HHS Public Access

Author manuscript

J Am Chem Soc. Author manuscript; available in PMC 2022 December 22.

Published in final edited form as:

J Am Chem Soc. 2021 December 22; 143(50): 21321–21330. doi:10.1021/jacs.1c09822.

Zwitterionic Phospholipidation of Cationic Polymers Facilitates Systemic mRNA Delivery to Spleen and Lymph Nodes

Shuai Liu,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States;

Xu Wang,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Xueliang Yu,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Qiang Cheng,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States;

Lindsay T. Johnson,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States;

Sumanta Chatterjee,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Di Zhang,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Sang M. Lee,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Yehui Sun,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States;

Corresponding Author : Daniel J. Siegwart – Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States; Daniel.Siegwart@UTSouthwestern.edu.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c09822.

Experimental details, synthesis procedures, structural characterization of ZPPs, pK_a measurements, polyplex size measurements, *in vitro* and *in vivo* mRNA delivery, cell type determination by flow cytometry analysis, *in vivo* toxicity analysis, and supplemental references (PDF)

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.1c09822

The authors declare no competing financial interest.

Ting-Chih Lin,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

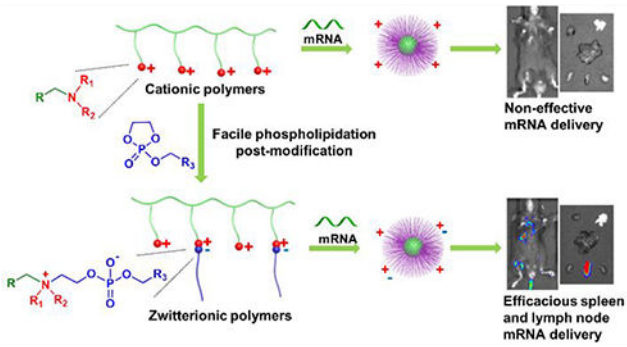
John L. Liu,
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Daniel J. Siegwart
Department of Biochemistry, Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States;

Abstract

Polymers represent a promising therapeutic platform for extrahepatic messenger RNA (mRNA) delivery but are hampered by low *in vivo* efficacy due to polyplex serum instability and inadequate endosomal escape following systemic administration. Here, we report the rational design and combinatorial synthesis of zwitterionic phospholipidated polymers (ZPPs) via cationic polymer postmodification by alkylated dioxaphospholane oxides to deliver mRNA to spleen and lymph nodes *in vivo*. This modular postmodification approach readily produces tunable zwitterionic species for serum resistance and introduces alkyl chains simultaneously to enhance endosomal escape, thereby transforming deficient cationic polymers to efficacious zwitterionic mRNA carriers without the need to elaborately synthesize functional monomers. ZPPs mediated up to 39 500-fold higher protein expression than their parent cationic counterparts *in vitro* and enabled efficacious mRNA delivery selectively in spleen and lymph nodes following intravenous administration *in vivo*. This zwitterionic phospholipidation methodology provides a versatile and generalizable postmodification strategy to introduce zwitterions into the side chains of cationic polymers, extending the utility of cationic polymer families for precise mRNA delivery and demonstrating substantial potential for immunotherapeutic applications.

Graphical Abstract



INTRODUCTION

Messenger RNA (mRNA)-based therapeutics have demonstrated utility for various applications, including vaccines against the SARS-CoV-2 virus, protein replacement therapy, cancer immunotherapy, and gene editing.[1–4] mRNA does not integrate into genomes, and

its transient effect in cytoplasm confers mRNA medicines low risk of mutagenesis, high safety, and versatile modularity to express a variety of different proteins of therapeutic interest.[5,6] Despite the great potential, mRNA medicines still face the substantial challenges of targeting specific organs/tissues via the bloodstream, highlighting the imperative demand for intelligent and targeted delivery systems.[7–10] Currently, most insights have been made through focusing on lipid nanoparticles (LNPs) to deliver mRNA,[11–14] where significant progress has been made for liver delivery and in intramuscularly injected vaccines. Less attention has been paid to polymers despite their attractive profiles of nonintegrating, scalable production, and large flexibility in chemical structure.[15–17] Additionally, polymers represent an alternative platform for delivery outside the liver due to their high tunability and adaptable functionality.[18,19] However, polymer mRNA polyplexes are, in general, less efficacious following systemic administration than LNPs, likely due to serum instability by nonspecific protein adsorption and inadequate endosomal escape.[20–22] Chemical pursuit of polymer architectures to access the physiochemical requirements to improve stability and endosomal escape is demanded and will enrich the diversity of mRNA delivery materials.

Although cationic polymers have been studied for decades as one of the major classes of nucleic acid delivery vehicles, many polymers with high *in vitro* activity fail to translate to *in vivo* applications and to the clinic as systemically administered medicines.[23,24] Cationic polymer/nucleic acids polyplexes are often formulated with an excess N:P ratio, leading to a net positive surface charge that can result in adsorption of negatively charged serum proteins that generate instability in the blood.[25–27] Additionally, endosomal escape remains challenging for lipid- and polymer-based nanoparticles, wherein FDA-approved LNPs likely mediate only 1–4% of encapsulated siRNA molecules into the cytoplasm.[28,29] Zwitterionic polymers offer a path forward toward some of these challenges due to the ability of zwitterions to interact with water molecules to form a sphere of hydration that can be useful for generating nonfouling surfaces, reducing serum protein adsorption, and increasing *in vivo* circulation time.[30–32] Previous efforts to incorporate zwitterionic functional groups into cationic polymers have largely relied on utilizing a limited set of zwitterionic monomers (e.g., 2-methacryloyloxyethyl phosphorylcholine (MPC) and 3-[[2-(methacryloyloxy)ethyl]-dimethylammonio]propionate (CBMA)) for direct polymerization.[33–36] This approach limits combinatorial chemical diversity, which is a requirement for discovering nucleic acid polyplexes that can overcome the numerous barriers to intracellular delivery *in vivo* including blood stability, tissue localization, cellular uptake, and endosomal escape.[37–39] Therefore, a versatile postpolymerization zwitterionic modification strategy of cationic polymers to simultaneously address challenges of blood instability and endosomal escape would be valuable to unlock further delivery utility of cationic polymers.

Herein, we report a zwitterionic phospholipidation function-alization strategy designed to transform cationic polymers into zwitterionic phospholipidated polymers (ZPPs) with improved serum stability and membrane fusion properties that together yield efficacious mRNA polyplexes capable of *in vivo* delivery to the spleen and lymph nodes. A library of 420 ZPPs were synthesized via fractional conjugation of different alkylated dioxaphospholane oxide molecules to 15 different cationic polymers. This simple postmodification approach yielded tunable zwitterionic, hydrophobic, and ionizable amino functionalities into polymer side chains for improvement of serum stability and endosomal

escape. This approach allowed us to further leverage our recent discovery that ionizable phospholipids (iPhos) LNPs utilize enhanced membrane fusion to increase endosomal escape.[40] In the current paper, we explore whether these lipid synthesis chemical reactions could be adapted to transform cationic polymers into modular ZPPs with improved properties. Following initial screening, ZPPs with zwitterions composed of one tertiary amine and one phosphate group mediated the highest mRNA expression. The best ZPP from the screen mediated up to 39 500-fold higher protein expression compared to its cationic precursor counterpart. Although some parent cationic polymers were effective for mRNA delivery to cells *in vitro*, all precursor cationic polymers were unable to deliver mRNA *in vivo*. Encouragingly, ZPPs derived from the PA6 cationic polymer precursor enabled ZPP mRNA accumulation in the spleen with high protein expression following mRNA translation. Better still, the top ZPP (PA6-4P14) mediated mRNA delivery not only to the spleen but also to lymph nodes following systemic administration, demonstrating substantial value for potential future use in immunotherapy applications. Because the modular zwitterionic phospholipidation strategy is applicable to a variety of cationic polymers, we envision that this method can be applied broadly to the field of polymer-based nucleic acid gene delivery for *in vivo* delivery of mRNA therapeutics.

