over time, indicating time-dependent, polymer-assisted internalization of Cy5-siRac1 into cells. In contrast, Cy5-siRac1 alone failed to internalize into cells, as indicated by the lack of Cy5-punctuated signal following 48 h of treatment.
APA:siPlk1 polyplexes downregulated Plk1 expression and specifically inhibited the proliferation of MDA-MB-231 and MCF-7 cells.

Plk1 is a serine/threonine kinase that has a pronounced role in the regulation of mitotic progression. Its overexpression was demonstrated in many tumor types, associated with high risk for metastasis and general poor prognosis, thus holding Plk1 as an attractive anti-cancer target. Our previous attempts to knock-down (KD) Plk1 expression in ovarian tumor model resulted in decreased tumor volume and prolonged mice survival. High levels of Plk1 were demonstrated also in breast cancer, while targeted delivery of siPlk1 to HER2-positive orthotopic breast cancer tumors reduced tumor growth, decreased metastases and prolonged mice survival. We have therefore evaluated APA:siPlk1 polyplexes for their in vitro silencing activity using the plasmid-based test system of dual luciferase reporter, on mammary adenocarcinoma cells (Figure 5). siPlk1 polyplexes silenced the expression of Plk1 gene to less than 50% of untreated cells, while minor non-specific gene silencing was observed at these concentrations (APA:siCtrl polyplex retained all and 70% of the original Plk1 expression in MCF-7 and MDA-MB-231 cells, respectively). However, the commercial transfection reagent Lipofectamine® 2000 (LF2000), a positive control, demonstrated significant non-specific silencing of circa 60% of untreated cells in MDA-MB-231 cells. In addition, efficient and specific Plk1 silencing was observed...
using Lipofectamine® 2000 of 99% compared with untreated cells. siPlk1 alone failed to silence gene’s expression in tested cell lines. In order to evaluate the ability of siPlk1-polyplexes to downregulate inherent protein’s expression, we further tested the expression of Plk1 protein in MDA-MB-231 and MCF-7 cells following treatment with APA:siPlk1 polyplexes by Western Blot analysis (Figure 5, B). We found that APA:siPlk1 polyplexes downregulated Plk1 protein expression to 65% and 36% compared with untreated MDA-MB-231 and MCF-7 cells respectively, while treatment with siPlk1 alone did not deplete protein’s expression in both cell lines. Non-specific effect of APA:siCtrl polyplex was demonstrated to some extent in MCF-7 cells (40% downregulation), although much smaller than that of the targeted Plk1 polyplex (64% downregulation). To demonstrate the ability of APA:siPlk1 polyplexes to inhibit the growth of mammary cancer cells, we evaluated their effect on the viability of MDA-MB-231 and MCF-7 cells. APA:siPlk1 treatment reduced the number of cells to 77%, 66% and 53% of untreated MDA-MB-231 and MCF-7 cells respectively, and to 45%, 26% and 31% of untreated MDA-MB-231 cells using similar concentrations.

Non-specific effect on the viability of cells was demonstrated in MDA-MB-231 cells, when APA:siCtrl polyplexes reduced cells number to 84%, 63% and 61% of untreated cells. This off-target effect, however, was lower than the effect of the targeted APA:siPlk1 polyplexes on MDA-MB-231 cells (Figure 5, C).

**Stability and toxicity of APA:siRNA polyplexes**

APA:siRNA polyplexes were evaluated for their compatibility in biological fluids. The polyanion heparin is a known indicator of the binding strength between siRNA and cationic polymers. Figure 6, A demonstrates the partial release of siRNA with the addition of 0.01 IU heparin and the complete replacement of the siRNA in the complex with the addition of 0.1 IU of heparin. We further evaluated the stability of APA:siRNA polyplexes in serum (Figure 6, B). The complexes were stable for 2 h, however, following 4 h, siRNA mobility was observed as indicated by a smear in the gel. Following 6 h of incubation in serum, the complexes were fully degraded, as shown by the appearance of a single band migrated to the same extent as free

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Figure 4. Cell internalization of APA:Cy5-siRac1 polyplexes. Time course internalization is indicated by the appearance of Cy5 clusters inside the cells following 4 h and the gradual increase in stains over time (scale bar = 30 μm).

