



New variants of poly(beta amino esters) to enhance delivery of nucleic acid medicines

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Doctor of Philosophy

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“You will face many defeats in life, but never let
yourself be defeated!”

– *Maya Angelou*

Table of Contents

Table of Contents.....	iii
General Abstract.....	vii
Declaration.....	x
Acknowledgements.....	xi
Statement of COVID impact.....	xiii
Publications	xiv
Credit authorship contribution statement.....	xv
List of Figures	xix
List of Abbreviations.....	xxvi
Chapter 1.....	1
1.1 Introduction	4
1.2 PBAE Synthesis	11
Table 1. Some selected examples of PBAEs in various applications.....	15
1.3 Delivery of nucleic acids.....	19
1.3.1 DNA	21
1.3.2 Messenger RNA.....	27
1.3.3 Self-amplifying mRNA	33
1.3.4 Therapeutic oligonucleotides.....	36
1.4 Conclusions and Research Challenges.....	38
1.5 Aims and Objectives.....	40
1.6 References.....	45
Chapter 2.....	53
2.1 Introduction	55
2.2 Materials and Methods	58
2.2.1 Materials	58
2.2.2 Instrumentation and Analysis	58
2.2.3 Polymer Synthesis and Characterisation	60

2.2.4	Synthesis of pDNA	61
2.2.5	Preparation of Polyplexes.....	62
2.2.6	Encapsulation Efficiency Using Gel Electrophoresis.....	62
2.2.7	In Vitro Experiments	63
2.2.8	In Vivo Experiments	66
2.2.9	Statistical analysis	69
2.3	Results and Discussion	69
2.3.1	Synthesis and Characterisation of PBAEs.....	69
2.3.2	Effect of PEG Density on Polyplex Physicochemical Properties	72
2.3.3	In Vitro Evaluation of pDNA Delivery	74
2.3.4	In Vivo Delivery of a SARS-CoV-2 Bivalent pDNA Vaccine	80
2.4	Conclusions.....	86
2.5	Acknowledgements	87
2.6	Author Contributions.....	88
2.7	References.....	89
2.8	Supporting Information	95
Chapter 3		97
3.1	Introduction	100
3.2	Materials and Methods	103
3.2.1	Materials	103
3.2.2	Instrumentation and Analysis	104
3.2.3	Polymer Synthesis and Characterisation	105
3.2.4	Synthesis of Fluorescently Labelled Polymers.....	108
3.2.5	Polymer Buffering Capacity Assay	108
3.2.6	Synthesis of saRNA	109
3.2.7	Preparation of Polyplexes.....	109
3.2.8	Encapsulation Efficiency Determination Using Ribogreen Assay.....	110
3.2.9	In vitro experiments	110
3.2.10	Statistical analysis	114

3.3	Results and discussion.....	115
3.3.1	Synthesis and Characterisation of PBAEs.....	115
3.3.2	Effect of pSAR Density on Polyplex Physicochemical Properties	118
3.3.3	In Vitro Evaluation of saRNA Delivery	120
3.3.4	Assessment of pSarylated polyplexes in colorectal cancer organoids 125	
3.4	Conclusions.....	126
3.5	Acknowledgements	128
3.6	Author Contributions.....	128
3.7	References.....	130
3.8	Supporting Information	135
Chapter 4		138
4.1	Introduction	140
4.2	Materials and Methods	144
4.2.1	Materials	144
4.2.2	Instrumentation and Analysis	145
4.2.3	Polymer Synthesis and Characterisation	146
4.2.4	Synthesis of Fluorescently Labelled Polymers.....	148
4.2.5	Polymer Buffering Capacity Assay	148
4.2.6	Synthesis of saRNA	149
4.2.7	Preparation of Polyplexes.....	149
4.2.8	Encapsulation Efficiency Using Ribogreen Assay.....	150
4.2.9	Determination of Drug Loading.....	150
4.2.10	Determination of Drug Loading Using Ultraviolet–visible	151
4.2.11	In vitro experiments.....	151
4.2.12	Statistical analysis.....	154
4.3	Results and discussion.....	155
4.3.1	Synthesis and Characterisation of PBAEs.....	155
4.3.2	Polyplex Physicochemical Properties of different end-capped polyplexes 157	

4.3.3	In Vitro Evaluation of saRNA Delivery	160
4.4	Conclusion	169
4.5	Acknowledgements	169
4.6	Author Contributions.....	170
4.7	References.....	171
4.8	Supporting Information	175
Chapter 5		179
5.1	Conclusions and future prospects	179

General Abstract

Nucleic acid technology is now acknowledged as a versatile and potent medical platform enabling transient protein expression for vaccines against infectious diseases and therapeutics targeting genetic disorders and cancer. The successful safety and efficacy demonstration of Moderna's SpikeVax and BioNTech-Pfizer's Comirnaty mRNA-based COVID-19 vaccines has greatly accelerated the field, with numerous nucleic acid vaccines and therapeutics now in clinical trials. While mRNA vaccines gained popularity during the COVID-19 crisis, the inherent stability of DNA in comparison to mRNA suggests that DNA technologies might find broader use, especially in low to middle income countries facing limitations in cold-chain storage accessibility.

A crucial aspect of effective nucleic acid medicine is a safe and efficient delivery system to safeguard the genetic sequence from endogenous nucleases and facilitate translocation into target cell cytosol for expression. Various nonviral delivery strategies, including lipid nanoparticles (e.g., liposomes, cubosomes) and polyelectrolyte complexes (polyplexes), have been explored for nucleic acid delivery. Polyplexes, self-assemblies of positively charged polymers condensing negatively charged nucleic acids into small nanoparticles (50-200 nm), are promising for nucleic acid delivery. Poly(beta-amino esters) (PBAEs), a class of polycations, are extensively investigated as nucleic acid delivery vectors due to their biodegradability via hydrolysis, allowing enhanced nucleic acid release. However, the polycationic content and the hydrophobic side-chains of some PBAEs' can induce cytotoxicity through membrane disruption, as well as compromise their colloidal stability and alter their potential immunogenicity profile limiting their clinical utility. Therefore, in this thesis, we

aimed to investigate new PBAE-based polymers through end-modification approaches, such as the conjugation of hydrophilic polymers or a ligand, to enhance their physicochemical properties. These modifications are intended to increase stability or enable targeted delivery, making them more effective as potential gene carriers.

Previous studies have demonstrated that coupling PBAEs with poly(ethylene glycol) (PEG) reduces surface cationic charges when complexed with nucleic acids, potentially mitigating toxicity and decreasing interactions with serum proteins. This approach has been shown to prolong systemic nanoparticle circulation time by shielding their surface from aggregation and phagocytosis, consequently increasing in-serum in vitro and in vivo transfection efficacy. Therefore, we focused on optimizing PBAE properties, particularly surface PEG density, for effective pDNA encapsulation and gene transfection in vitro. Then, selected formulations were further evaluated in vivo to investigate their potential as a SARS-CoV-2 pDNA vaccine and revealed that one formulation led to strong antigen specific T-cell responses.