RESULTS AND DISCUSSION

Rational Design and Synthesis of ZPPs.

Substantial efforts have been made to circumvent obstacles to *in vivo* gene delivery, such as nanoparticle instability in blood, low cellular uptake, and insufficient endosomal escape.[22,41,42] Although zwitterions have been reported to improve serum resistance,[33,35] broad pursuit of zwitterionic architectures to address delivery barriers has been hindered by limitations in chemical reactions to synthesize zwitterionic polymers and their inherently limited structural flexibility. The most common route to zwitterionic polymers has been direct monomer first polymerization via a set of zwitterionic monomers (e.g., MPC and CBMA).[33–36] To increase chemical diversity, we focused here on developing a facile postmodification method to partially introduce zwitterions into the side chains of precursor cationic polymers. Our recent work demonstrated that dioxaphospholane oxide molecules could react with primary, secondary, and tertiary amines to yield a variety of small molecule zwitterionic phospholipids.[40] Further inspired by this modular chemical reaction, we envisioned that alkylated dioxaphospholane oxides could also be applied to polymers, wherein ring-opening of side chain amines in cationic polymers would enable tunable degrees of zwitterionic, alkyl, and amino groups into the resulting polymers via a combinatorial approach. In this way, the chemical species and fraction of each group can be readily accessed through adjustment of dioxaphospholane oxide/amine molar ratio in the feed.

To explore this concept, we employed reversible addition–fragmentation chain transfer (RAFT) polymerization because this controlled radical polymerization (CRP) method produces polymers with well-defined molecular weight and molecular weight distribution. RAFT polymerization of glycidyl methacrylate (GMA) using 2-cyano-2-propyl benzodithioate (CPDB) as a chain transfer agent (CTA) yielded a polymer with an

epoxide side chain for further modification. We then synthesized 15 different cationic polymers (PAn, n = 1–15, around 8 repeating units) via ring-opening of the side chain epoxide by different amines (An) (Figures S1 and S2). Afterward, the combinatorial reaction of PAn with alkylated dioxaphospholane oxide molecules (Pm) was performed, yielding a combinatorial library of 420 ZPPs (termed PAn-xPm) (Figures 1, S3, and S4), where "x" represents the number of Pm molecules functionalized on each PAn molecule. Varying the starting amine types gave rise to different species of zwitterions: phosphate-quaternary amine, phosphate-tertiary amine, and phosphate-multiple amine zwitterions (Figure S5). Moreover, this modular, postmodification approach not only produced zwitterionic structures for serum resistance but also introduced alkyl chains simultaneously aiding cellular uptake and endosomal escape. Through adjustment of the amine types, alkyl lengths in amines and Pm molecules, structurally flexible ZPPs with controlled hydrophilic zwitterion species and hydrophobic alkyl chain lengths were obtained. We envision that this zwitterionic phospholipidation approach could, in principle, be extended to many other cationic polymer precursors to yield unique zwitterionic structures, potentially broadening the chemical diversity of zwitterionic polymers for nucleic acid delivery applications.

ZPPs with Flexible Zwitterion Structures Showed Efficient and Safe *in Vitro* mRNA Delivery.

Transfection competency is largely associated with gene vector chemical structures.[11,43] To study the structure and activity relationships (SAR), ZPPs were utilized to deliver firefly luciferase (Fluc) mRNA to an ovarian cancer cell line (IGROV1). For initial screening, P9 molecules were conjugated to PA1–PA15 cationic polymers to yield PAn-xP9, and 5 wt % PEG-lipid was added for increased stability and smaller sizes.[21,44] Consequently, PA6 and PA7 derived ZPPs showed efficient protein expression; better still, zwitterion introduction reduced the cytotoxicity of cationic polymers (Figure S6). Next, we examined an alternative helper additive, amphiphilic Pluronic F127,[20] into the polyplexes, which exhibited higher mRNA delivery efficacy compared to PEG-lipid (Figures S6 and S7). Therefore, surface stabilizing F127 was used in subsequent screening experiments.

To prepare a larger combinatorial ZPP library, Pm molecules with different alkyl lengths were further used to control the hydrophobic residues of zwitterionic polymers. From the heat map of *in vitro* screening, the zwitterion species of ZPPs greatly influenced the delivery activity, with phosphate-tertiary amine zwitterions (PA5-xPm to PA7-xPm) demonstrating higher efficacy compared to phosphate-quaternary amine (PA1-xPm to PA4-xPm) and phosphate-multiple amine zwitterions (PA8-xPm to PA15-xPm) (Figure 2A–C). In PA5-xPm to PA7-xPm ZPPs, the tertiary amines would not be protonated at physiological pH; however, these tertiary amines could be protonated in the acidic endosomal environment to give the pH-switchable zwitterion. PA5, PA6, and PA7 derived ZPPs contained ionizable amine groups that could exhibit proton sponge effects at acidic endosomal pH; moreover, hydrophobic side tails beside the amine groups and phospholipid moieties could aid endosome membrane fusion. These functional groups might function in combination to facilitate the endosomal escape, therefore mediating enhanced mRNA delivery efficacy. Among the efficient ZPPs (PA5-xPm to PA7-xPm), tail length at the amine side was critical and the hit rates (RLU > 10 000) reached 89% for 4–6 carbon lengths (Figure

2D). In one aspect, zwitterionic phospholipidation transformed the noneffective PA5 and PA6 cationic polymers to efficacious mRNA vehicles, verifying the aforementioned theory about optimized zwitterions overcoming obstacles faced during gene delivery. Top ZPP vector mediated up to 39 500-fold higher mRNA delivery efficacy compared to its cationic counterpart (Figure S7). In another aspect, zwitterion introduction reduced the toxic positive charges that resulted in cytotoxicity of cationic polymers (Figures 2E and S8), without affecting the mRNA encapsulation efficiency (Figure 2F). It was worth noting that excess zwitterions introduced into the cationic polymers could decrease the delivery efficacy, demonstrating the significance of balancing zwitterionic and cationic groups. We further characterized key physicochemical properties of ZPPs, including pK_a and critical micelle concentration (CMC) (Figure S9 and Table S1). Zwitterion incorporation could decrease the pK_a of cationic polymers from ~8.0 to ~6.5, which is known to benefit *in vivo* mRNA delivery.[10,45] These observations highlight the advantages of zwitterionic phospholipidation modification of cationic polymers in safety and efficacy, which might be beneficial to overcoming delivery barriers and applicable for *in vivo* applications.

ZPPs Outperformed Parent Cationic Polymers in Serum Resistance, Cellular Uptake, and Endosomal Escape.

To date, substantial gene delivery systems have been explored; however, the chemical pursuit of nonviral polymer vectors have mostly rested on cationic architectures with limited serum stability and endosomal membrane disruption.[20–22] To examine the potential of *in vivo* delivery, serum resistance of ZPP mRNA polyplexes was first evaluated. Positive surface charges of nanoparticles can potentially adsorb negatively charged serum proteins to induce instability and rapid clearance by the reticuloendothelial system (RES).[25,46] As expected, zwitterion introduction resulted in positive to negative surface transition of nanoparticles, which showed stability in the presence of fetal bovine serum (FBS) (Figures 3A and S10). Consequently, ZPPs PA6-4P9 and PA6-4P14 encapsulated mRNA to formulate negatively charged polyplexes and mediated high transfection efficacy even at a high FBS concentration of 30% (Figure 3B). This robust efficacy could be attributed to not only serum stability but also high cellular uptake and endosomal escape of polyplexes.

