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Citation: Kowalski, Piotr S. et al. "Ionizable Amino-Polyesters Synthesized via Ring Opening Polymerization of Tertiary Amino-Alcohols for Tissue Selective mRNA Delivery." Advanced materials (2020): e1801151 © 2020 The Author(s)

As Published: https://dx.doi.org/10.1002/ADMA.201801151

Publisher: Wiley

Persistent URL: https://hdl.handle.net/1721.1/125442

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Published in final edited form as:

Adv Mater. ; : e1801151. doi:10.1002/adma.201801151.

Ionizable amino-polyesters synthesized via ring opening polymerization of tertiary amino-alcohols for tissue selective mRNA delivery

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Supporting Information

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

Conflict of interest

U. Capasso Palmiero, P.S. Kowalski and D.G. Anderson filed a patent for the development of the described amino-polyesters for drug delivery

Keywords

nucleic acid delivery; messenger RNA; polymeric nanoparticles; polyesters; biomaterials

Polymers have been widely used in biology and medicine for the development of delivery systems for variety of therapeutic payloads, including small molecules, proteins and nucleic acids[1]. Delivery of RNA-based therapeutics, in particular, allows for both transient control of gene and protein expression as well as permanent editing of the genomic DNA with the advent of CRISPR, providing a therapeutic platform suitable for addressing a wide range of diseases[2]. Thus far, most of the polymeric carriers have been developed for delivery of short RNAs (~7–14 kDa), such as antisense oligonucleotides (ASO) and short interfering RNA (siRNA), while delivery of significantly larger messenger RNA (mRNA) molecules (~600–1000 kDa) to various tissues poses an additional challenge[3, 4].

Cationic polymers have been developed for RNA delivery given their ability to electrostatically condense nucleic acids into nanoparticles[5]. Examples of those studied for *in vivo* delivery of RNAs include derivatives of polyethyleneimine (PEI), poly(amido-amine) (PAMAM), polylysine, chitosan and poly-beta amino-esters (PBAEs)[6-11]. However, concerns regarding limited efficacy and toxicity of the above and many other cationic polymers remain[12, 13]. High net-positive charge and the inability to degrade under physiological conditions or yielding potentially toxic degradation products, which can accumulate in the body at hazardous levels, are the sources of potential toxicity. In addition, synthesis of well-defined amine bearing polymers by commonly employed step growth polymerization (e.g. Michael addition), polycondensation, or nucleophilic substitution of epoxides is challenging, which can lead to polymers with broad molecular weight distribution and difficulties with purification[14, 15].

Ring opening polymerization (ROP), is a versatile method for the synthesis of polymers with controlled molecular weight and low dispersity[16]. Notably, polyesters prepared by ROP of lactones and lactides, including polycaprolactone (PCL), polylactide (PLA) or polyglycolide (PGA) have been widely used clinically[17]. Adopting ROP for the synthesis of amine-containing polyester for nucleic acid delivery, however, faces several synthetic challenges related to incompatibility of the primary and secondary amines with the ROP as well as the lack of naturally occurring amine bearing lactone monomers. To date, reported methods involve multi-step synthesis requiring protection/deprotection after polymerization or post-polymerization modification of functionalized polyesters, both approaches suffering from issues with conversion, scalability, and polymer-chain degradation[18]. Methods utilizing ROP of amine-containing lactone monomers require multi-step synthesis of functional monomers[19]. In addition, it can be difficult to a priori predict biological compatibility of these polymers and their corresponding degradation products, such as lipocationic hydroxy acids [19, 20], in contrast to polyester degradation products composed of readily available lactones. Many of these lactones are generally recognized as safe substances (GRAS) by the American Food and Drug Administration (FDA)[21] and are utilized in food and biomedical applications or found in natural products. Such lactones were used by Zhou et al. for preparation of poly(amine-co-esters) via combined enzymatic ring opening and

polycondensation co-polymerization of lactones in the presence of tertiary amines and dialkyl-diester, catalyzed by Candida antarctica lipase B (CALB)[22]. These high molecular weight terpolymers were used for DNA transfection into cells showing low toxicity.

To develop degradable materials for the delivery of mRNA that would address the aforementioned challenges, we synthesized a new type of ionizable amino-polyesters (APEs) via controlled ROP of lactones with tertiary amino-alcohols. We generated a diverse library of APEs using one step synthesis with control over the number of repeating monomer units, by varying the stoichiometry between the alkoxy bearing initiator and the lactone monomer, yielding degradable polymers with low dispersity (Đ < 1.4) and high yields. We then sought to characterize these APEs and their ability to deliver mRNA, as well as explore their structure-activity relationship.

To this end, a library of 37 APEs was synthesized via ROP of readily available lactones in the presence of tertiary amino-alcohols as initiators and triazabicyclodecene (TBD) as a catalyst, in a one synthetic step (Figure 1A, Table S1). To maximize biodegradability of the APEs we incorporated amino-alcohols containing tertiary amines unable to open a lactone ring during polymerization, in order to avoid formation of amide bonds, which are generally less susceptible to hydrolysis and typically enzymatically degraded[23]. A majority of tertiary amino-alcohols were commercially available, while the custom aminonalcohols (e.g. H and I, Figure 1A) were synthesized in two synthetic steps by reduction of an ester intermediate with $LiAlH_4$, as described in the materials and method section (Supplementary Information). We selected amino-alcohols with different number of tertiary amines (N; 1–4) and alkoxy groups (ROH; 1–4 and 6) as the presence of ionizable amines, charge density and the polymer structure (linear vs branched) have been previously indicated as important factors to confer effective nucleic acid delivery[24-26]. To investigate the effects of polyester molecular weight and the monomer lipophilicity on mRNA transfection, APEs were synthesized with the degree of polymerization (q) corresponding to three and five lactone repeating units, using lactone monomers with a varying length of the alkyl side chain, including caprolactone (CL, no side chain; C0), dodecalactone (DD, seven carbon side chain; C7), and tetradecalactone (TD, nine carbon side chain; C9).

A study of the ROP kinetics initiated by the amino-alcohol B (N=2, ROH=4), showed a linear correlation between the number-average molecular weight (Mn) of the polymers and the monomer conversion (Figure 1B), which is expected from a well-controlled ROP. Similarly, a linear correlation between Mn and the degree of polymerization demonstrated good control over APEs molecular weight (Figure 1C). All synthesized APEs displayed a narrow molecular weight distribution (Đ < 1.4), with Mn and *q* close to the theoretical values (Table S1 and S2, Supporting Information). Obtained APEs had lower dispersities than the previously reported amine bearing polyesters used for nucleic acid delivery, including lipocationic polyesters synthesized via methyl lithium initiated ROP of amine bearing lactones (Đ > 1.6)[19], poly(amine-co-esters) prepared by enzymatic catalysis of lactones with tertiary amines and dialkyl-diester (Đ > 1.8)[22], or polyesters synthesized by step growth polymerization (e.g. PBAE; Đ > 2)[9]. Low dispersity is often a desired feature of materials intended for biomedical applications as it helps to overcomes challenges associated with clinical translation, including batch-to-batch reproducibility, quality control

and implementing good manufacturing processes (GMP)[27]. Above 80% monomer conversion was reached for the majority of polymers (Table S2, Supporting Information). Most of the reported APEs were synthesized at a scale above 1 g (M&M section, Supporting Information).