In addition, PBAEs were further investigated via end-capping with polysarcosine (pSar) as an alternative to PEGylation, and as potential RNA carriers. Then, the efficacy of newly synthesised formulations was explored using human-derived colon colorectal organoids. The selected formulations displayed high transfection ability within the core of organoids indicating high penetration of these nanoparticles. As a result, these findings indicate that pSarylated PBAEs have the potential to serve as a promising component material for enhancing delivery of nucleic acid therapeutics.

Further optimization of PBAEs as enhanced gene delivery vehicles was achieved by conjugating a Toll-like receptor 7 (TLR7) agonist (loxoribine) to enhance the robustness of immune response. Synthesised polymers were formulated using RNA and investigated in vitro using different types of cell lines. TLR activation assays showed that loxoribine-conjugated formulations can efficiently agonise TLR7 with some synergy observed between loxoribine and the delivered RNA. Consequently, these findings suggest the potential of adjuvanted polyplexes for enhanced mRNA vaccines.

Overall, the studies in this thesis revealed that the choice of end-capping agent significantly impacts PBAE-based polymer properties and effectiveness in nucleic acid delivery. The findings emphasized the importance of surface properties in polymer delivery systems, showing potential for vaccination and tumor delivery.

Declaration

Unless otherwise acknowledged, the work presented in this thesis is my own. No part has been submitted for any other degree at The University of Nottingham or any other institution. This work was supported by Turkish Ministry of Education.

This doctoral dissertation was conducted under the supervision of:

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Statement of COVID impact

During my PhD journey, I faced unprecedented challenges due to the COVID-19 pandemic. Starting my doctoral studies during this time meant dealing with restrictions, especially when it came to accessing laboratory facilities and resources. These limitations made academic research much tougher to progress as usual.

Despite the disruptions caused by the pandemic, I didn't give up on my academic goals. Instead, I adapted to the situation by exploring alternative research methods, collaborating through virtual platforms, and making the most of remote resources. Although not having direct access to the lab initially slowed me down, I found creative ways to work around these obstacles with determination. The journey was definitely tough, filled with uncertainty and adaptation. But through sheer dedication and perseverance, I managed to successfully complete my studies.

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Publications

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List of Figures

Figure 1.1 Activation of CD8+ and CD4+ T cells by antigen-presenting dendritic cells triggers distinct immune responses. Dendritic cells expressing antigens on MHC class I molecules activate CD8+ T cells, leading to their differentiation into cytotoxic T lymphocytes (CTLs) through interleukin signalling. CTLs, in turn, target and kill virally infected, cancerous, or intracellular bacteria-harboring cells. Meanwhile, dendritic cells presenting antigens on MHC class II molecules activate CD4+ T cells, resulting in the formation of TH1 and TH2 cells through distinct interleukin pathways. TH1 cells produce interferon-gamma, further enhancing CTL activation, while both TH1 and TH2 cells stimulate B cells to produce antibodies, augmenting the immune response against targeted cells.....	10
Figure 1.2: Synthesis of PBAEs (a) linear (b) branched polymer synthesis using different types of amines and diacrylates.....	19
Figure 1.3. Schematic diagram illustrating the diverse cargos carried by PBAE polyplexes (DNA, mRNA, saRNA, siRNA, small molecule drug) and their intracellular fates: DNA polyplexes deliver DNA to the nucleus, where it is transcribed into mRNA by RNA polymerase and then translated by ribosomes to produce the target protein; mRNA polyplexes release mRNA into the cytoplasm for direct translation by ribosomes; saRNA polyplexes utilize a self-amplifying replicon to generate multiple mRNA copies, leading to increased protein production; siRNA polyplexes induce RNA interference through its complexation with RNA-induced silencing complex (RISC), silencing specific genes and reducing target protein production; small molecule drug polyplexes target specific proteins/receptors to elicit pharmacological effects.	21
Figure 1.4: (A) Polymer synthesis scheme and (B) associated monomers. Diacrylate-D and amine-A monomers were copolymerized to create acrylate-terminated base polymers (i) which were then amine-capped using excess ethylenediamine (E, ii) and reacted with allyl- α -D-mannopyranoside (ADM) to create mannose-capped polymers (iii). Respective polymers are named by their monomers and mannose content. For example, reacting diacrylate monomer D4 with amine monomer A4 results in the synthesis of polymer 'D4A4.' 'D4A4-Man' represents the mannosylated variant. (C) Fluorescent microscopy of top-performing polymers. Images depict EGFP-positive RAW264.7 cells resulting from transfection using naked pDNA (1), JET-PEI (2), D4A4 (3), and D4A4-Man (4). Reprinted with permission from. Jones, C. H. <i>et al.</i> (2015) 'Mannosylated poly (beta-amino esters) for targeted antigen presenting cell immune modulation', <i>Biomaterials</i>	24

Figure 1.5: (A) Mannose receptor-targeted mRNA nanoparticles efficiently transfect M2 macrophages. Design of macrophage-targeted polymeric NPs formulated with mRNAs encoding key regulators of macrophage polarization. The particles consist of a PbAE-mRNA polyplex core coated with a layer of PGA-Di-mannose, which targets the particles to mannose receptors (CD206) expressed by M2-like macrophages. Also depicted is the synthetic mRNA encapsulated in the NP, which is engineered to encode the reprogramming transcription factors. (B) Transmission electron microscopy of a population of NPs (scale bar 200 nm) and a single NP (inset, scale bar 50 nm). (C) Size distributions measured using a NanoSight NS300 instrument. (D-E) T cells contribute to anti-tumor effects achieved with macrophage-programming nanoparticles. Nanoparticle-mediated macrophage programming increases T cell recruitment into tumor lesions. Shown are representative confocal images of peritoneal metastases of ID8 ovarian cancer cells in the mesentery. Tissues were collected after 6 biweekly i.p. injections of PBS or IRF5/IKK β NPs (50 μ g mRNA/dose), and were stained for the indicated lymphocyte- and myeloid-markers. Tu = Tumor, Mes = Mesentery. Scale bar: 100 μ m. Reprinted with permission from. Zhang, F *et al.* (2019) 'Genetic programming of macrophages to perform anti-tumor functions using targeted mRNA nanocarriers', *Nature communications*. 30

Figure 1.6: (A) A library of 196 PBAEs were synthesised combinatorially from 28 base acrylate terminated polymers and seven end-cap monomers. Polymers were screened in 384-well plates in C2C12 cells for transfection at a dose of 1 ng of eGFP SAM per well at two w/w ratios. Each cell of the heatmap shows mean of two wells of a 384-well plate. Transfection of myoblasts using eGFP SAM was strongly improved compared to 5mou eGFP mRNA used at the same dose. (B) Structures of lead polymers for intramuscular administration and representative IVIS images with the top two nanoparticle formulations compared to naked SAM. Reprinted with permission from. Wilson, D. R.; *et al.* (2018) 'Biodegradable STING agonist nanoparticles for enhanced cancer immunotherapy. *Nanomedicine: Nanotechnology, Biology and Medicine*..... 35

Figure 1.7: Overview of undertaken work. PBAE-based polymer synthesis and end modifications. Polyplex formation utilising pDNA or saRNA. In vitro analysis of synthesised complexes. Application of these formulations..... 42

Figure 2.1. Schematic representation of PBAE synthesis, polyplex assembly, in vitro analysis and in vivo antigen response of a bivalent SARS-Cov-2 pDNA vaccine..... 57