Although electrostatic interaction between polyplexes and cell membranes was decreased, phospholipidated materials were hypothesized to compensate by increasing membrane fusion with biological membranes to benefit polyplex cellular uptake and endosomal escape. To examine this hypothesis, a fluorescence resonance energy transfer (FRET) assay was utilized to determine the membrane fusion and rupture ability of ZPPs.[34,47] A pair of FRET probes, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2–1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-PE), were incorporated into the same endosomal mimicking liposomes, resulting in increased rhodamine fluorescence intensity because of FRET to rhodamine. Once membranes fused by ZPPs, attenuated rhodamine signal would be observed due to the larger FRET pair distance. Figure 3C showed that phospholipidation significantly improved the membrane fusion and endosomal rupture of ZPPs compared to unmodified cationic counterparts. Following this, confocal fluorescence images confirmed that PA6-4P14 mRNA polyplexes exhibited higher cellular uptake than the cationic PA6 parent counterpart (Figure

3D). Additionally, PA6-4P14 mRNA polyplexes mediated efficacious mRNA escape from endosomes. These results demonstrate the advantages of phospholipidation modification in enhancing the serum stability, cellular uptake, and endosomal escape of nanoparticles.

In Vivo SAR Study of ZPPs for mRNA Delivery to Spleen.

The spleen consists of a variety of immune cell populations, making splenic delivery appropriate for immunotherapy applications.[18,48] Since most mRNA carriers are only efficient in liver hepatocytes after intravenous (iv) administration,[13,14,49] development of spleen delivery materials is an important goal receiving increasing attention. As one aspect of this process, we recently showed that incorporation of negatively charged lipids into LNPs aids spleen tropism.[50] Moreover, compared to short RNAs (e.g., siRNAs and miRNAs), longer mRNAs likely require weaker electrostatic binding by delivery materials to facilitate mRNA release and enhance delivery efficacy.[40,51] Incorporating neutral monomers (e.g., zwitterionic and hydrophobic monomers) for polymerization could also reduce the electrostatic interaction toward RNA.[33,52] In this study, we employed a facile postmodification strategy to introduce zwitterions into cationic polymers, which might satisfy the aforementioned advantages. The chemical zwitterion structure of ZPPs formed by negative phosphate introduction may possess inherent advantages in transfecting splenic cells.

To examine this rationale, PA6-4P9 was first used for mRNA delivery *in vivo*, with the help of two surface stabilizing polymers F127 and PEG-lipid (Figures S11 and S12). PA6-4P9 with 2.5 wt % F127 showed the highest mRNA delivery efficacy, which was thus used for the following studies. Afterward, 26 cationic PAn and zwitterionic ZPPs were selected based on *in vitro* results and further evaluated for Fluc mRNA delivery *in vivo* after iv injection (mRNA, 0.1 mg/kg) (Figure 4A). Although efficient *in vitro*, PA7 did not mediate mRNA expression *in vivo* at all. In contrast, zwitterionic ZPPs showed significantly higher efficacy compared to their cationic counterparts (Figure 4A,B). From further study of SAR, alkyl length of amines affected mRNA expression: PA6 (4 carbon length) derived ZPPs outperformed PA5 (3 carbon length) and PA7 (6 carbon length) derivatives, demonstrating the importance of hydrophobicity and hydrophilicity balance for *in vivo* mRNA delivery (Figure 4C). It is worth noting that PA6 was not efficacious even *in vitro*, but PA6 derived ZPPs mediated efficient protein expression both *in vitro* and *in vivo* (Figure 4D). These results showed the significant role phospholipidation modification played in endowing and enhancing polymer vectors potency for mRNA delivery.

We next selected the optimized PA6-4P14 to determine the correlation between nanoparticle accumulation and protein expression. PA6-4P14 mRNA polyplexes biodistributed to several organs (spleen, liver, and lungs) with the highest accumulation in the spleen (Figure 4E,F and Figure S13A). This result was consistent with prior studies related to size and composition for spleen uptake (Figures S14 and S15).[44,53] Additionally, negative phosphate group introduction into ZPPs decreased the nanoparticle surface charge and pK_a compared to cationic polymer based polyplexes, which was also beneficial to *in vivo* efficacy and spleen tropism (Figures 3A and S16). Consistently, protein expression was observed selectively in spleen, showing orders of magnitude higher efficacy than other organs

(Figures 4G, S13B, and S17). PA6-4P14 mRNA polyplexes exhibited a dose dependent delivery, where protein expression increased with the increasing mRNA dose from 0.1 mg/kg to 0.25 mg/kg (Figures 4H–J and S18). The polyplexes here mediated robust Fluc expression in spleen (roughly 10^7 photons s^{-1} cm^{-2} sr^{-1}) at a low mRNA dose of 0.1 mg/kg. These results demonstrate that zwitterionic phospholipidation methodology holds great potential for transforming deficient cationic polymers to efficacious mRNA carriers for spleen delivery *in vivo*.

ZPPs Enabled Lymph Nodes Delivery after Systemic Administration.

Beyond the spleen, lymph nodes are another important target for cancer immunotherapy and vaccine development.[11,54] Most approaches have focused on lymph node drainage following local intramuscular injection.[55] It remains challenging to achieve lymph node delivery following systemic iv administration. Encouragingly, ZPPs with specific zwitterion structures and numbers showed potential of lymph node transfection. We evaluated the mRNA delivery capability of ZPPs to lymph nodes by recording the whole body images of mice after Fluc mRNA delivery (0.1 mg/kg) and luciferin intraperitoneal injection (Figure 5A). Balancing the amphoteric and amphiphilic functionalities, rationally designed PA6-4Pm with four zwitterions and four long alkyl tails delivered mRNA to produce protein in many distant areas of lymph nodes. This targeting capability seemed restricted to a narrow area of chemical structures (Figure S19). Following up through examination of *ex vivo* organs, PA6-4Pm efficiently mediated protein expression in spleen and lymph nodes (Figure 5B,C and Figure S20). The optimal PA6-4P14 efficiently induced mRNA delivery to diverse lymph nodes, including iliac, axillary, and mesenteric lymph nodes (Figure 5D,E). To study cell type transfection, we utilized an activatable Cre-LoxP mouse model (Ai14) that contains the LoxP flanked stop cassette, preventing the expression of tdTomato protein. tdTomato will be expressed only in successfully transfected cells via translating Cre-recombinase (Cre) protein to delete the stop cassette.[40,50] PA6-4P14 delivered Cre mRNA successfully to lymph nodes, with nearly 6% of all $CD4^+$ T cells transfected. In addition, mRNA was delivered to $CD8^+$ T cells and macrophages (Figures 5F, S21, and S22). Moreover, PA6-4P14 delivered Cre mRNA to spleen cells, including $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages. *In vivo* toxicity was not observed after PA6-4P14 mRNA polyplexes administration (Figures S23 and S24). These results highlight the potential of ZPPs for future immunotherapeutic applications.