To date, lipid nanoparticles (LNPs) present the most clinically advanced delivery platform for RNA therapeutics [2, 10]. Lipid excipients have been shown to reduce aggregation of the NPs, provide enhanced stability at physiological condition and promote the endosomal escape [28-30]. Therefore, we prepared APE-LNPs by mixing each ionizable amino-polyester with helper lipids, including 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol (Chol), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (C14-PEG_{2000}) and mRNA (Figure 2A). Nanoparticles were formulated at an 8:1 ratio of tertiary amines in the polyester to mRNA phosphate groups (N/P). A library of APE-LNPs formulated with mRNA encoding Firefly luciferase (FLuc) was screened in HeLa cells to identify most potent candidates for the delivery of mRNA (Figure 2B). We found eight APE-LNPs that were able to effectively transfect HeLa cells, including I-DD3, I-TD3, A-TD3, B-DD3, A-DD5, B-TD3, B-DD5 and H-TD3. We observed that amino-polyesters composition, in particular the ones incorporating lactones contain alkyl side chains (TD, DD), strongly correlated with an effective mRNA delivery as compared to APEs composed of caprolactone (CL) lacking an alkyl side chain, that showed low delivery efficacy. Moreover, the presence of amino-alcohols with a higher number of tertiary amines in the APE composition had a positive effect on their transfection efficiency, with the top performing APEs containing four and two amines (A-TD3, B-DD3 and I-DD3). In contrast, increasing number of lactone repeating units from three to five did not significantly improve transfection and in some cases reduced the potency of the APEs (e.g. A-TD3 vs A-TD5, Figure 2B). Similarly, the number of initiator alkoxy groups and the length of the lactone alkyl side chain (C7 vs C9) appeared to have no impact on the APE-LNPs performance. Limited transfection efficacy of CL-based APEs indicates a significant role of side chain lipophilicity, that may influence the ability of APEs to condense into nanoparticles or impact their stability and interaction with the cell membranes. Further studies are needed to investigate the effect of alkyl side chain length (e.g. C3–6) as well as the dimension of lactone ring (e.g. 5–16-membered) on APEs properties and their performance.

The top performing APEs comprised of different initiators (I-DD3, A-TD3, B-DD3) were selected for further studies. Three different batches of each amino-polyester were independently synthesized to investigate batch-to-batch reproducibility. Polymers were characterized by ^{1}H-NMR (Figure S1 and Table S2, Supplementary information) and gel-permeation chromatography (GPC). All APEs showed high batch-to-batch reproducibility, low dispersities ($Đ < 1.3$) and unimodal molecular weight distributions (Figure 2C and Table 1). mRNA nanoparticles prepared for each polymer batch by microfluidic mixing were mono-disperse, with diameters below 100 nm, and the zeta potential (ζ) ranging from −2 to 10 mV, as characterized by dynamic light scattering (Table 1 and Figure S2A). This data reflects the diversity of APEs platform and low batch-to-batch variability of both the polymers and their corresponding LNPs, demonstrating the importance of reproducibility of the polymer properties for producing the high-quality lipid nanoparticles. Cryo-electron

microscopy revealed a distinct morphology of each APE-LNP, that differed from a multi-lamellar lipoplex-like structure typically observed for RNA LNPs (Figure 2D and S3). This finding shows that the polymer structure, dictated by the amino-alcohol, is important for the organization of the RNA into LNPs, which might influence the physicochemical properties of APE-LNPs and play a role in transfection. The efficacy of cell transfection with APE-LNPs containing FLuc mRNA was reproducible between different polymer batches and the nanoparticles were well tolerated, as demonstrated by no noticeable decrease in cell viability (Figure S2, Supporting Information). I-DD3 LNPs showed the most potent mRNA delivery to HeLa cells among the selected APEs, and when compared to commercially available mRNA transfection reagents FLuc expression mediated by I-DD3 LNPs was nearly five-fold greater than Stemfect and matched the expression of Lipofectamine MessengerMax (Figure 2E). To the best of our knowledge, reported mRNA cell transfection efficiency displayed by I-DD3 LNPs is unparalleled by other polyester carriers developed thus far for systemic mRNA delivery [9, 31].

To better understand the differences in mRNA delivery efficacy between the top performing APEs as well as the limited efficacy of CL-based polymers, we investigated the uptake of the nanoparticles into HeLa cells using Cy5 labeled mRNA. We found that uptake of A-CL3, B-CL3 and I-CL3 was significantly lower as compared to A-TD3, B-DD3 and I-DD3, showing that the limited performance of CL-based APEs results from impaired ability of their nanoparticles to cross cell membranes (Figure 2F). CL-based APE-LNPs were highly polydisperse as compared to their TD and DD analogues (PDI > 0.3, data not shown), thus decreased uptake could result from impaired formation and stability of the nanoparticles that may lead to aggregation in cell medium and hinder interaction with the cells. In particular, we found that A-CL3 and B-CL3 nanoparticles had limited stability when stored in PBS (pH 7.4) at 4^{o} C and over time crashed out from the solution. In contrast, TD and DD-based APEs could be stored in the same conditions for at least two weeks without any measurable change in nanoparticles properties and their transfection activity (Figure S2A, B, Supporting Information). In addition, the presence of hydrophobic alkyl side chains in the composition of TD and DD-based APEs might influence the ability of their nanoparticles to interact with the lipid cell membranes, as suggested by higher uptake into the cells (Figure 2F, G). Surprisingly, A-TD3 LNPs were more effectively taken up by HeLa cells as compared to I-DD3 LNPs, despite the latter exhibiting much higher FLuc mRNA expression (Figure S2, supporting information), which suggest that I-DD3 LNPs may be superior in promoting the endosomal escape due to the presence of two additional tertiary-amines[3, 32] (Figure 2G, diffused Cy5 mRNA pattern).