Figure 2.2. Synthesis and characterisation of PBAEs. (A) Synthetic scheme for step-growth Michael-addition polymerisation of BDD, 5-AP and mPEG-SH to produce PEG-PBAE-PEG triblock copolymer. First, acylate-terminated base polymer was synthesised by polymerisation of monomer BDD and 5-AP. In the second step, base

polymer was further terminated with 0.8 kDa mPEG-SH. (B) Molecular weights of PBAEs determined using GPC and (C) the molecular weight distribution of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG. (D) Buffering capacity of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG compared to polyethyleneimine (PEI) and negative control of a 0.1 M NaCl_(aq) solution. (E) ¹H NMR spectra of pBDD-5APm OH-(pBDD-5AP)-OH showing disappearance of the acrylate peaks at 6-7 ppm indicating complete capping with either 5-amino-1-butanol for OH-(pBDD-5AP)-OH or PEG-SH for PEG-(pBDD-5AP)-PEG (deuterated DMSO was used). 71

Figure 2.3. Formulation of PBAEs with pDNA to produce polyplex nanoparticles. (A) Schematic representation of pDNA formulation with OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG or a 1:1 w/w mixture of OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG termed 'mix' in pH 5.0 25 mM sodium acetate at a final pDNA concentration of 10 µg/mL. (B) Particle size (bars) and PDI (floating points) and (C) Zeta potential for the OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG and 'mix' polyplexes at a PBAE/pDNA w/w ratio of 32, 64 and 128 determined by dynamic light scattering at 25°C. Bars represent the mean ± SD for 3 independent formulations. (D) Representative transmission electron micrograph of PEG-(pBDD-5AP)-PEG polyplexes and (E) measured particle size distribution from five separate TEM images. (F) Encapsulation efficiency of pDNA within polyplexes evaluated using agarose gel retardation assay. 74

Figure 2.4. Metabolic activity and transfection efficiency of PBAE derived pDNA polyplexes. Effect of PBAE polyplexes formulated with firefly luciferase encoding pDNA at PBAE/pDNA w/w ratios of 32, 64 and 128 on metabolic activity (A and B) and transfection efficiency (C and D) on HEK293T cells (A and C) and DC2.4 cells (B and D). Cells were treated with polyplexes containing pDNA at a concentration of 500 ng/mL (100 ng/well) in serum-free Opti-MEM. Metabolic activity was compared against Triton X (positive control) and untreated cells (negative control) and calculated by normalizing metabolic activity to untreated cells. In vitro transfection efficiency was analysed after 24 h post-transfection using the Promega ONE-GLO luciferase assay and compared against Lipofectamine 2000 (positive control) and naked pDNA (negative control) expressed as relative light units (RLU) for luciferase expression. Bars represent the mean ± SD (n=3). (E) Representative live cell confocal microscopy images of HEK293T cells 4 h post-transfection with an eGFP encoding pDNA formulated with OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG or a 1:1 w/w mixture of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG termed 'mix' at a PBAE/pDNA w/w ratio of 64. Cells were stained with Hoechst 33342 CellMask™ Plasma Membrane Stain Deep Red to visualise the nucleus and cell membrane respectively. Images are

displayed as individual channels and a merged signal. Scale bar corresponds to 20 μm (x40). Note: the contrast of the images has been enhanced for clarity, original images can be found in the supporting information (Figure S3.2). 77

Figure 2.5. Cellular uptake of PBAE derived pDNA polyplexes. (A-C) Imaging cytometry (A) histograms of fluorescence intensity and internalisation for DC2.4 cells after 30 mins, 1 h, 2 h and 4 h following treatment with FITC-labelled PEG-(pBDD-5AP)-PEG polyplexes at a PBAE/pDNA w/w ratio of 64 at 500 ng/mL pDNA. Untreated fluorescence intensity histogram can be found in the supporting information (Figure S2.3) Data were analysed using IDEAS software. (B and C) Representative images for imaging cytometry for untreated and treated group after 4 h. (D) Representative merged live cell confocal microscopy images of DC2.4 cells treated pDNA polyplexes derived from FITC-labelled PEG-(pBDD-5AP)-PEG at a PBAE/pDNA w/w ratio of 64 after 1 h and 4 h at 100 ng/well (500 ng/mL) pDNA. Scale bar 10 μm (40x). Note: the contrast of the images has been enhanced for clarity, original images can be found in the supporting information (Figure S2.4)..... 79

Figure 2.6. In vivo transfection, immunisation performance and tolerability following intramuscular administration of PBAE polyplexes containing a SARS-CoV-2 bivalent pDNA vaccine. (A) Schematic of the dosing schedule. (B) Antigen-specific T cell responses were quantified by IFN- γ ELISpot with splenocytes isolated from vaccinated mice 21 days following intramuscular administered 5 μg pDNA bivalent SARS-CoV-2 vaccine (encoding for both spike (S1) and nucleocapsid (N) proteins) complexed with OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG or a 1:1 w/w mixture of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG termed 'mix' at a PBAE/pDNA w/w ratio of 64. These were compared against a positive control of gold particle associated gene gun delivered vaccine. Doses were administered in a prime-boost regime, with 3 identical injections spaced 7 days apart. (C) Representative images of IFN- γ spot-forming cells (D) Animal body weight data over the time course of the experiment. (E) Serum N and (F) S1 antibody response 21 d after vaccination as determined by an ELISA assay. (G) Histopathological analysis of liver and kidney to investigate tissue damage for the non-treated, positive control and 'mix' polyplexes. H&E and TUNEL staining was utilised for the analyses of general tissue structure and apoptotic cells respectively. Scale bar 20 μm and 100 μm for H&E and TUNEL images respectively the percentage of apoptotic cells in the (H) liver and (I) kidney histopathology was quantified using QuPath software. Bars represent the mean $\pm$ standard deviation (n=3). Statistical analyses were performed with a two-way ANOVA with Tukey multiple comparisons post-hoc test with level of statistical significance represented as $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$ 82

Figure 3.1. Schematic representation of PBAE synthesis, polyplex assembly, in vitro analysis and the cellular uptake and transfection efficiency in 3D colorectal cancer organoids. 103

Figure 3.2. Synthesis and characterisation of PBAEs. (A) Synthetic scheme for step-growth Michael-addition polymerisation of BDD, 5-AP and pSar-Cystamine to produce pSar-PBAE-pSar triblock copolymer. ^1H NMR (400 MHz, DMSO-d_6) spectra of pBDD-5AP, pSar-Cystamine, and pBDD-5AP-pSar. *DPs calculated from ^1H NMR spectra as described in the main text. (B) Molecular weights of PBAEs determined using GPC and (C) the molecular weight distribution of OH-(pBDD-5AP)-OH and pSAR-(pBDD-5AP)-pSAR. (D) Buffering capacity of OH-(pBDD-5AP)-OH and pSar-(pBDD-5AP)-pSar compared to polyethylene imine (PEI) and negative control of a 0.1 M $\text{NaCl}_{(\text{aq})}$ solution..... 117