CONCLUSIONS

A combinatorial library of ZPPs via zwitterionic phospholipidation of cationic polymers was developed for mRNA delivery. This facile phospholipidation postmodification method provides a versatile and practical approach to transform deficient cationic polymers to efficacious zwitterionic polymers as mRNA carriers. This strategy introduced tunable zwitterions with controllable species and numbers for serum resistance and incorporated hydrophobic alkyl chains simultaneously to benefit endosomal escape. As a result, ZPPs outperformed parent cationic polymers in key attributes such as nanoparticle serum stability, cellular uptake, and endosomal escape, without sacrificing mRNA encapsulation capability. ZPPs mediated mRNA delivery for high protein expression in high serum concentrations

in vitro and *in vivo*. Encouragingly, ZPPs led to efficacious mRNA delivery selectively in the spleen and lymph nodes following iv administration, demonstrating significant potential for future use in cancer immunotherapy and vaccine development. We envision that the generalizable zwitterionic phospholipidation methodology described here can be expanded to a wealth of cationic polymers with primary, secondary, and tertiary amine groups in the future to open new avenues for polymer-based mRNA therapeutic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

D.J.S. acknowledges financial support from the National Institutes of Health (NIH), National Institute of Biomedical Imaging and Bioengineering (NIBIB) (Grant R01 EB02519201A1), the Cystic Fibrosis Foundation (CFF) (Grant SIEGWA18XX0), the Cancer Prevention and Research Institute of Texas (CPRIT) (Grant RP190251), the American Cancer Society (ACS) (Grant RSG-17-012-01), and the Welch Foundation (Grant I-1855). S.M.L. acknowledges support from NIH (Grant T32GM127216). The authors acknowledge the UTSW Small Animal Imaging Shared Resource, the Moody Foundation Flow Cytometry Facility, and the UTSW Tissue Resource, which are supported in part by the National Institutes of Health, National Cancer Institute Support Grant P30 CA142543.

REFERENCES

(1). Hajj KA; Whitehead KA Tools for translation: non-viral materials for therapeutic mRNA delivery. Nat. Rev. Mater 2017, 2, 17056.

(2). Pardi N; Hogan MJ; Porter FW; Weissman D mRNA vaccines - a new era in vaccinology. Nat. Rev. Drug Discovery 2018, 17, 261–279. [PubMed: 29326426]

(3). Kong N; Tao W; Ling X; Wang J; Xiao Y; Shi S; Ji X; Shajii A; Gan ST; Kim NY; Duda DG; Xie T; Farokhzad OC; Shi J Synthetic mRNA nanoparticle-mediated restoration of p53 tumor suppressor sensitizes p53-deficient cancers to mTOR inhibition. Sci. Transl. Med 2019, 11, No. eaaw1565. [PubMed: 31852795]

(4). Mulligan MJ; Lyke KE; Kitchin N; Absalon J; Gurtman A; Lockhart S; Neuzil K; Raabe V; Bailey R; Swanson KA; Li P; Koury K; Kalina W; Cooper D; Fontes-Garfias C; Shi P-Y; Türeci Ö; Tompkins KR; Walsh EE; Frenck R; Falsey AR; Dormitzer PR; Gruber WC;Şahin U; Jansen KU Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. Nature 2020, 586, 589–593. [PubMed: 32785213]

(5). Sahin U; Kariko K; Tureci O mRNA-based therapeutics developing a new class of drugs. Nat. Rev. Drug Discovery 2014, 13, 759–780. [PubMed: 25233993]

(6). Kaczmarek JC; Kowalski PS; Anderson DG Advances in the delivery of RNA therapeutics: from concept to clinical reality. Genome Med 2017, 9, 60. [PubMed: 28655327]

(7). Servick K mRNA's next challenge: Will it work as a drug? Science 2020, 370, 1388–1389. [PubMed: 33335042]

(8). Kowalski PS; Rudra A; Miao L; Anderson DG Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery. Mol. Ther 2019, 27, 710–728. [PubMed: 30846391]

(9). Wei T; Cheng Q; Farbiak L; Anderson DG; Langer R; Siegwart DJ Delivery of Tissue-Targeted Scalpels: Opportunities and Challenges for In Vivo CRISPR/Cas-Based Genome Editing. ACS Nano 2020, 14, 9243–9262. [PubMed: 32697075]

(10). Zhang D; Atochina-Vasserman EN; Maurya DS; Huang N; Xiao Q; Ona N; Liu M; Shahnawaz H; Ni H; Kim K; Billingsley MM; Pochan DJ; Mitchell MJ; Weissman D; Percec V One-Component Multifunctional Sequence-Defined Ionizable Amphiphilic Janus Dendrimer Delivery Systems for mRNA. J. Am. Chem. Soc 2021, 143, 12315–12327. [PubMed: 34324336]

(11). Miao L; Li L; Huang Y; Delcassian D; Chahal J; Han J; Shi Y; Sadtler K; Gao W; Lin J; Doloff JC; Langer R; Anderson DG Delivery of mRNA vaccines with heterocyclic lipids increases

anti-tumor efficacy by STING-mediated immune cell activation. Nat. Biotechnol 2019, 37, 1174–1185. [PubMed: 31570898]

(12). Hou X; Zhang X; Zhao W; Zeng C; Deng B; McComb DW; Du S; Zhang C; Li W; Dong Y Vitamin lipid nanoparticles enable adoptive macrophage transfer for the treatment of multidrugresistant bacterial sepsis. Nat. Nanotechnol 2020, 15, 41–46. [PubMed: 31907443]

(13). Lee SM; Cheng Q; Yu X; Liu S; Johnson LT; Siegwart DJ A Systematic Study of Unsaturation in Lipid Nanoparticles Leads to Improved mRNA Transfection In Vivo. Angew. Chem., Int. Ed 2021, 60, 5848–5853.

(14). Yu X; Liu S; Cheng Q; Wei T; Lee S; Zhang D; Siegwart DJ Lipid-Modified Aminoglycosides for mRNA Delivery to the Liver. Adv. Healthcare Mater 2020, 9, 1901487.

(15). McKinlay CJ; Vargas JR; Blake TR; Hardy JW; Kanada M; Contag CH; Wender PA; Waymouth RM Charge altering releasable transporters (CARTs) for the delivery and release of mRNA in living animals. Proc. Natl. Acad. Sci. U. S. A 2017, 114, E448–E456. [PubMed: 28069945]

(16). Patel AK; Kaczmarek JC; Bose S; Kauffman KJ; Mir F; Heartlein MW; DeRosa F; Langer R; Anderson DG Inhaled Nanoformulated mRNA Polyplexes for Protein Production in Lung Epithelium. Adv. Mater 2019, 31, 1805116.

(17). Kowalski PS; Capasso Palmiero U; Huang Y; Rudra A; Langer R; Anderson DG Ionizable amino-polyesters synthesized via ring opening polymerization of tertiary amino-alcohols for tissue selective mRNA delivery. Adv. Mater 2018, 30, 1801151.

(18). McKinlay CJ; Benner NL; Haabeth OA; Waymouth RM; Wender PA Enhanced mRNA delivery into lymphocytes enabled by lipid-varied libraries of charge-altering releasable transporters. Proc. Natl. Acad. Sci. U. S. A 2018, 115, 5859–5866.

(19). Abd Elwakil MM; Gao TL; Isono T; Sato Y; Elewa YHA; Satoh T; Harashima H Engineered epsilon-decalactone lipomers bypass the liver to selectively in vivo deliver mRNA to the lungs without targeting ligands. Mater. Horiz 2021, 8, 2251–2259. [PubMed: 34846429]

(20). Yan Y; Xiong H; Zhang X; Cheng Q; Siegwart DJ Systemic mRNA Delivery to the Lungs by Functional Polyester-based Carriers. Biomacromolecules 2017, 18, 4307–4315. [PubMed: 29141136]

(21). Kaczmarek JC; Patel AK; Kauffman KJ; Fenton OS; Webber MJ; Heartlein MW; DeRosa F; Anderson DG Polymer–lipid nanoparticles for systemic delivery of mRNA to the lungs. Angew. Chem 2016, 128, 14012–14016.