We next explored the capability of the selected APE-LNPs for in vivo mRNA delivery. First, we investigated the biodistribution of A-TD3, B-DD3 and I-DD3 LNPs formulated with Cy5-labeled mRNA in C57BL/6 mice. Mice were injected intravenously via tail vein with mRNA nanoparticles at 0.6 mg kg^{-1}. Interestingly, we found that the distribution through the major organs varied significantly depending on the polymer composition. A-TD3-LNPs preferentially accumulated in the liver, 80% of total fluorescence, while I-DD3 LNPs showed significantly higher accumulation in the lungs as compared to other LNPs, 20% of the total fluorescence (Figure 3A, C and S4). B-DD3 LNPs distributed more readily into the spleen, and heart. In vivo mRNA expression was studied using APE-LNPs containing FLuc

mRNA, injected via tail vein into mice at 0.6 mg kg^{-1}. We found that mRNA expression corroborated the preferential accumulation of the APE-LNPs through the organs (Figure 3B, D and S4). The highest percentage of total FLuc expression was found in the liver for A-TD3, in the spleen for B-DD3 and in the lungs for I-DD3. Despite the accumulation of all LNPs in the liver both B-DD3 and I-DD3 showed significantly higher FLuc expression in the spleen and lungs, respectively. For B-DD3 LNPs 80% of the total FLuc expression was found in the spleen, whereas for I-DD3 LNPs over 60% of total FLuc luminescence originated from the lungs. Thus far, little is known about the mechanism governing preferential uptake of the nanoparticles into different tissue and selective transfection of different cell types. Ionizable lipid nanoparticles were shown to accumulate in the liver hepatocytes by Apo lipoprotein E (ApoE) mediated uptake[33], while surface charge has been linked with the nanoparticle uptake into the lungs and spleen[34, 35]. Since all developed APEs have been formulated with an identical lipid composition and N/P ratios, we hypothesized that the polymers structure and their properties (e.g. lipophilicity, pKa) dictate the organization into RNA LNPs, subsequently influencing nanoparticles surface charge and their ability to interact with the serum proteins, that could result in the observed differences in the uptake and in vivo efficacy. Recent studies provided an evidence of the protein corona formation on the nanoparticle pharmacokinetics and the preferential uptake in vivo by the immune cells, via surface-deposited active complement factor 3 (C3), while in vitro studies revealed the importance of the surface charge and lipophilicity for preferential uptake by the endothelial cells [36-38]. Importantly, we demonstrated that developed APE-LNPs are a versatile platform that enables selective delivery of mRNA to different tissues. Both tissue selectivity and in vivo efficacy of the APEs could be further optimized by fine tuning APE-LNP composition using methodologies such as definitive screening and fractional factorial designs, as previously demonstrated for ionizable lipid nanoparticles[39] or by active targeting, recently shown with crosslinked chimaeric polymersomes obtained by end-conjugation of PEI with well-defined polyesters [40].

Similarly to in vitro studies, I-DD3 LNPs showed the highest potency of mRNA delivery, as reflected by the levels of FLuc mRNA expression (Figure 3D and S4). When compared to commercially available and clinically evaluated[41, 42] in vivo-jetPEI, a cationic polymer for nucleic acid delivery known for high levels of lung transfection after systemic administration[43], I-DD3 LNPs provided nearly 10-fold higher FLuc mRNA expression in the lungs. To our best knowledge it distinguishes I-DD3 LNPs as the most effective degradable polymeric nanoparticles for mRNA delivery to the lungs reported to date (Figure S5, Supporting Information). Vivo-jetPEI has been used as control in a number of studies evaluating systemic nucleic acid delivery, including our prior work with PBAEs [9, 44], therefore it is a suitable benchmark for assessing transfection efficacy of APE-LNPs. Moreover, APE-LNPs containing scrambled mRNA were shown to be well tolerated in vivo even at the higher doses of 1 mg kg^{-1}, as reflected by the negligible weight loss, up to 4% body mass, 24 hours post-systemic administration into animals (Figure S6B, Supporting Information). In addition, we did not find signs of liver toxicity after administration of 1 mg kg^{-1} of APE-LNPs. As compared to PBS treated mice, slightly elevated level of liver enzyme alanine aminotransferase (ALT) was observed for I-DD3 LNPs, however, serum levels for all measured liver enzymes, including Aspartate Aminotransferase (ASL),

Alkaline phosphatase (ALP) and ALT were within the normal healthy range[45] for all tested APE-LNPs (Figure S6A, Supporting Information).

Identification of the cell types that express the mRNA in vivo is vital for predicting potential applications of mRNA delivery system. Therefore, to identify which cell types are transfected by the APE-LNPs in their designated organs, we used Ai14 mouse model with a Cre-activatable tdTomato knock-in reporter allele containing loxP-flanked STOP cassette (LSL-Tom). Ai14 Mice were injected via tail vein with the APE-LNPs encapsulating mRNA encoding Cre recombinase enzyme at 0.6 mg kg^{-1}. This model allowed us to visualize with high sensitivity any cells that expressed Cre mRNA, by turning on the robust production of red fluorescent protein (tdTomato) in these cells[35]. Immuno-fluorescent staining with specific cell markers were performed to identify transfected cells in the context of the whole tissues (Figure 4). Cell transfected by A-TD3 LNPs in the liver were solely identified as hepatocytes based on their cuboidal morphology, large size, and often binucleated nature[46], mainly due to lack of available hepatocyte specific markers. For B-DD3 LNPs we found a substantial number of tdTomato-positive cells residing in the marginal zone of the spleen in a close proximity to CD169-positive macrophages, that we identified as CD31+ endothelial cells (Figure S7, Supplementary Information). In addition, a co-localization of tdTomato signal within the spleen red pulp with CD11c-positive cells and CD169-positive cells indicates the ability of B-DD3 nanoparticles to transfect antigen presenting cells (APC) such as dendritic cells and macrophages. The majority of tdTomato-positive cell in the lungs transfected by I-DD3 LNPs were identified as CD31+ microvascular endothelial cells, primarily alveolar capillaries and venules. Some co-localization with PDPN-1-positive type I epithelial cells was also observed, while no transfection of cobblestone-like type II bronchi epithelium was found (Figure 4, br). The ability of APE-LNPs to selectively transfect various cell types with mRNA therapeutics can be important for potential therapeutic applications. Hepatocytes have been utilized as primary target of mRNA carriers for production of various proteins, including human FIX [47, 48], erythropoietin [49, 50], therapeutic antibodies for different applications[51, 52] and most recently for therapeutic genome editing using CRISPR-Cas9[53, 54]. mRNA transfection of splenic APCs has been utilized to induce antigen-specific T-cell responses for cancer immunotherapy[34] while lung endothelium has been shown to play an important role in the pathophysiology of many inflammatory diseases[55-57] and could serve as a potential target for treatment of acute respiratory distress syndrome (ARDS)[58], pulmonary hypertension[59, 60] and cancer [6, 61].