Figure 3.3. Formulation of PBAEs with saRNA to produce polyplex nanoparticles. (A) Schematic representation of saRNA formulation with OH-(pBDD-5AP)-OH, pSar-(pBDD-5AP)-pSar or different mixtures, called 'mix' of pSar-(pBDD-5AP)-pSar and OH-(pBDD-5AP)-OH as a weight% which is termed as 0%-pSar, 20%-pSar, 50%-pSar, 80%-pSar and 100%-pSar. The nanoparticles were prepared in pH 5.0 25 mM sodium acetate at a final saRNA concentration of 10 $\mu\text{g/mL}$. (B) Zeta potential and (C) particle size for the OH-(pBDD-5AP)-OH, pSar-(pBDD-5AP)-pSar and 'mix' polyplexes at a PBAE/saRNA w/w ratio of 32, 64 and 128 determined by dynamic light scattering at 25°C. Bars represent the mean $\pm$ SD ($n=3$). (D) Encapsulation efficiency of saRNA within polyplexes evaluated using Ribogreen assay. (E) Representative transmission electron micrograph of all polyplexes (Scale bars correspondence to 200 nm)..... 119

Figure 3.4. Metabolic activity and transfection efficiency of PBAE derived saRNA polyplexes. Effect of PBAE polyplexes formulated with firefly luciferase encoding saRNA at PBAE/saRNA w/w ratios of 32, 64 and 128 on metabolic activity (A and B) and transfection efficiency (C and D) on HEK293T cells (A and C) and Caco-2 cells (B and D). Cells were treated with polyplexes containing saRNA at a concentration of 500 ng/mL (100 ng/well) in serum-free Opti-MEM. Metabolic activity was compared against Triton X (positive control) and untreated cells (negative control) and calculated by normalizing metabolic activity to untreated cells. In vitro transfection efficiency was analysed after 24 h post-transfection using the Promega ONE-GLO luciferase assay and compared against Lipofectamine Messenger MAXTM (positive control) and naked saRNA (negative control) expressed as relative light units (RLU) for luciferase expression. Bars represent the mean $\pm$ SD ($n=3$). 122

Figure 3.5. Cellular uptake of PBAE derived saRNA polyplexes. (A-B) Imaging cytometry histograms of fluorescence intensity and internalisation for Caco-2 cells after

2h and 4 h following treatment with FITC-labelled 50%-pSar polyplexes at a PBAE/saRNA w/w ratio of 128 at a concentration of 500 ng/mL saRNA. Data were analysed using IDEAS software. (C) Representative confocal microscopy images of Caco-2 cells treated saRNA polyplexes derived from FITC-labelled pSar-(pBDD-5AP)-pSar at a PBAE/saRNA w/w ratio of 128 for 50%-pSar polyplexes after 4 h at 100 ng/well (500 ng/mL) saRNA. Scale bar 10 μ m (63x). 124

Figure 3.6. Analysis of GFP expression of 50%-pSar in human colon colorectal cancer organoids using confocal microscopy after 24 and 48 h (40X – scale bar 100 μ m).126

Figure 4.1. Schematic representation of PBAE synthesis, polyplex assembly, analysis of in vitro transfection and toxicity profiles and the evaluation of TLR7 activation. . 144

Figure 4.2. Synthesis and characterisation of PBAEs. (A) Synthetic scheme for step-growth Michael-addition polymerisation of BDD, 5-AP (Step 1) then end-capped utilizing 2,2'-(Ethylenedioxy)bis(ethylamine) (Step 2) and 3-Mercaptopropanyl-N-hydroxysuccinimide ester (Step 3) to produce LOX-end-capped PBAE (Step 4). (B) Molecular weights of PBAEs determined using GPC and (C) the molecular weight distribution of pBDD-5AP, H₂N-(pBDD-5AP)-NH₂, SH-(pBDD-5AP)-SH and LOX-(pBDD-5AP)-LOX. (D) Buffering capacity of H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX compared to polyethyleneimine (PEI) and negative control of a 0.1 M NaCl(aq) solution..... 157

Figure 4.3. Formulation of PBAEs with saRNA to produce polyplex nanoparticles. (A) Schematic representation of saRNA formulation with H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX prepared in pH 5.0 25 mM sodium acetate at a final saRNA concentration of 10 μ g/mL. (B) Particle size and (C) zeta potential for the H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX polyplexes at a PBAE/saRNA w/w ratio of 32, 64 and 128 determined by dynamic light scattering at 25°C. Bars represent the mean $\pm$ SD (n=3). (D) Encapsulation efficiency of saRNA within polyplexes evaluated using Ribogreen assay. (E) Representative transmission electron micrograph of the polyplexes H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX..... 159

Figure 4.4. Metabolic activity and transfection efficiency of PBAE derived saRNA polyplexes. Effect of PBAE polyplexes formulated with firefly luciferase encoding saRNA at PBAE/saRNA w/w ratios of 32, 64 and 128 on metabolic activity (A and B) and transfection efficiency (C and D) on HEK293T cells (A and C) and DC2.4 cells (B and D). Cells were treated with polyplexes containing saRNA at a concentration of 500 ng/mL (100 ng/well) in serum-free Opti-MEM. Metabolic activity was compared against Triton X (positive control) and untreated cells (negative control) and calculated by normalizing metabolic activity to untreated cells. In vitro transfection efficiency was analysed after 24 h post-transfection using the Promega ONE-GLO luciferase assay

and compared against Lipofectamine Messenger MAX™ (positive control) and naked saRNA (negative control) expressed as relative light units (RLU) for luciferase expression. (E-F) RLU for H₂N-(pBDD-5AP)-NH₂ and H₂N-(pBDD-5AP)-NH₂ + free LOX dilutions (3-100 µg/mL) in HEK293T and DC2.4 cells. Bars represent the mean ± SD (n=3). 162

Figure 4.5. TLR7 Activation Assay using HEK-BLUE cells. (A) Schematic representation of nanoparticle uptake and TLR7 activation. (B) The determination of the TLR7 activation capacity of amine and loxoribine-end-capped polymers and nanoparticles (w/w 128) compared to controls in HEK-BLUE cells. The concentration of free lox was 25 µg/mL (C) Metabolic activity of the amine and loxoribine-end-capped polymers and nanoparticles (w/w 128) compared to controls in HEK-BLUE cells. Bars represent the mean ± SD (n=3). 165

Figure 4.6. Cellular uptake of PBAE derived saRNA polyplexes. (A) Imaging flow cytometry images for DC2.4 cells after 2 h and 4 h following treatment with FITC-labelled LOX-(pBDD-5AP)-LOX polyplexes at a PBAE/saRNA w/w ratio 128 at 500 ng/well (500 ng/mL) (Scale bar 10 µm) (B) histograms of fluorescence intensity and (C) median fluorescence values Data were analysed using IDEAS software and Kaluza. (D) Representative live cell confocal microscopy images of HEK293T and DC2.4 cells 2 h post-transfection with a firefly luciferase encoding saRNA formulated with LOX-(pBDD-5AP)-LOX at a PBAE/saRNA w/w ratio of 128 at 100 ng/well (500 ng/mL) saRNA. Scale bar 10 µm (63x). 168