(22). Jiang Y; Lu Q; Wang Y; Xu E; Ho A; Singh P; Wang Y; Jiang Z; Yang F; Tietjen GT; Cresswell P; Saltzman WM Quantitating Endosomal Escape of a Library of Polymers for mRNA Delivery. Nano Lett 2020, 20, 1117–1123. [PubMed: 32003222]

(23). Cheng Y; Yumul RC; Pun SH Virus-inspired polymer for efficient in vitro and in vivo gene delivery. Angew. Chem., Int. Ed 2016, 55, 12013–12017.

(24). Liu S; Gao Y; Zhou D; Zeng M; Alshehri F; Newland B; Lyu J; O'Keeffe-Ahern J; Greiser U; Guo T; Zhang F; Wang W Highly branched poly(β-amino ester) delivery of minicircle DNA for transfection of neurodegenerative disease related cells. Nat. Commun 2019, 10, 3307. [PubMed: 31341171]

(25). Zhou J; Liu J; Cheng CJ; Patel TR; Weller CE; Piepmeier JM; Jiang Z; Saltzman WM Biodegradable poly(amine-co-ester) terpolymers for targeted gene delivery. Nat. Mater 2012, 11, 82–90.

(26). Mastrobattista E; Hennink WE Polymers for gene delivery: Charged for success. Nat. Mater 2012, 11, 10–12.

(27). Zhou D; Cutlar L; Gao Y; Wang W; O'Keeffe-Ahern J; McMahon S; Duarte B; Larcher F; Rodriguez BJ; Greiser U; Wang W The transition from linear to highly branched poly(betaamino ester)s: Branching matters for gene delivery. Sci. Adv 2016, 2, No. e1600102. [PubMed: 27386572]

(28). Wittrup A; Ai A; Liu X; Hamar P; Trifonova R; Charisse K; Manoharan M; Kirchhausen T; Lieberman J Visualizing lipid formulated siRNA release from endosomes and target gene knockdown. Nat. Biotechnol 2015, 33, 870–876. [PubMed: 26192320]

(29). Gilleron J; Querbes W; Zeigerer A; Borodovsky A; Marsico G; Schubert U; Manygoats K; Seifert S; Andree C; Stöter M; Epstein-Barash H; Zhang L; Koteliansky V; Fitzgerald K;

Fava E; Bickle M; Kalaidzidis Y; Akinc A; Maier M; Zerial M Image-based analysis of lipid nanoparticle–mediated siRNA delivery, intracellular trafficking and endosomal escape. Nat. Biotechnol 2013, 31, 638–646. [PubMed: 23792630]

(30). Jiang SY; Cao ZQ Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications. Adv. Mater 2010, 22, 920–932. [PubMed: 20217815]

(31). Ladd J; Zhang Z; Chen S; Hower J; Jiang S Zwitterionic polymers exhibiting high resistance to nonspecific protein adsorption from human serum and plasma. Biomacromolecules 2008, 9, 1357–1361. [PubMed: 18376858]

(32). Zhang L; Cao Z; Bai T; Carr L; Ella-Menye JR; Irvin C; Ratner BD; Jiang S Zwitterionic hydrogels implanted in mice resist the foreign-body reaction. Nat. Biotechnol 2013, 31, 553–556. [PubMed: 23666011]

(33). Jackson MA; Werfel TA; Curvino EJ; Yu F; Kavanaugh TE; Sarett SM; Dockery MD; Kilchrist KV; Jackson AN; Giorgio TD; Duvall CL Zwitterionic nanocarrier surface chemistry improves siRNA tumor delivery and silencing activity relative to polyethylene glycol. ACS Nano 2017, 11, 5680–5696. [PubMed: 28548843]

(34). Li Y; Cheng Q; Jiang Q; Huang Y; Liu H; Zhao Y; Cao W; Ma G; Dai F; Liang X; Liang Z; Zhang X Enhanced endosomal/lysosomal escape by distearoyl phosphoethanolamine polycarboxybetaine lipid for systemic delivery of siRNA. J. Controlled Release 2014, 176, 104–114.

(35). Wen Y; Zhang Z; Li J Highly Efficient Multifunctional Supramolecular Gene Carrier System Self-Assembled from Redox Sensitive and Zwitterionic Polymer Blocks. Adv. Funct. Mater 2014, 24, 3874–3884.

(36). Peng H; Ji W; Zhao R; Lu Z; Yang J; Li Y; Zhang X Ph sensitive zwitterionic polycarboxybetaine as a potential non-viral vector for small interfering RNA delivery. RSC Adv 2020, 10, 45059–45066. [PubMed: 35516239]

(37). Siegwart DJ; Whitehead KA; Nuhn L; Sahay G; Cheng H; Jiang S; Ma M; Lytton-Jean A; Vegas A; Fenton P; Levins CG; Love KT; Lee H; Cortez C; Collins SP; Li YF; Jang J; Querbes W; Zurenko C; Novobrantseva T; Langer R; Anderson DG Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery. Proc. Natl. Acad. Sci. U. S. A 2011, 108, 12996–13001. [PubMed: 21784981]

(38). Green J; Langer R; Anderson D A combinatorial polymer library approach yields insight into nonviral gene delivery. Acc. Chem. Res 2008, 41, 749–759. [PubMed: 18507402]

(39). Akinc A; Zumbuehl A; Goldberg M; Leshchiner ES; Busini V; Hossain N; Bacallado SA; Nguyen DN; Fuller J; Alvarez R; Borodovsky A; Borland T;Constien R; de Fougerolles A; Dorkin JR; Narayanannair Jayaprakash K; Jayaraman M; John M; Koteliansky V; Manoharan M; Nechev L; Qin J; Racie T; Raitcheva D; Rajeev KG; Sah DW; Soutschek J; Toudjarska I; Vornlocher HP; Zimmermann TS; Langer R; Anderson DG A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. Nat. Biotechnol 2008, 26, 561–569. [PubMed: 18438401]

(40). Liu S; Cheng Q; Wei T; Yu X; Johnson LT; Farbiak L; Siegwart DJ Membrane-destabilizing ionizable phospholipids for organ-selective mRNA delivery and CRISPR–Cas gene editing. Nat. Mater 2021, 20, 701–710. [PubMed: 33542471]

(41). Liu S; Zhou D; Yang J; Zhou H; Chen J; Guo T Bioreducible zinc(II)-coordinative polyethylenimine with low molecular weight for robust gene delivery of primary and stem cells. J. Am. Chem. Soc 2017, 139, 5102–5109. [PubMed: 28322564]

(42). Wang H; Wang Y; Wang Y; Hu J; Li T; Liu H; Zhang Q; Cheng Y Self-Assembled Fluorodendrimers Combine the Features of Lipid and Polymeric Vectors in Gene Delivery. Angew. Chem., Int. Ed 2015, 54, 11647–11651.

(43). Xiong H; Liu S; Wei T; Cheng Q; Siegwart DJ Theranostic dendrimer-based lipid nanoparticles containing PEGylated BODIPY dyes for tumor imaging and systemic mRNA delivery in vivo. J. Controlled Release 2020, 325, 198–205.