APEs represent significant improvement in terms of the control of the molecular weight and molecular weight dispersity as compared to other polymeric materials previously reported for RNA delivery such as poly-beta-aminoesters (PBAEs), 7c1, ionizable amphiphilic dendrimers and PEI [9, 12, 44, 62]. Novel design of the current system, employing tertiary aminoalcochols as ROP initiators and biocompatible lactone monomers, allows one step synthesis of branched polymers and ensures the degradation products with well-established biocompatibility (hydroxyacids), that distinguishes APEs from polymers that either lack degradability or yield degradation products with unknown biocompatibility. Importantly, while polymers like PEI, PBAEs and recently reported functional polyesters show effective mRNA transfection predominantly in the lung tissue [9, 31], we demonstrated that APEs display tissue and cell selective delivery to lung endothelium, liver hepatocytes and antigen

presenting cells in the spleen with several fold greater efficacy of mRNA lung transfection for I-DD3 as compared to PEI and top preforming PBAEs, relative to jetPEI control. All of the above demonstrate the versatility of APE platform for mRNA delivery and highlight the potential for clinical translation.

In summary, we report on the design, synthesis and characterization of new ionizable amino-polyesters designated for tissue-selective mRNA delivery. We demonstrated that utilizing lactones and tertiary amino alcohols to initiate ROP is an effective and reproducible method to make ionizable amino-polyesters with low dispersity and at high yields. We explored the structure-function relationships within this class of polymers by generating the combinatorial library of APEs and identified polymers, which formulated into LNPs elicited potent mRNA expression both in vitro and in vivo with low toxicity. Importantly, this study not only demonstrates that the developed APE-LNPs are a versatile platform that enables selective delivery of mRNA to different tissues and cells, including lung endothelium, liver hepatocytes and APCs in the spleen but also shows the importance of employing controlled polymerization in the design of new polymeric nanomaterials to improve in vivo nucleic acid delivery.

Experimental section

Animal experiments.

All animal studies were approved by the MIT Institutional Animal Care and Use Committee and were consistent with local, state, and federal regulations as applicable. For biodistribution studies, APE-LNPs containing Cy5-labeled mRNA were injected intravenously into female C57BL/6 mice (Charles River Labs, 18–22 g) via tail vein (0.6 mg kg^{-1}). Mice were sacrificed 2h after nanoparticle administration and the organs were isolated and imaged with an IVIS Spectrum in vivo imaging system (Perkin Elmer, Waltham, MA), using a 640/680 excitation/emission narrow band filters. For the efficacy studies, APE-LNPs containing FLuc mRNA were injected as described above (0.6 mg kg^{-1}). 6 h after injection of the nanoparticles, mice were injected intraperitoneally with 130 μL of D-luciferin (30 mg mL^{-1} in PBS, Perkin Elmer). After 10 min, mice were sacrificed by CO_2 asphyxiation and the organs were isolated and imaged with an IVIS Spectrum in vivo imaging system. Both luminescence and florescence signals were quantified using Living Image software v4.4 (Perkin Elmer). For the evaluation of the toxicity, APE-LNPs containing scrambled mRNA were injected as described above (1 mg kg^{-1}). After 24 h, blood samples were collected via cheek bleeds, subsequently mice were sacrificed by CO_2 asphyxiation and organs were collected. Blood samples were allowed to coagulate for 20 min at RT and were subsequently centrifuged at 3,000 x g for 20 min at 4 ^{o}C to retrieve serum. Serum liver enzyme concentrations were measured with a Beckman Olympus AU400Serum Chemistry Analyzer by Charles River Laboratories (Wilmington, MA).

Statistical analysis.

Statistical analysis of the results was performed by a two-tailed un-paired Student’s t-test, assuming equal variances to compare two replicate means, or One-Way ANOVA followed

by Bonferroni post-hoc analysis to compare multiple replicate means. Differences were considered significant when $p<0.05$

Detail description of the materials, instrumentation as well as synthetic and experimental procedures can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

P.S. Kowalski and U. Capasso Palmiero contributed equally to this work. U. Capasso Palmiero acknowledges financial support through a Roberto Rocca Doctoral fellowship. P.S. Kowalski acknowledges funding from the Juvenile Diabetes Research Foundation (JDRF) postdoctoral fellowship Grant 3-PDF-2017–383-A-N. This work was supported in part by the Defense Advanced Research Projects Agency (Grant W32P4Q-13–1-0011) The authors also thank the Nanotechnology Materials and Flow cytometry Core Facilities at the Koch Institute at MIT and W.M. Keck Biological Imaging Facility at the Whitehead Institute.

References

[1]. Kamaly N, Yameen B, Wu J, Farokhzad OC, Chem Rev 2016, 116, 2602. [PubMed: 26854975]

[2]. Kaczmarek JC, Kowalski PS, Anderson DG, Genome Med 2017, 9, 60. [PubMed: 28655327]

[3]. Patel S, Ashwanikumar N, Robinson E, DuRoss A, Sun C, Murphy-Benenato KE, Mihai C, Almarsson O, Sahay G, Nano Lett 2017, 17, 5711. [PubMed: 28836442]

[4]. Stewart MP, Lorenz A, Dahlman J, Sahay G, Wiley Interdiscip Rev Nanomed Nanobiotechnol 2016, 8, 465. [PubMed: 26542891]

[5]. Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin RJ, Anderson DG, Nature Reviews Genetics 2014, 15, 541.

[6]. Dahlman JE, Barnes C, Khan OF, Thiriot A, Jhunjunwala S, Shaw TE, Xing Y, Sager HB, Sahay G, Speciner L, Bader A, Bogorad RL, Yin H, Racie T, Dong Y, Jiang S, Seedorf D, Dave A, Sandhu K. Singh, Webber MJ, Novobrantseva T, Ruda VM, Lytton-Jean AK, Levins CG, Kalish B, Mudge DK, Perez M, Abezgauz L, Dutta P, Smith L, Charisse K, Kieran MW, Fitzgerald K, Nahrendorf M, Danino D, Tuder RM, von Andrian UH, Akinc A, Panigrahy D, Schroeder A, Koteliansky V, Langer R, Anderson DG, Nat Nanotechnol 2014, 9, 648. [PubMed: 24813696]

[7]. Chahal JS, Khan OF, Cooper CL, McPartlan JS, Tsosie JK, Tilley LD, Sidik SM, Lourido S, Langer R, Bavari S, Ploegh HL, Anderson DG, Proc Natl Acad Sci U S A 2016.