List of Abbreviations

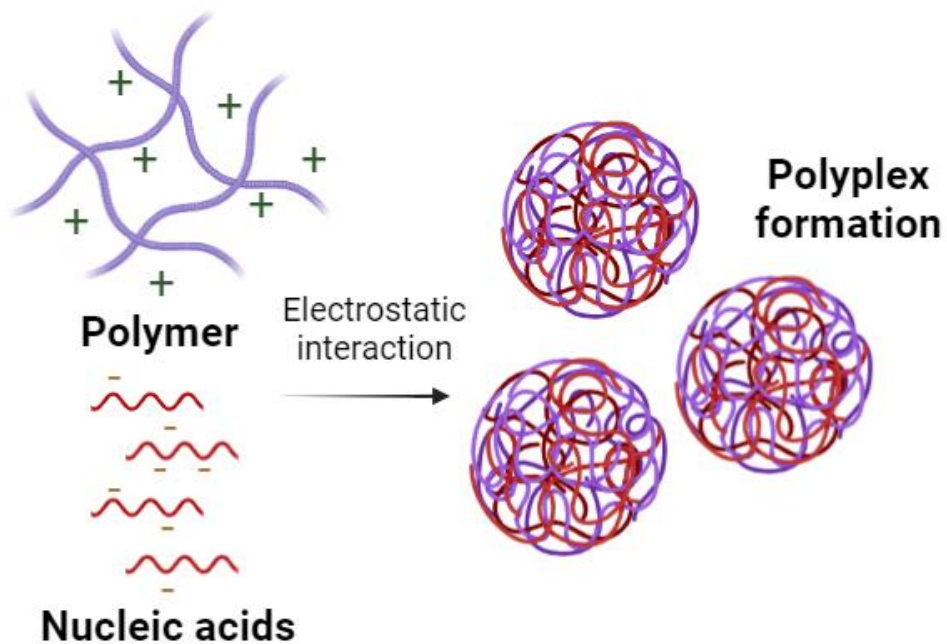
Column1	Column2
5-AP	5-aminopentan-1-ol
AAV	Adeno-associated viral
ACN	Acetonitrile
APC	Antigen-presenting cell
ASO	Antisense oligonucleotides
BDD	1,4-butanediol diacrylate
Boc-Sar	N-Boc-sarcosine
Đ	Dispersity (Expressed by the ratio of Mw/ Mn)
DCM	Dichloromethane
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DP	Degree of polymerisation
DRI	Differential refractive index
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GPC	Gel permeation chromatography
GRAS	Generally Regarded as Safe
H&E	Haematoxylin and eosin
HEK BLUE	Human TLR7 transfected HEK cells
HEK293T	Human embryonic kidney 293T
im	Intramuscular
LB	Luria broth

LNPs	Lipid nanoparticles
LOX	Loxoribine
LS	light scatter
m1ψ	Methylpseudouridine
miRNA	microRNA
Mn	Number average molecular weight
mPEG-SH	methoxy-PEG-thiol
mRNA	Messenger RNA
Mw	Weight average molecular weight
N	Nucleocapsid
NaOAc	Sodium acetate
NCA-Sar	Sarcosine N-carboxyanhydride
NMR	Nuclear magnetic resonance spectroscopy
OGM	Organoid Growth Medium
OM-PBAEs	Oligopeptide endmodified PBAEs
PBAEs	Poly(beta-amino esters)
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
PEI	Polyethylenimine
PGA	Poly(glutamic acid)
PRRs	Pathogen recognition receptors
pSar	Polysarcosine
RES	Reticuloendothelial system
RLU	Relative light units
RNAi	RNA interference
RT	Room temperature
S1	Spike

saRNA	Self-amplifying mRNA
SCID	Severe combined immunodeficiency
SEC	Size-exclusion chromatography
siRNA	small interfering RNA
TAE	Tris-acetate-EDTA
TEA	Triethylamine
THF	Tetrahydrofuran
THP	Tris-(2-hydroxyethyl) phosphine
TLR7	Toll-like receptor 7
TLRa	Toll-like receptor agonists
VS	Viscometry

Chapter 1

Introduction: Poly(beta amino esters) as gene delivery vehicles



Abstract

Nucleic acids play fundamental roles in life processes, serving as carriers of genetic information and regulators of protein expression. Harnessing their therapeutic potential, particularly in addressing diseases like cancer and immune-related disorders, has been a focus of medical research. However, the vulnerability of naked nucleic acids to degradation and their limited ability to penetrate cell membranes present significant hurdles for effective therapeutic application. Delivery vehicles, such as lipid nanoparticles (LNPs) and cationic polymers, are essential for transporting nucleic acids into cells. While LNPs have seen clinical success, alternative approaches are sought due to intellectual property constraints and chemical limitations. Poly(beta amino ester) (PBAE) materials emerge as promising candidates, with versatility and control over chemistry, making these polymers suitable for various delivery challenges. PBAEs, with their ionizable amine groups, form polyplex nanoparticles through electrostatic interactions with nucleic acids. Since their introduction in gene delivery in 2000, PBAEs have garnered attention for their tunable chemistry and efficient nucleic acid delivery. This has led to a surge in research exploring PBAEs for diverse therapeutic applications, including cancer immunotherapies and vaccines. This review provides insights into PBAE synthesis, structural considerations, and formulation strategies for nucleic acid delivery. It highlights recent advancements, potential avenues for development, and persistent challenges in PBAE-based nucleic acid delivery systems. In summary, PBAEs represent a promising avenue for overcoming intracellular barriers and realising the therapeutic potential of nucleic acids.

Continued research and innovation in PBAE design and application hold the key to unlocking the full scope of nucleic acid-based therapies for diverse medical needs.

1.1 Introduction

Nucleic acids exhibit many purposes for the propagation and function of life, most commonly for information storage via DNA in the genome, or as relays to produce functional proteins (though mRNA and tRNAs) from the stored genetic information. Gene therapy began to take shape in the late 20th century with pioneering experiments that aimed to introduce functional genes into cells to correct genetic disorders. The first clinical protocol for gene therapy was approved in 1989, leading to the first successful procedure in 1990 when a four-year-old girl with a severe immune disorder called severe combined immunodeficiency (SCID) was treated with gene therapy [1]. Doctors at the National Institutes of Health used a retroviral vector to deliver a functional copy of the gene responsible for her condition into her white blood cells, effectively restoring her immune system function. Despite setbacks, including a tragic death in a clinical trial, the field has progressed with hundreds of clinical trials and promising outcomes. The potential benefits of gene therapy to treat currently incurable disorders fuel ongoing research and development in this area. Today, gene therapy holds promise for treating a wide range of genetic disorders, as well as diseases such as cancer and neurodegenerative conditions. Ongoing research aims to improve the safety and efficacy of gene therapy approaches, making them viable options for treating a growing number of patients.

Nucleic acids have been a critical target for medicine for decades, using small molecule drugs which can disrupt the structure or chemistry of DNA [2]. These

concepts have been the cornerstone for treatment of cancers and other diseases. More recently, nucleic acids themselves have been recognised as a therapeutic modality [3]. Examples include mRNA and plasmid DNA to instruct expression of a desired proteins or miRNA, siRNA and antisense oligonucleotides that can be employed to suppress expression of disease related proteins [4, 5]. These nucleic acid technologies have created the possibility for selective therapies which can reduce the off-target effects which can be observed with conventional small molecule therapeutics and contribute to their side effects and poor tolerability.