(44). Kaczmarek JC; Kauffman KJ; Fenton OS; Sadtler K; Patel AK; Heartlein MW; DeRosa F; Anderson DG Optimization of a Degradable Polymer–Lipid Nanoparticle for Potent Systemic

Delivery of mRNA to the Lung Endothelium and Immune Cells. Nano Lett 2018, 18, 6449–6454. [PubMed: 30211557]

(45). Zhang D; Atochina-Vasserman EN; Maurya DS; Liu M; Xiao Q; Lu J; Lauri G; Ona N; Reagan EK; Ni H; Weissman D; Percec V Targeted Delivery of mRNA with One-Component Ionizable Amphiphilic Janus Dendrimers. J. Am. Chem. Soc 2021, 143, 17975–17982. [PubMed: 34672554]

(46). Guo S; Huang L Nanoparticles Escaping RES and Endosome: Challenges for siRNA Delivery for Cancer Therapy. J. Nanomater 2011, 2011, 742895.

(47). Zhang Y; Arrington L; Boardman D; Davis J; Xu Y; DiFelice K; Stirdivant S; Wang W; Budzik B; Bawiec J; et al. The development of an in vitro assay to screen lipid based nanoparticles for siRNA delivery. J. Controlled Release 2014, 174, 7–14.

(48). Fenton OS; Kauffman KJ; Kaczmarek JC; McClellan RL; Jhunjhunwala S; Tibbitt MW; Zeng MD; Appel EA; Dorkin JR; Mir FF; et al. Synthesis and biological evaluation of ionizable lipid materials for the in vivo delivery of messenger RNA to B lymphocytes. Adv. Mater 2017, 29, 1606944.

(49). Miao L; Lin J; Huang Y; Li L; Delcassian D; Ge Y; Shi Y; Anderson DG Synergistic lipid compositions for albumin receptor mediated delivery of mRNA to the liver. Nat. Commun 2020, 11, 2424. [PubMed: 32415122]

(50). Cheng Q; Wei T; Farbiak L; Johnson LT; Dilliard SA; Siegwart DJ Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR–Cas gene editing. Nat. Nanotechnol 2020, 15, 313–320. [PubMed: 32251383]

(51). Cheng Q; Wei T; Jia Y; Farbiak L; Zhou K; Zhang S; Wei Y; Zhu H; Siegwart DJ Dendrimer-based lipid nanoparticles deliver therapeutic FAH mRNA to normalize liver function and extend survival in a mouse model of Hepatorenal Tyrosinemia Type I. Adv. Mater 2018, 30, 1805308.

(52). Werfel TA; Jackson MA; Kavanaugh TE; Kirkbride KC; Miteva M; Giorgio TD; Duvall C Combinatorial optimization of PEG architecture and hydrophobic content improves ternary siRNA polyplex stability, pharmacokinetics, and potency in vivo. J. Controlled Release 2017, 255, 12–26.

(53). Blanco E; Shen H; Ferrari M Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nat. Biotechnol. 2015, 33, 941–951. [PubMed: 26348965]

(54). Liang F; Lindgren G; Sandgren KJ; Thompson EA; Francica JR; Seubert A; De Gregorio E; Barnett S; O'Hagan DT; Sullivan NJ; Koup RA; Seder RA; Loré K Vaccine priming is restricted to draining lymph nodes and controlled by adjuvant mediated antigen uptake. Sci. Transl. Med 2017, 9, No. eaal2094. [PubMed: 28592561]

(55). Reddy ST; Swartz MA; Hubbell JA Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol 2006, 27, 573–579. [PubMed: 17049307]

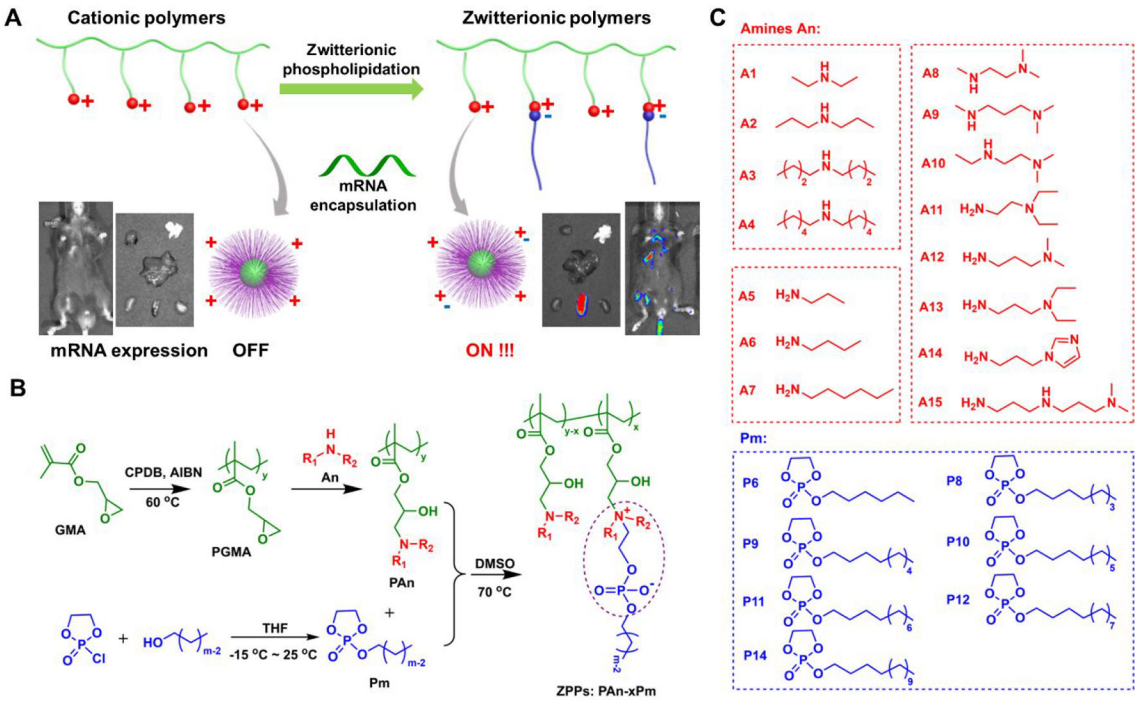


Figure 1.

A combinatorial library of ZPPs was synthesized by phospholipidation of cationic polymers for *in vivo* mRNA delivery. (A) Phospholipidation transformed cationic polymers to efficient zwitterionic mRNA carriers, which enabled protein expression in the spleen and lymph nodes. (B) The synthetic route toward zwitterionic polymers PAn-xPm is shown. Cationic polymers PAn and alkylated dioxaphospholane oxide molecules Pm were prepared separately, then reacted to yield PAn-xPm. “x” in “PAn-xPm” is defined as the number of Pm molecules functionalized on each cationic polymer. (C) 15 amines and 7 Pm molecules were used for combinatorial PAn-xPm synthesis.