[8]. Mahiny AJ, Dewerth A, Mays LE, Alkhaled M, Mothes B, Malaeksefat E, Loretz B, Rottenberger J, Brosch DM, Reautschnig P, Surapolchai P, Zeyer F, Schams A, Carevic M, Bakele M, Griese M, Schwab M, Nürnberg B, Beer-Hammer S, Handgretinger R, Hartl D, Lehr C-M, Kormann MSD, Nat Biotechnol 2015, 33, 584. [PubMed: 25985262]

[9]. Kaczmarek JC, Patel AK, Kauffman KJ, Fenton OS, Webber MJ, Heartlein MW, DeRosa F, Anderson DG, Angew Chem Int Ed Engl 2016, 55, 13808. [PubMed: 27690187]

[10]. Kauffman KJ, Webber MJ, Anderson DG, Journal of Controlled Release 2015, 240, 227. [PubMed: 26718856]

[11]. Pack DW, Hoffman AS, Pun S, Stayton PS, Nat Rev Drug Discov 2005, 4, 581. [PubMed: 16052241]

[12]. Lv H, Zhang S, Wang B, Cui S, Yan J, J Control Release 2006, 114, 100. [PubMed: 16831482]

[13]. Akhtar S, Benter I, Adv Drug Deliv Rev 2007, 59, 164. [PubMed: 17481774]

[14]. Green JJ, Langer R, Anderson DG, Acc Chem Res 2008, 41, 749. [PubMed: 18507402]

[15]. Molla MR, Levkin PA, Advanced Materials 2016, 28, 1159. [PubMed: 26608939]

[16]. Huayu Tian ZT, Zhuang Xiuli, Chen Xuesi, Jing Xiabin, Prog Polym Sci, 37, 237.

[17]. Jerome C, Lecomte P, Adv Drug Deliv Rev 2008, 60, 1056. [PubMed: 18403043]

[18]. Jing Haa SE, Siegwart Daniel J., Synlett 2016, 27, 2285.

[19]. Hao J, Kos P, Zhou K, Miller JB, Xue L, Yan Y, Xiong H, Elkassih S, Siegwart DJ, J Am Chem Soc 2015, 137, 9206. [PubMed: 26166403]

[20]. Blake TR, Waymouth RM, J Am Chem Soc 2014, 136, 9252. [PubMed: 24946200]

[21]. U.S. Food and Drug Administration, CFR - Code of Federal Regulations Title 21, www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=172. Updated 4 01 2017

[22]. Zhou J, Liu J, Cheng CJ, Patel TR, Weller CE, Piepmeier JM, Jiang Z, Saltzman WM, Nat Mater 2011, 11, 82. [PubMed: 22138789]

[23]. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM, Chem Rev 1999, 99, 3181. [PubMed: 11749514]

[24]. Eltoukhy AA, Siegwart DJ, Alabi CA, Rajan JS, Langer R, Anderson DG, Biomaterials 2012, 33, 3594. [PubMed: 22341939]

[25]. Whitehead KA, Dorkin JR, Vegas AJ, Chang PH, Veiseh O, Matthews J, Fenton OS, Zhang Y, Olejnik KT, Yesilyurt V, Chen D, Barros S, Klebanov B, Novobrantseva T, Langer R, Anderson DG, Nat Commun 2014, 5, 4277. [PubMed: 24969323]

[26]. Rehman Z, Zuhorn IS, Hoekstra D, J Control Release 2013, 166, 46. [PubMed: 23266451]

[27]. Ragelle H, Danhier F, Preat V, Langer R, Anderson DG, Expert Opin Drug Deliv 2017, 14, 851. [PubMed: 27730820]

[28]. Hoekstra D, Rejman J, Wasungu L, Shi F, Zuhorn I, Biochem Soc Trans 2007, 35, 68. [PubMed: 17233603]

[29]. Allen TM, Cullis PR, Adv Drug Deliv Rev 2013, 65, 36. [PubMed: 23036225]

[30]. Mui BL, Tam YK, Jayaraman M, Ansell SM, Du X, Tam YY, Lin PJ, Chen S, Narayanannair JK, Rajeev KG, Manoharan M, Akinc A, Maier MA, Cullis P, Madden TD, Hope MJ, Mol Ther Nucleic Acids 2013, 2, e139. [PubMed: 24345865]

[31]. Yan Y, Xiong H, Zhang X, Cheng Q, Siegwart DJ, Biomacromolecules 2017.

[32]. ur Rehman Z, Hoekstra D, Zuhorn IS, ACS Nano 2013, 7, 3767. [PubMed: 23597090]

[33]. Akinc A, Querbes W, De S, Qin J, Frank-Kamenetsky M, Jayaprakash KN, Jayaraman M, Rajeev KG, Cantley WL, Dorkin JR, Butler JS, Qin L, Racie T, Sprague A, Fava E, Zeigerer A, Hope MJ, Zerial M, Sah DW, Fitzgerald K, Tracy MA, Manoharan M, Koteliansky V, Fougerolles A, Maier MA, Mol Ther 2010, 18, 1357. [PubMed: 20461061]

[34]. Kranz LM, Diken M, Haas H, Kreiter S, Loquai C, Reuter KC, Meng M, Fritz D, Vascotto F, Hefesha H, Grunwitz C, Vormehr M, Hüsemann Y, Selmi A, Kuhn AN, Buck J, Derhovanessian E, Rae R, Attig S, Diekmann J, Jabulowsky RA, Heesch S, Hassel J, Langguth P, Grabbe S, Huber C, Türeci Ö, Sahin U, Nature 2016, 534, 396. [PubMed: 27281205]

[35]. Kauffman KJ, Oberli MA, Dorkin RJ, Hurtado JE, Kaczmarek JC, Bhadini S, Wyckoff J, Langer R, Jaklenec A, Anderson DG, Molecular Therapy - Nucleic Acids 2017.

[36]. Shen L, Tenzer S, Storck W, Hobernik D, Raker VK, Fischer K, Decker S, Dzionek A, Krauthäuser S, Diken M, Nikolae A, Maxeiner J, Schusterg P, Kappel C, Verschoor A, Schild H, Grabbe S, Bros M, Journal of Allergy and Clinical Immunology 2018.

[37]. Bertrand N, Grenier P, Mahmoudi M, Lima EM, Appel EA, Dormont F, Lim JM, Karnik R, Langer R, Farokhzad OC, Nat Commun 2017, 8, 777. [PubMed: 28974673]

[38]. Voigt J, Christensen J, Shastri VP, Proc Natl Acad Sci U S A 2014, 111, 2942. [PubMed: 24516167]

[39]. Kauffman KJ, Dorkin JR, Yang JH, Heartlein MW, DeRosa F, Mir FF, Fenton OS, Anderson DG, Nano Lett 2015, 15, 7300. [PubMed: 26469188]

[40]. Zou Y, Zheng M, Yang W, Meng F, Miyata K, Kim HJ, Kataoka K, Zhong Z, Adv Mater 2017, 29.