The vulnerability of naked nucleic acid to degradation by endogenous nucleases and poor association with cell membranes necessitates the use of a delivery vehicle to transport these fragile molecules to their intracellular targets [6]. Additionally, macrophages and the reticuloendothelial system swiftly eliminate them [6]. Typically, the half-life of DNA and RNA in vivo ranges from a few minutes to a couple of hours. Strong electrostatic repulsion occurs between DNA or RNA and the negatively charged phospholipid bilayer at the membrane of a cell, posing a considerable challenge for cellular uptake of the gene. Hence, choosing a safe and reliable transport carrier is essential for delivering nucleic acids to target cells and tissues in clinical research.

In general, transporters are categorised into viral and non-viral vectors. Viral vectors, such as retroviruses, adenoviruses, and lentiviruses, are often more efficient than non-viral vectors across various cell lines due to their ability to tightly package and release nucleic acids within or from their capsids. They also possess the capability to overcome a range of extracellular and intracellular delivery barriers or defence mechanisms within targeted cells [7]. However,

concerns regarding severe off-target immunogenicity, inflammatory response, and toxicity exist with viral vectors. On the other hand, non-viral vectors, which utilise cationic lipids, polymers, and peptides with chemical properties resembling those of viral vectors, can compact and deliver nucleic acids in a similar manner [8]. Besides, they are generally considered safer than viral vectors since their artificial design is typically not immediately recognised by the immune system. Additionally, their chemical structure is controllable, making them easier to scale up and synthesize commercially. However, non-viral carriers still face certain challenges that need to be addressed.

The majority of nanocarriers engage with the cell membrane electrostatically, facilitated by the presence of anionic cell surface proteoglycans, and subsequently enter the cells [9]. Cell uptake typically involves energy-dependent processes such as clathrin- and caveolae-mediated endocytosis, which are regulated by internalised molecules within the cell. Additionally, uptake may occur in phagocytes like macrophages, dendritic cells, and neutrophils. Following cellular uptake, endosomes pose a significant intracellular barrier to gene transfection due to the presence of multiple nucleases and a low-pH environment [10, 11]. Despite numerous efforts to develop effective vectors for delivering DNA and RNA, their ability to escape endosomes generally remains below 4% [12]. Microfilaments and microtubules within the cytoplasm can profoundly impact the transport of DNA and RNA. The nuclear membrane, a phospholipid bilayer that separates the cytoplasm from the nucleus, contains nuclear pores that are small and highly selective for macromolecules such as DNA and RNA [13]. Exogenous DNA relies on the rupture of the nuclear membrane during cell mitosis to enter the nucleus and

undergo transcription. Therefore, the development of safe and efficient gene delivery vectors, while a promising approach for effectively overcoming various intra- and extracellular barriers during gene delivery, remains challenging.

The most popular delivery systems are lipid nanoparticles (LNPs) which are 50-200 nm self-assembled particles comprised of various lipid components. These operate by condensing nucleic acids into core-shell structures, protecting them and enhancing translocation across the cell membrane. Despite LNPs being the most clinically advanced delivery approach, given their application in Onpattro (an LNP-siRNA therapy for hereditary transthyretin-mediated amyloidosis) and the mRNA COVID-19 vaccines, SpikeVax and Comirnaty, there is a need for alternative delivery approaches [14]. LNPs currently have a tight intellectual property space and may not possess the chemical diversity for all delivery challenges. Cationic polymers have garnered increasing interest due to their ability to easily modify their physicochemical properties through various strategies, such as altering chemical composition, molecular weight, or architecture, to provide multiple functions [15]. Since their first use as a non-viral vector in 1965 by Vaheri and Pagano, employing diethylaminoethyl functionalised dextran, a wide range of cationic polymers have been explored [16].

Notably, poly(beta amino ester)s (PBAE)s have emerged as one of the most promising options [17]. PBAEs are highly versatile biodegradable polymer scaffolds which offer control over the polymer backbone chemistry, pendant chain and end-group which have been used in a variety of applications. The ionisable amine in the polymer backbone can be protonated at physiological pH or lower into polycations, allowing PBAEs to complex via electrostatic

interactions with negative charged nucleic acids to form polyelectrolyte complexes (sometimes called polyplex nanoparticles). These materials were first reported for gene delivery in 2000, when D. Lynn and R. Langer published their two pioneering studies using a broad range of diacrylate and amine monomers to produce high throughput combinatorial libraries, which were then screened for effectiveness in DNA delivery in vitro [18]. Following the foundation of PBAEs in the gene delivery field, hundreds of reports focusing on PBAEs as effective delivery systems for different nucleic acid cargos can now be found [19, 20]. This explosion on to the gene delivery scene is largely due to the ability to tune the materials chemistry and encode specified nucleic acid release mechanisms which cannot easily be achieved for other polymer or lipid delivery approaches.

The progression of nucleic acid therapeutics towards different applications, such as cancer immunotherapies and vaccines has therefore led to the investigation of various aspects, such as efficacy, safety and mechanism of action. The immune system has developed complex defence mechanisms against exogenous threats, consisting of the innate and adaptive immune systems [21, 22]. The innate immune system is the first line of defence, providing general protection and activating the adaptive immune system. The adaptive immune system generates pathogen-specific responses, taking time to become effective due to rounds of proliferation and selection of reactive cells. A key feature of adaptive immunity is the generation of immunological memory through T cells and B cells, which express unique antigen-specific receptors (Activation of CD8⁺ and CD4⁺ T cells by antigen-presenting dendritic cells triggers distinct immune responses. Dendritic cells expressing antigens on

MHC class I molecules activate CD8⁺ T cells, leading to their differentiation into cytotoxic T lymphocytes (CTLs) through interleukin signalling. CTLs, in turn, target and kill virally infected, cancerous, or intracellular bacteria-harboring cells. Meanwhile, dendritic cells presenting antigens on MHC class II molecules activate CD4⁺ T cells, resulting in the formation of TH1 and TH2 cells through distinct interleukin pathways. TH1 cells produce interferon-gamma, further enhancing CTL activation, while both TH1 and TH2 cells stimulate B cells to produce antibodies, augmenting the immune response against targeted cells. (Figure 1.1). These receptors undergo gene rearrangement during early lymphocyte development to ensure uniqueness [23].

T cells develop in the thymus, where they interact with major histocompatibility complex (MHC) molecules (Figure 1.1) [24]. MHC class I molecules present endogenous antigens to CD8⁺ T cells, while MHC class II molecules present exogenous antigens to CD4⁺ T cells. B cells form in the bone marrow, and both T and B cells undergo central tolerance to eliminate cells with autoreactive or non-functional receptors [25]. Naïve cells that pass central tolerance migrate to secondary lymphoid organs, where they encounter antigens, undergo further differentiation, and are subjected to peripheral tolerance to eliminate non-functional or autoreactive cells [26].