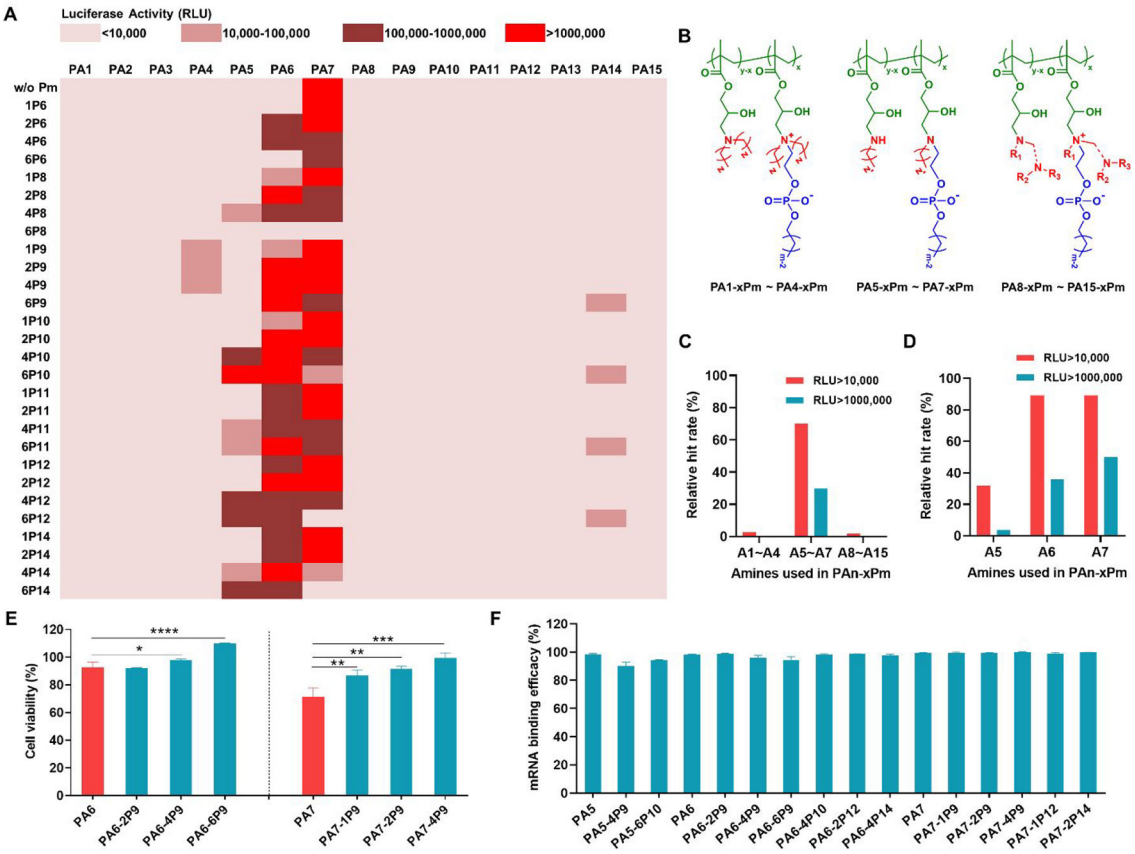


Figure 2.

Structure–activity relationships emerged for PAn-xPm polymers that deliver mRNA *in vitro*. (A) Heat map of Fluc mRNA expression mediated by PAn-xPm in IGROV1 cells, plotted in relative light units (RLU) ($n = 3$). (B) Chemical structures of PAn-xPm polymers derived from different species of amines at physiological pH. (C) Relative hit rate of PAn-xPm with different kinds of zwitterions. (D) Relative hit rate of Pan-xPm with the zwitterion composed of one tertiary amine and one phosphate group. The alkyl chain length of starting amines affected mRNA delivery efficacy. (E) Cell viability of PAn and representative PAn-xPm mRNA polyplexes (mean ± sd, $n = 3$). Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test: ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (F) mRNA binding efficiency of PAn and representative PAn-xPm mRNA polyplexes (mean ± sd, $n = 3$).

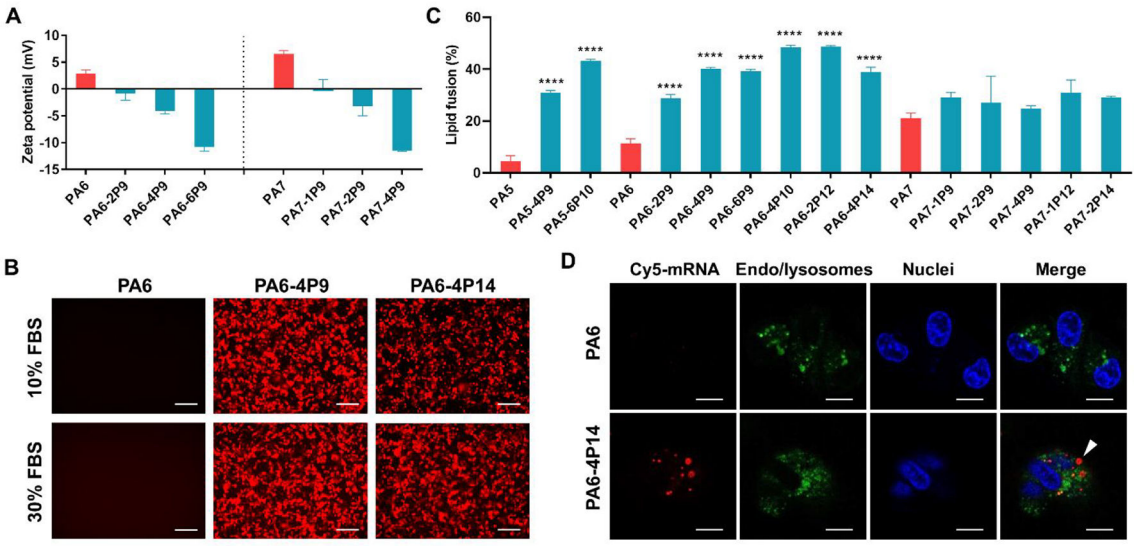


Figure 3.

Zwitterionic phospholipidation modification endowed cationic polymers with increased serum resistance and endosomal escape ability. (A) ζ potential of PAn and PAn-xPm mRNA polyplexes (mean ± sd, $n = 3$). The polymer/mRNA weight ratio was fixed at 20/1 (w/w). (B) PA6, PA6-4P9, and PA6-4P14 delivered mCherry mRNA to IGROV1 cells at different FBS concentrations (mRNA, 50 ng/well). Scale bar: 200 μm. (C) Lipid fusion and endosomal escape of polyplexes were determined by a FRET assay at pH 5.5 (mean ± sd, $n = 3$). Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test compared to respective unmodified PAn groups: ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (D) Endosomal escape and cellular uptake fluorescence images of IGROV1 cells treated with PA6 and PA6-4P14 Cy5-mRNA polyplexes for 4 h. Cy5-mRNA (red), endo/lysosomes (green), and nuclei (blue) were shown in the images. The white arrow indicated mRNA escape from endo/lysosomes. Scale bar: 10 μm.