[41]. Taljaard M, Ward MR, Kutryk MJ, Courtman DW, Camack NJ, Goodman SG, Parker TG, Dick AJ, Galipeau J, Stewart DJ, Am Heart J 2010, 159, 354. [PubMed: 20211295]

[42]. Ohana P, Schachter P, Ayesh B, Mizrahi A, Birman T, Schneider T, Matouk I, Ayesh S, Kuppen PJ, de Groot N, Czerniak A, Hochberg A, J Gene Med 2005, 7, 366. [PubMed: 15521051]

[43]. Bragonzi A, Dina G, Villa A, Calori G, Biffi A, Bordignon C, Assael BM, Conese M, Gene Ther 2000, 7, 1753. [PubMed: 11083497]

[44]. Capasso Palmiero U, Kaczmarek JC, Fenton OS, Anderson DG, Adv Healthc Mater 2018, e1800249. [PubMed: 29761648]

[45]. Otto GP, Rathkolb B, Oestereicher MA, Lengger CJ, Moerth C, Micklich K, Fuchs H, Gailus-Durner V, Wolf E, Hrabe de Angelis M, J Am Assoc Lab Anim Sci 2016, 55, 375. [PubMed: 27423143]

[46]. Baratta JL, Ngo A, Lopez B, Kasabwalla N, Longmuir KJ, Robertson RT, Histochem Cell Biol 2009, 131, 713. [PubMed: 19255771]

[47]. Ramaswamy S, Tonnu N, Tachikawa K, Limphong P, Vega JB, Karmali PP, Chivukula P, Verma IM, Proceedings of the National Academy of Sciences 2017, 114.

[48]. DeRosa F, Guild B, Karve S, Smith L, Love K, Dorkin JR, Kauffman KJ, Zhang J, Yahalom B, Anderson DG, Heartlein MW, Gene Ther 2016, 23, 699. [PubMed: 27356951]

[49]. Thess A, Grund S, Mui BL, Hope MJ, Baumhof P, Fotin-Mleczek M, Schlake T, Molecular Therapy 2015, 23, 1456. [PubMed: 26050989]

[50]. Kormann MSD, Hasenpusch G, Aneja MK, Nica G, Flemmer AW, Herber-Jonat S, Huppmann M, Mays LE, Illenyi M, Schams A, Griese M, Bittmann I, Handgretinger R, Hartl D, Rosenecker J, Rudolph C, Nat Biotechnol 2011, 29, 154. [PubMed: 21217696]

[51]. Stadler CR, Bahr-Mahmud H, Celik L, Hebich B, Roth AS, Roth RP, Kariko K, Tureci O, Sahin U, Nat Med 2017, 23, 815. [PubMed: 28604701]

[52]. Pardi N, Secreto AJ, Shan X, Debonera F, Glover J, Yi Y, Muramatsu H, Ni H, Mui BL, Tam YK, Shaheen F, Collman RG, Karikó K, Danet-Desnoyers GA, Madden TD, Hope MJ, Weissman D, Nat Commun 2017, 8, 14630. [PubMed: 28251988]

[53]. Yin H, Song CQ, Suresh S, Wu Q, Walsh S, Rhym LH, Mintzer E, Bolukbasi MF, Zhu LJ, Kauffman K, Mou H, Oberholzer A, Ding J, Kwan SY, Bogorad RL, Zatsepin T, Koteliansky V, Wolfe SA, Xue W, Langer R, Anderson DG, Nat Biotechnol 2017, 35, 1179. [PubMed: 29131148]

[54]. Yin H, Song C-QQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Koteliansky V, Wolfe SA, Langer R, Xue W, Anderson DG, Nat Biotechnol 2016, 34, 328. [PubMed: 26829318]

[55]. Augustin HG, Koh GY, Science 2017, 357.

[56]. Molema G, in Endothelial Dysfunction and Inflammation, Vol. 2 (Eds: Dauphinee SM, Karsan A), Birkhäuser, Basel 2010, 15.

[57]. Kowalski PS, Leus NG, Scherphof GL, Ruiters MH, Kamps JA, Molema G, IUBMB Life 2011, 63, 648. [PubMed: 21766413]

[58]. Brenner JS, Bhamidipati K, Glassman PM, Ramakrishnan N, Jiang D, Paris AJ, Myerson JW, Pan DC, Shuvaev VV, Villa CH, Hood ED, Kiseleva R, Greineder CF, Radhakrishnan R, Muzykantov VR, Nanomedicine 2017, 13, 1495. [PubMed: 28065731]

[59]. Dickinson MG, Kowalski PS, Bartelds B, Borgdorff MA, van der Feen D, Sietsma H, Molema G, Kamps JA, Berger RM, Cardiovasc Res 2014, 103, 573. [PubMed: 25028387]

[60]. Budhiraja R, Tuder RM, Hassoun PM, Circulation 2004, 109, 159. [PubMed: 14734504]

[61]. Dudley AC, Cold Spring Harb Perspect Med 2012, 2, a006536. [PubMed: 22393533]

[62]. Khan OF, Zaia EW, Yin H, Bogorad RL, Pelet JM, Webber MJ, Zhuang I, Dahlman JE, Langer R, Anderson DG, Angew Chem Int Ed Engl 2014, 53, 14397. [PubMed: 25354018]