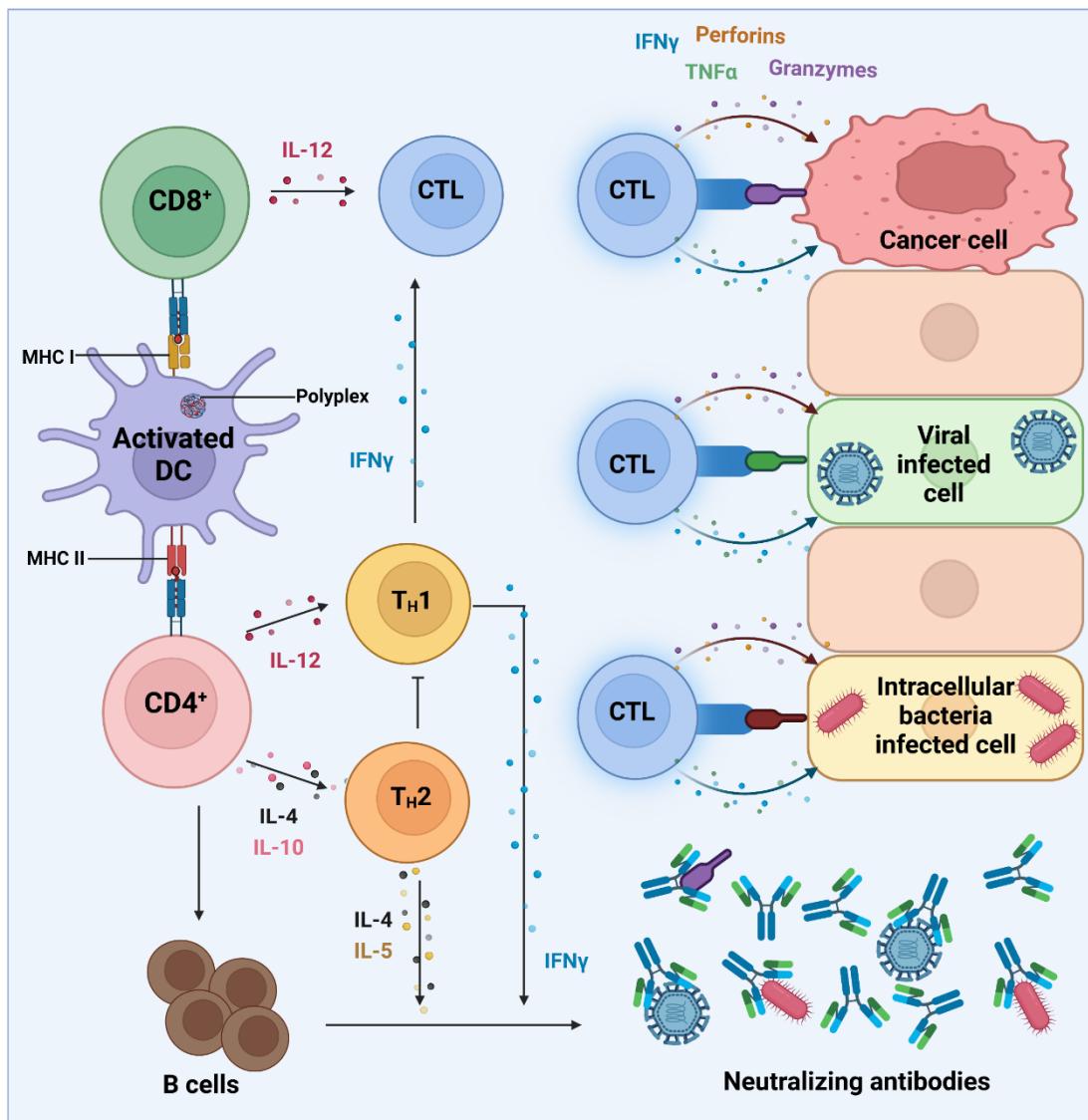


Figure 1.1 Activation of CD8⁺ and CD4⁺ T cells by antigen-presenting dendritic cells triggers distinct immune responses. Dendritic cells expressing antigens on MHC class I molecules activate CD8⁺ T cells, leading to their differentiation into cytotoxic T lymphocytes (CTLs) through interleukin signalling. CTLs, in turn, target and kill virally infected, cancerous, or intracellular bacteria-harboring cells. Meanwhile, dendritic cells presenting antigens on MHC class II molecules activate CD4⁺ T cells, resulting in the formation of TH1 and TH2 cells through distinct interleukin pathways. TH1 cells produce interferon-gamma, further enhancing CTL activation, while both TH1 and TH2 cells stimulate B cells to produce antibodies, augmenting the immune response against targeted cells.

In this section, as a potential gene delivery vehicle of PBAES, we present a brief overview of the synthesis and their structures. In addition, the types of PBAE-based formulations are explained. Finally, we discuss some of the

literature of PBAE design for nucleic acid delivery related applications, including recent advances, potential avenues for development, and persistent challenges.

1.2 PBAE Synthesis

The most popular way to synthesise PBAEs is aza-Michael addition polymerisation between amines and diacrylate esters. This is a simple synthetic protocol, and the reaction does not lead to many side products, so the final product does not require extensive purification and may in some cases be used directly for biological assays [27]. Another advantage of aza-Michael addition polymerisation is the vast availability of commercial monomers as different di-, tri- and tetra-acrylates as well as amines. Therefore, this highly atom-efficient polymerisation enables facile production of a large library of PBAEs for screening delivery of a desired cargo for a specified application. Many hundreds of PBAEs have been synthesised and tested as delivery vectors for nucleic acids, which have been summarized in numerous review papers [17, 28-33]. Beside nucleic acid delivery, numerous other macromolecules and small drugs have been successfully delivered by PBAEs [34-37].

Aza-Michael addition, the mechanism behind PBAE synthesis takes place rapidly even at room temperature [38, 39]. However, as polymer synthesis requires nearly quantitative conversion of monomer reactive groups, reactions are usually conducted at elevated temperature (40 - 110°C) to shorten the time to completion to around 12-24 h. Importantly, as aza-Michael addition polymerisation obeys third order kinetics, its rate is strongly dependent on a substrate concentration. Therefore, a high concentration of monomers is

required to achieve the desired monomer conversion within a reasonable time. Another advantage of a high monomer concentration is a reduction in the side product content. All step growth polymerisations, including aza-Michael addition polymerisation, suffer from the formation of undesired macrocyclic compounds [40-42], and the contribution of macrocyclization reaction over polymerisation is promoted at low monomer concentration [43, 44]. Usually, these macrocycles may be easily detected on a size-exclusion chromatography (SEC) trace as very narrow peaks at low molecular weight and may be removed via dialysis [45-47]. However, this creates the need for additional purification steps, therefore, it is more convenient to prevent the formation of macrocycles by applying as high concentration of monomer as possible. However, the high concentration of the growing polymer may lead to very high viscosity of the reaction mixture.