Figure 5. Downregulation of Plk1 expression in MDA-MB-231 and MCF-7 cells following treatment with APA:siPlk1 polyplexes. (A) Western Blot analysis showing 65% and 36% downregulation in MDA-MB-231 and MCF-7 cells respectively. (B) APA:siCtrl polyplexes demonstrated 40% downregulation in MCF-7 cells. (C) APA:siPlk1 polyplexes reduced cell viability to 77%, 66% and 53% in MDA-MB-231 and to 45%, 26% and 31% in MCF-7 cells respectively.

Figure 6. Stability of APA:siRNA polyplexes. (A) Partial release of siRNA with 0.01 IU heparin and complete replacement with 0.1 IU. (B) Complexes were stable for 2 h, following 4 h, siRNA mobility was observed. Following 6 h of incubation, complexes were fully degraded.
siRNA. STORM image confirmed the low serum stability of APA:siRNA polyplexes, demonstrating lower degree of colocalized APA and siRNA following 1 h of incubation in 10% serum (Figure S3) compared with freshly made polyplexes (Figure 3, F). Red blood cells are the first large compartment in the plasma to interact with IV-injectable drugs. In order to assess the toxicity of APA:siRNA polyplexes, ex vivo red blood cell (RBC) lysis assay was performed (Figure 6, C). APA concentration of up to 1000 μg/mL did not cause hemolysis, based on the extent of released hemoglobin. Notably, this concentration is ~25-folds of the dose used for the following in vivo experiments (marked in red arrow below the x axis). Similar results were obtained with dextran (negative control) and the lower concentrations of SDS, while higher concentrations of SDS, > 40 μg/mL, caused extensive hemolysis.
APA:siRac1 polyplexes selectively accumulated in MDA-MB-231 mammary tumors

To further evaluate the ability of APA to promote tumor accumulation of siRNA following systemic IV administration, the levels of siRac1 in tumors were quantified. Rac1 is a member of the Rho small GTPases that is known to regulate cellular motility. Its expression was linked with epithelial-mesenchymal transition (EMT), tumor metastasis and invasion, but not with proliferation, thus killing effect is not traditionally attributed to siRac1 treatment. Therefore, in this study we have used siRac1 to study tumor accumulation while avoiding cellular killing. Nu/nu tumor-bearing mice were treated with 1.5 mg/kg (siRNA-equivalent) APA:siRNA polyplexes for 3 sequential days, followed by tumor resection on the fourth day. Results demonstrate ~17-fold accumulation of siRac1 in mice treated with APA:siRac1 polyplexes, while APA:siCtrl polyplexes did not cause siRac1 accumulation (Figure 7, D). To demonstrate the selective accumulation of APA:siRNA in ectopic SC tumors, our polyplexes were IV injected at 4 mg/kg dose to A549 lung carcinoma SC tumor bearing mice. As shown in Figure S4, A, siRac1 demonstrated ~35-folds accumulation compared to mice treated with PBS. mRNA silencing to ~0.43 folds (57%) was demonstrated in human mRNA, while murine Rac1 mRNA was not depleted following treatment (Figure S4, B). To establish plasma circulation time of formulated (with vehicle) versus unformulated (without vehicle) siRNA, single IV injection of APA:siRac1 polyplexes or siRac1 alone was given to nu/nu tumor bearing mice, followed by collection of small volumes of blood samples from the tail (Figure S4, C). PK measurements revealed rapid siRNA clearance from plasma with a decrease to ~4% of the initial plasma level within 30 min, and to ~0.7% within 2 h. However, this amount of siRNA was ~3-fold higher than the plasma levels of siRNA in the non-formulated siRNA treated mice. Interestingly, at 24 h, the level of siRNA in the plasma of APA:siRac1-injected mice was 23-fold higher than the level in plasma of mice injected with free siRNA. Short plasma half-life of APA:siRNA polyplexes might be attributed to the low stability of the complexes in plasma, as shown in Figures 6, B and S3, leading to exposure of siRNA and rapid elimination. However, the accumulation of siRNA in tumors despite rapid plasma clearance indicates an efficient and rapid tumor uptake. One major obstacle in the therapeutic use of siRNA is the segregation of siRNA-based formulations in reticuloendothelial system (RES).
organs such as liver, spleen and lungs.\textsuperscript{52} In order to follow the biodistribution of our polyplexes, APA:CsCy5-siRac1 polyplexes were IV injected to mice bearing fluorescently-labeled tumors. Twenty-four hours following single injection of APA:CsCy5-siRac1 at 1.5 mg/kg dose, mice were imaged, followed by organs resection and Cy5 fluorescence quantification. As it is difficult to subtract the fluorescence of mCherry from Cy5 due to partial spectral overlapping, it was more reliable to measure and quantify directly the amount of siRac1 in the tumor, thus we relied on the RT-PCR quantification of the resected tumors to determine the accumulation extent (Figure 7, D). Distribution of Cy5-siRNA to other organs demonstrated high signal in the kidneys, probably due to renal excretion of polyplexes, and much lower signal (about hundredth fold) from the lungs. The rest of the organs: liver, heart and spleen exhibited negligible Cy5-siRac1 signal (Figure 7, B-C). The body distribution analysis confirmed that polyplexes’ body elimination was attributed to renal excretion, rather than the mononuclear phagocytic system (MPS) organs uptake (liver, spleen and lymph nodes),\textsuperscript{39} resulting in no indicated toxicity, as demonstrated by RBC lysis assay and the normal healthy increase in mice body weight (Figures 6, C and 8, A, respectively).