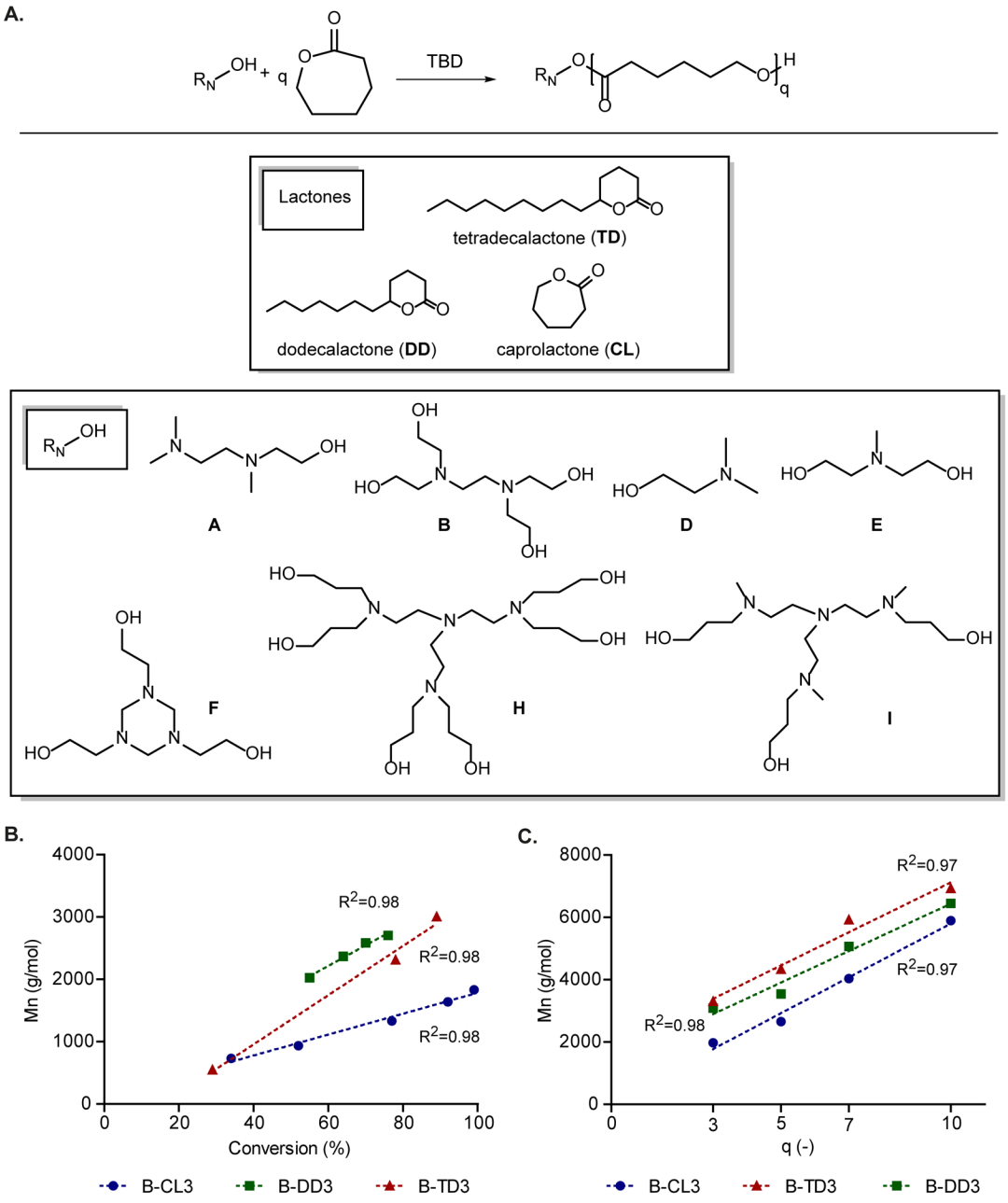

Figure 1.

Schematic synthesis of the combinatorial library of 37 amino-polyesters (A). Kinetics of the ring opening polymerization of lactones initiated by a tertiary amino-alcohol. (B) Plot of Mn of the polymer versus monomer conversion; (C) Plot of Mn of the polymer versus the degree of polymerization (q).

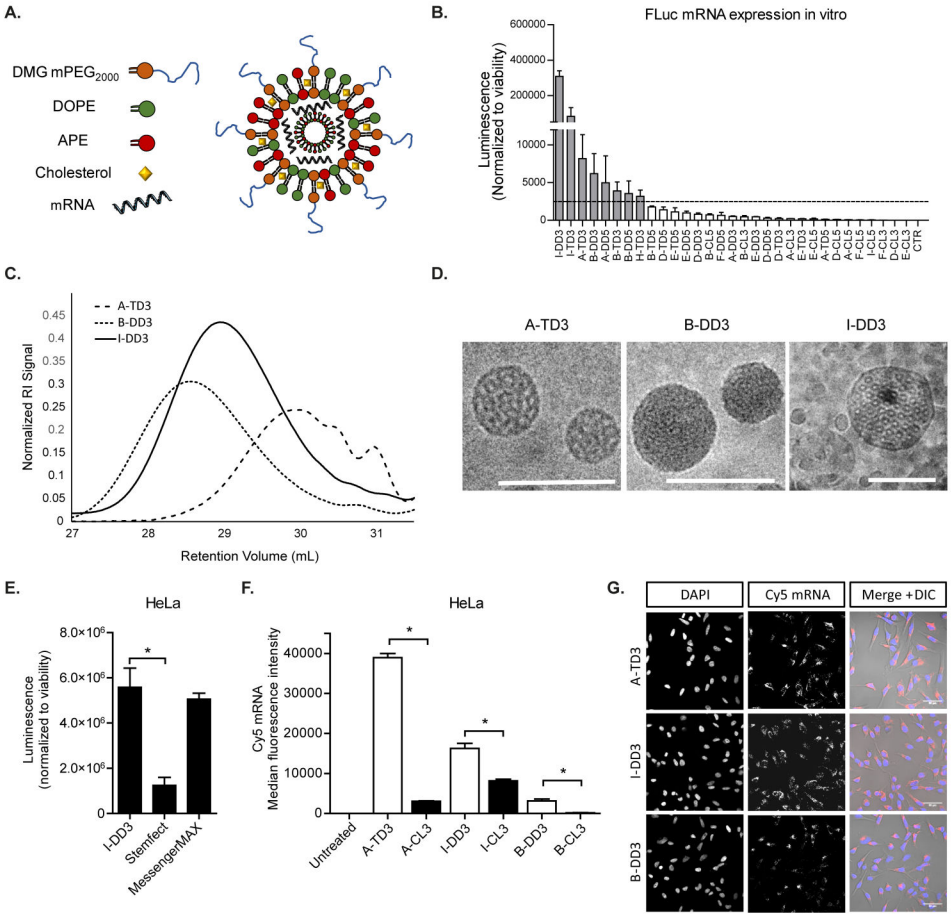

Figure 2.

In vitro evaluation of APEs. (A) Schematic representation of the APE-LNP. (B) In vitro library screening of APE nanoparticles containing FLuc mRNA in HeLa cells. Data are presented as mean relative luminescence ± SD; n = 4; Dotted line represent threshold for selection of the top performing candidates; (C) Representative GPC traces for the selected top performing APEs (I-DD3, A-TD3 and B-DD3); (D) Representative Cryo-TEM images of I-DD3, A-TD3 and B-DD3 nanoparticles. Scale bar represents 100 nm; (E) Comparison of the FLuc mRNA delivery efficacy between I-DD3 LNP and commercial transfection reagents. Cells were transfected for 24 h with 50 ng of FLuc mRNA. Data are presented as mean relative luminescence ± SD; n = 4; (E) Flow cytometry analysis of the uptake of APE LNPs containing Cy5 mRNA, 24 h after transfection. Data are presented as mean ± SD; n = 3; (F) Representative images of the uptake of APE LNPs into HeLa cells. Nuclei are stained with DAPI (Blue), Cy5 mRNA (Red), DIC, differential interference contrast. Scale bar represents 50 μm. * $p < 0.05$.