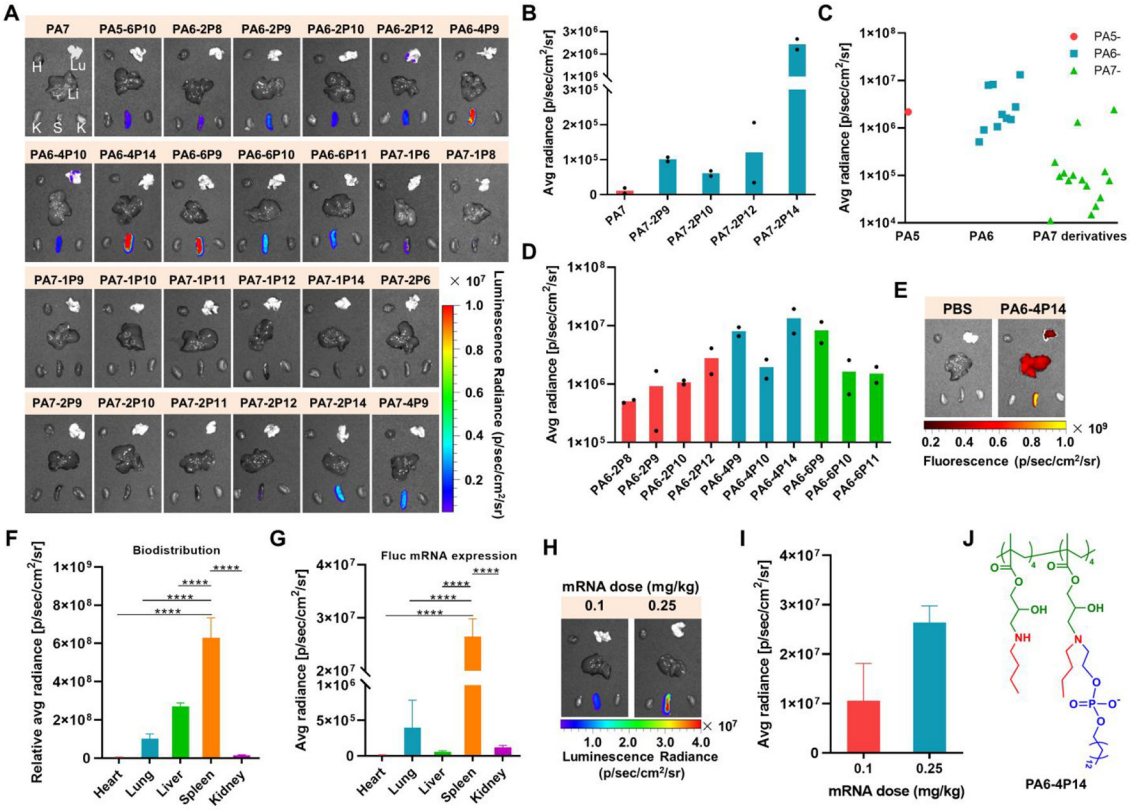


Figure 4.

Chemical structure alteration of ZPPs controlled mRNA delivery to the spleen *in vivo*. (A) *In vivo* structure–activity studies of mRNA delivery by PAn and PAn-xPm polymers (Fluc mRNA, 0.1 mg/kg, iv, 6 h, $n = 2$ for initial screening): H, heart; Lu, lung; Li, liver; K, kidney; S, spleen. (B) ZPPs outperformed their cationic counterparts in spleen mRNA translation to Fluc expression. (C) Average (avg) radiance quantification of PAn-xPm mRNA polyplexes. (D) Balance of amine and zwitterionic groups mediated higher mRNA delivery. Four Pm molecules modified on each PA6 polymer showed better performance compared to two and six Pm molecule functionalization. (E) Biodistribution images of selected PA6-4P14 Cy5-mRNA polyplexes (Cy5-mRNA, 0.25 mg/kg). (F) Biodistribution quantification of PA6-4P14 Cy5-mRNA polyplexes (Cy5-mRNA, 0.25 mg/kg). Relative avg radiance was calculated by deduction of PBS control group. The polyplexes accumulated the most in the spleen. (G) Quantification of PA6-4P14 mediated Fluc mRNA expression *in vivo*. The majority of protein expression occurred in spleen. Data in parts F and G are presented as the mean ± sd ($n = 3$), and statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. (H) PA6-4P14 mRNA polyplexes exhibited dose dependent mRNA delivery efficacy. (I) PA6-4P14 delivered mRNA at different doses *in vivo* (mean ± sd, $n = 3$). (J) Chemical structure of top ZPP polymer PA6-4P14 at physiological pH.

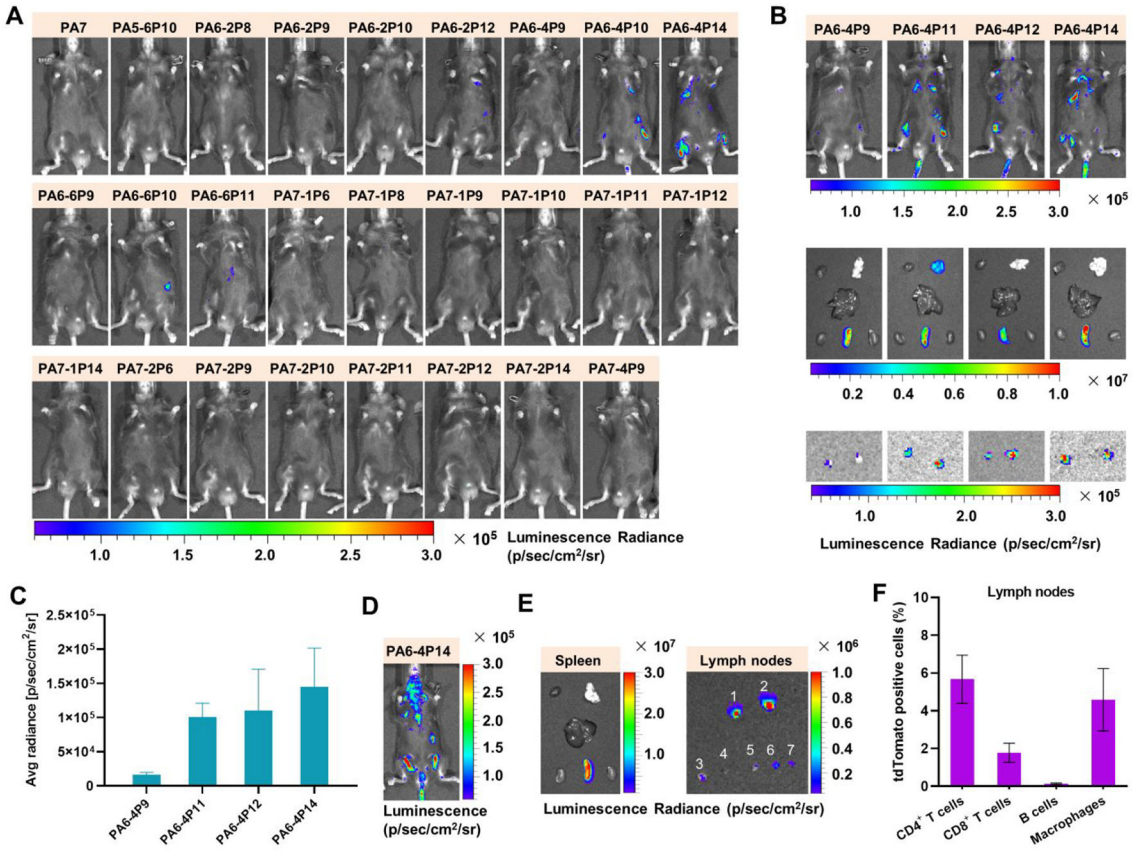


Figure 5.

Optimal ZPPs exhibited potential for lymph node transfection following systemic administration. (A) *In vivo* mRNA delivery screening by PAn-xPm mRNA polyplexes (Fluc mRNA, 0.1 mg/kg). All body images were recorded 6 h after iv injection of C57BL/6 mice. (B) PA6-4Pm polymers demonstrated superior mRNA expression in lymph nodes and spleen (Fluc mRNA, 0.1 mg/kg). Iliac lymph nodes were imaged here. (C) Quantification of Fluc protein expression by PA6-4Pm mRNA polyplexes in lymph nodes. (D) Whole body images of lymph node transfection following PA6-4P14 mRNA polyplex delivery at the dose of 0.25 mg/kg. (E) *Ex vivo* organ images of protein expression following PA6-4P14 mRNA polyplex delivery (Fluc mRNA, 0.25 mg/kg). mRNA expression occurred specifically in the spleen and in lymph nodes: (1, 2) iliac lymph nodes; (3, 4) axillary lymph nodes; (5–7) mesenteric lymph nodes. (F) Flow cytometry analysis of lymph node cells 48 h after iv injection: tdTomato+ $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages were quantified (mean ± sd, $n = 3$).