Encouraged by the targeted accumulation of APA:siRac1 polyplexes, we further evaluated the anti-tumor efficacy of the polyplexes in MDA-MB-231 mammary model. siPlk1 was previously demonstrated to be an efficacious anti-tumorigenic siRNA,\textsuperscript{21,48} thus tumor-bearing mice were treated with APA:siPlk1 or APA:siCtrl polyplexes. The minor inhibition in tumor growth obtained by APA:siPlk1 polyplexes was not significant ($P = 0.697$, Figure 8, B). Moreover, the survival rate of APA:siPlk1 treated mice was lower than that of APA:siCtrl treated mice, although in a non-significant manner ($P = 0.592$, Figure 8, C). Nevertheless, albeit the polyplex accumulated
preferentially in the tumor, it did not induce specific mRNA degradation, as shown by lack of rapid amplification of cDNA end (RACE) products in both APA:siRac1 and APA:siCtrl treated groups (Figure 8, D).

Discussion

The development of an efficacious and safe nano-sized delivery system for oligonucleotides is a crucial step to exploit the potential of RNAi in clinical practice. Several polyaminated delivery systems have been developed and underwent preclinical evaluation, but many of them are affected by non-specific activity and systemic toxicity. In addition, the high concentrations required for in vivo delivery compromise nanoparticles’ colloidal stability resulting in large aggregates and hamper the clinical translation of such therapeutic modality. In an attempt to overcome these limitations, we developed a PGA based delivery system bearing cationic residues for the electrostatic interaction with oligonucleotides and alkyl residues to increase the colloidal stability of nanoparticles. As reflected by $^1$H NMR and MAL^S analyses, efficient conjugation of ethylenediamine and hexylamine moieties to the PGA backbone was controlled by the equivalents of reactants. The obtained amphiphilic polymer formed potent polyplex with siRNA that was further characterized in the relevant concentration and conditions used for in vivo administration. This polyplex was found to bear narrow polydispersity and desired size to exploit the EPR effect. We hypothesize that the micellar-like morphology observed in TEM and Cryo-TEM was due to macromolecular rearrangement in which the hydrophobic tails (the hexylamine moieties) turn inwards, exposing the positively charged amines to complex with siRNA on the surface of a round-shaped particle. The active polyplex showed excellent in vitro gene knockdown together with functional inhibition of gene-of-target. Biocompatibility evaluation revealed that the polyplex is safe for IV administration. Desired binding strength between APA and siRNA was demonstrated by heparin-induced release of siRNA with the addition of 0.01-0.1 IU, which corresponds to concentrations of approximately 0.3-3 mg/mL. Han et al demonstrated the importance of optimal binding strength between siRNA and its polymeric delivery vehicle to a successful in vivo gene KD. Optimized polyplexes released siRNA by the addition of 0.2-0.4 mg/mL heparin and possessed good balance between serum-protection and cytoplasmic release, further reflected in effective in vitro gene silencing and in vivo antitumor efficacy. APA:siRNA polyplexes’ preferential accumulation in tumor tissue was further demonstrated in two animal models.