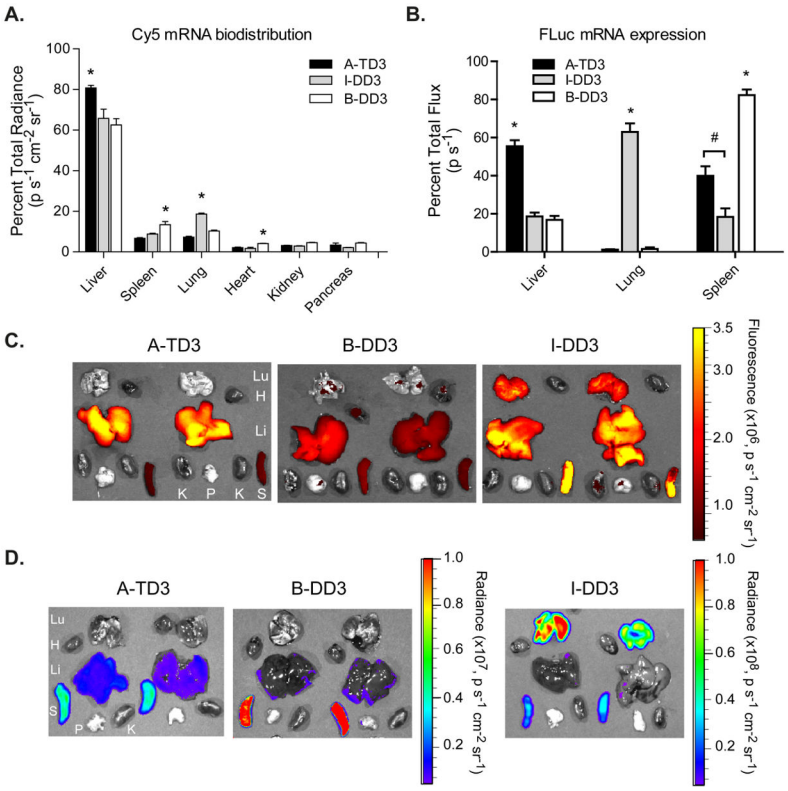

Figure 3.

In vivo evaluation of APEs. C57BL/6 mice were injected via tail vein with 0.6 mg kg^{-1} of APE-LNPs containing mRNA encoding Firefly luciferase (FLuc) or Cy5 labeled mRNA and imaged by IVIS after 6h. (A) Quantification of Cy5 mRNA tissue distribution. Data are presented as mean ± SD; n = 3; (B) Quantification of FLuc mRNA expression in selected tissues. Data are presented as mean ± SD; n = 3; (C) Representative images of tissue distribution of Cy5 mRNA, Lu - lungs, H - heart, Li - liver, S - spleen, K - kidney, P – pancreas; (D) Representative images of FLuc mRNA expression within the tissues. * $p < 0.05$.

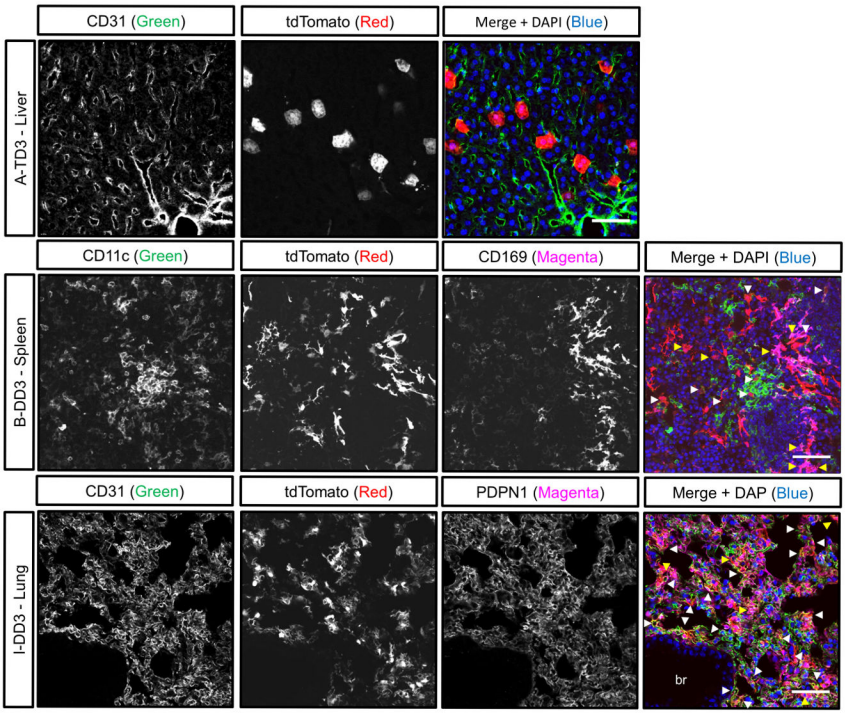

Figure 4.

Cellular selectivity analysis of APE-LNPs in Ai14/Cre mRNA mouse model. Ai14 mice carrying a Cre-activatable tdTomato knock-in reporter allele (LSL-Tom), were injected via tail vein with 0.6 mg kg^{-1} of APE-LNPs containing mRNA encoding for Cre recombinase. Immunofluorescence data set shows representative confocal microscopy images of liver, spleen and lung tissues stained for specific cell markers. CD31 – endothelial cell marker, CD169 – macrophage restricted adhesion molecule, CD11c – dendritic cell marker, PDPN1 – Type I alveolar epithelium marker, br - bronchi. Cell nuclei were stained with DAPI, cells transfected with Cre mRNA express TdTomato. Original magnification 25x, scale bar represent 50 μm. Arrowheads indicate the co-localization of green and red signals (white) or red and magenta signals (orange).

Table 1.

Characterization of the selected top performing APEs and their LNPs.

APE	Mn[a] (kDa)	Đ[a] (−)	Diameter[b] (nm)	PDI[b] (−)	ζ[b] (mV)	mRNA EE[c] (%)
A-TD3	1.39 ±0.02	1.23 ±0.01	65 ±3	0.140 ±0.03	8.9 ±3.4	97 ± 0.7
B-DD3	3.03 ±0.29	1.27 ±0.04	82 ±18	0.136 ±0.03	−2.1 ±3.3	93 ± 0.5
I-DD3	2.53 ±0.2	1.21 ±0.05	100 ±10	0.151 ±0.02	10.0 ±2.1	98 ± 0.2

Data are presented as mean ±SD from three independently synthesized bathes of each polymer (n=3)

[a]- characterized by Gel-permeation chromatography

[b]- characterized by dynamic light scattering

[c]- characterized by Quant-iT RiboGreen

EE - encapsulation efficacy, PDI – polydispersity index, ζ - zeta potential, Đ – dispersity, Mn – number average molecular weight