Figure 8. In vivo anti-tumor activity and safety of APA:siPlk1 polyplexes following 9 repeated intravenous injections to mammary tumor bearing mice. (A) Maintenance of weight following repeated and extended intravenous treatments with APA:siPlk1 polyplexes. (B) The progression of MDA-MB-231 tumors following intravenous treatments with APA:siPlk1 polyplex, APA:siCtrl polyplex or glucose was similar ($n=5$). (C) Kaplan-Meier survival plot for all treated groups. (D) RACE products hybridized with specific probe indicating no specific mRNA cleavage was obtained by APA:siRac1 polyplexes.
Nevertheless, the polyplexes with siPlk1 failed to show any in vivo anticancer activity. In their review, Wilhelm et al describe the low delivery efficiency of nanoparticles to tumors as a major obstacle to an efficient anti-cancer effect. They report that only 0.6%-0.7% of intratumorally-injected spherical, neutrally charged and <100 nm sized nanoparticles actually reach the tumor, from which the effective dose is even smaller due to interactions with tumor stroma and other extracellular barriers. We hypothesize that while our polyplexes demonstrated preferred properties such as high reproducibility, suitable size and narrow polydispersity that further resulted in desired complexation strength, low toxicity and satisfactory in vitro gene silencing, the effective dose reaching the tumor cells was insufficient in order to induce significant gene silencing and inhibition of tumor growth, mainly due to polyplexes’ low serum stability. Local delivery of these polyplexes (APA:siPlk1) can overcome this limitation. Alternatively, stability of our polyplexes was recently improved by complexation with a combination of siRNA and miRNA as opposed to siRNA alone. Additional in vivo investigation using local intratumor administration will be performed in the future. The PEG shield improved the stability of the nanoparticles in serum while de-shielding in response to tumor’s acidity enabled high tumor uptake. Thus, treatment with PEG-shielded siPlk1-carrying nanoparticles resulted in 70% downregulation of mRNA in tumors. In response, significant reduction in tumor volume was obtained. Interestingly, no downregulation of Plk1 mRNA or the desired effect on tumor volume was shown in mice treated with non-shielded particles. As one of the reasons for the low accumulation of IV administered nanoparticles in tumors is the low extravasation through endothelial cells, Zuo et al suggested combining treatment with the TGF-β type I receptor inhibitor LY364947, which is known for its ability to improve vascular permeability by reducing the coverage of pericytes on blood vessels. Prior intraperitoneal (IP) injection of LY364947 facilitated the accumulation of siPlk1 in tumors, while siPlk1-loaded cationic lipid nanoparticles had limited penetration into the core of MDA-MB-231 intra-mammary tumors. Thus, the synergistic effect of siPlk1 nanoparticles and LY364947 resulted in significant tumors’ shrinkage compared with just siPlk1 nanoparticles treatment. Arrest of Plk1 pathway is a promising approach to combat cancer since depletion of Plk1 gene causes “mitotic catastrophe” and tumor-growth cessation. Unfortunately, hitting a single target to block tumor growth and dissemination is often scant to achieve a therapeutic response. Therefore, the combination of siPlk1 with conventional anticancer drugs for the treatment of breast cancer was proposed. The addition of Plk1 inhibition to chemotherapy (doxorubicin and cyclophosphamide) prevented tumor relapse in triple negative breast cancer (TNBC) xenografts. Sun et al developed Micelleplex loaded with both siPlk1 and the chemotherapeutic drug paclitaxel (PTX). This IV administered micelleplex significantly reduced tumor volume in the metastatic breast cancer tumor model MDA-MB-435 (later defined as melanoma). The effect of tumor shrinkage was much greater than the effect of each micelleplex-siPlk1 or micelleplex-PTX separately, or the combination of micelleplex carrying either PTX or siPlk1 separately. Hence, we postulate that improving the biostability of our polyplexes via smart shielding alongside increased doses, in addition to combination of siPlk1 with drugs that will target key, but different, cellular pathways in tumor-stroma-endothelial cells crossstalk, will maximize the anti-cancer effect of the IV administered APA:siRNA polyplexes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2017.10.012.

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