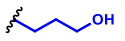
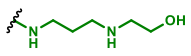
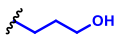
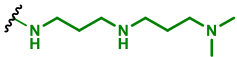
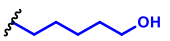
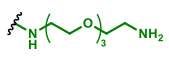
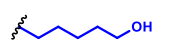
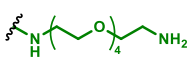
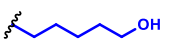
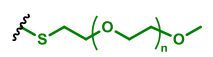
Another crucial parameter regarding PBAE synthesis through aza-Michael addition polymerisation is solvent selection. First and foremost, all monomers and polymeric products should be fully soluble in the chosen solvent, as precipitation of oligomers and short polymers may suppress formation of high molecular weight polymers. In addition, low solubility of monomers requires a high solvent volume, thus reducing monomer concentration which slows down the reaction and increases cyclic side product formation [37, 38]. The most popular solvents for aza-Michael addition polymerisation are CH_2Cl_2 [48, 49], THF [18], DMF [50], and DMSO [51, 52]. Alcohols are rather avoided due to hydrogen bond formation between solvent and amines, which lowers their reactivity toward acrylates slowing down aza-Michael addition. Considering the potential toxicity of the residual solvent in the final polymer, DMSO is often

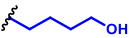
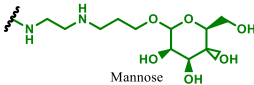
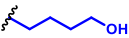
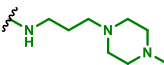
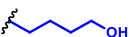
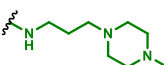
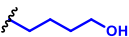
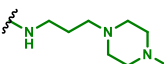
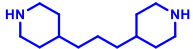
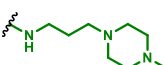
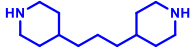
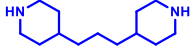
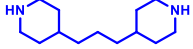
chosen due to its relatively low toxicity. Additionally, the most common way of handling PBAEs is a preparation of a concentrated stock solution in DMSO, which is subsequently diluted in a proper buffer. Therefore, DMSO remaining in the polymer after precipitation out of a reaction mixture does not impact subsequent biological assays. Another popular approach is aza-Michael addition polymerisation without solvent, which is possible because most monomers are liquids [53, 54]. Such an approach allows for the highest monomers concentration thus the highest reaction rate and the lowest fraction of cyclic by-products.

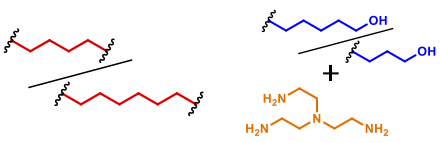
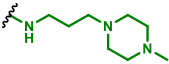
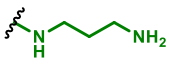
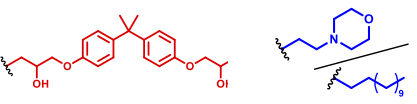
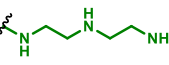
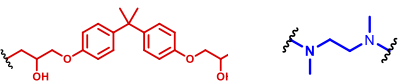
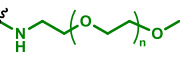
It has been shown that the type of terminal groups [55-57] and the molar mass of PBAEs [19, 58] have enormous impacts on the delivery efficacy and toxicity, therefore these parameters need to be carefully fine-tuned for a targeted application. In table 1, some examples of PBAEs were used in the literature using different monomers and for the delivery of various cargo types and targeted applications were summarised. In the aza-Michael addition polymerisation process, these may be easily controlled by a simple manipulation of monomer ratio, and the degree of polymerisation (DP) of the final product may be estimated using Carother's equation [59]. However, one should keep in mind that this prediction assumes a quantitative monomer conversion into the polymer without side reactions. In general, the longest polymer may be obtained using monomers at the exact stoichiometric ratio, and an increasing excess of one monomer decreases the molecular weight of the product terminated with the monomer used in the excess. The most popular way for the end-group modification with both small molecules and other polymers (end-capping) is conducting an aza-Michael addition polymerisation

with an excess of bis-acrylic monomers leading to polymers terminated with activated double bonds. The resultant acrylate end-groups are convenient for further functionalization via thia-Michael and azo-Michael additions [60-62]. It is important to not leave uncapped acrylic end groups as these have been shown to limit nucleic acid delivery [19].

Table 1. Some selected examples of PBAEs in various applications

$R_4 \left(\text{CH}_2 \text{CH}_2 \text{C}(=\text{O}) \text{O} \text{R}_1 \text{O} \text{C}(=\text{O}) \text{CH}_2 \text{CH}_2 \text{N}(\text{R}_3) \text{CH}_2 \text{CH}_2 \text{C}(=\text{O}) \text{O} \text{R}_1 \text{O} \text{C}(=\text{O}) \text{CH}_2 \text{CH}_2 \right)_n R_4$							
No.	R1	R2 / R3	R4	Cargo type	Target cells	Therapeutic target	Ref.
1. ^a				cDNA encoding sTRAIL protein	HepG2 cancer cells	Liver cancer - reprogramming of liver cells to locally secrete TRAIL	[63]
2. ^a				pDNA encoding 4-1BBL and interleukin-12	B16-F10 melanoma cells and xenograft tumor	Antitumoral – stimulation of T and NK cells	[64]
3. ^{a,b}				RP-182 peptide + DNA encoding ErbB2-specific CAR-T	Macrophages	Brain tumor – reprogramming of M2-like macrophages to M1-like phenotype	[65]
4. ^{a,b}				mRNA encoding OVA and TRP2	Dendritic cells	Vaccination - reprogramming cells to present specific epitope (OVA, TRP2) to be recognised by T cells	[66]
5. ^a				pDNA encoding the spike and the nucleocapsid of SARS-CoV-2	Dendritic cells (DC2.4)	Vaccination - reprogramming cells to present SARS-CoV-2 epitopes	[67]

6. ^a			 Mannose	pDNA encoding OVA	RAW264.7, macrophages	Vaccination - reprogramming cells to present specific epitope (OVA)	[68] [69]
7. ^a				mRNA encoding M1- polarizing transcription factor TAMs	Ovarian cancer, melanoma, glioblastoma, macrophages	Cancer cells - reprogramming of M2-like macrophages to M1-like phenotype	[54]
8. ^a				Cyclic dinucleotides (CDNs)	B16 melanoma tumors	Cancer cells – stimulation of the cytosolic (STING) signaling pathway	[70]
9.				DNA encoding CAR + targeting antibody	T-cells	Introducing of leukemia- targeting CAR genes into T-cell	[71]
10.				Antigens MHC's (Kb1g-SI, Db1g-gp100 and Kb1g-TRp2) and anti-CD28	T-cells	Antitumor – T-cell activation as an artificial antigen presenting cells	[72]
11. ^{a,b}			-----	Reporter mRNA encodin luciferase	Dendritic cells (DC2.4)	Proof-of-concept for delivery to CD2.4	[73]
12.			-----	Control dicer-substrate siRNA + immunostimulatory effect of PBAE itself	RAW264.7 and RAW-Dual, macrophage cells	Activation of TLR and NF- KB pathway	[74]
13.			-----	CpG oligonucleotide (CpG-ODNA)	Macrophages	Antitumor - Activation of TLR9	[75]

14. ^a			mRNA encoding a reporter protein (eGFP) + immunostimulatory effect of PBAE itself	Mouse bone marrow-derived dendritic cells (BMDCs)	Activation of antigen-specific immune response by PBAE itself	[76]
15. ^{a,b}			reporting mRNA encoding Cre	Lung Endothelium	Proof-of-concept for lung delivery	[77-79]
16. ^{a,b}			saRNA encoding rabies virus glycoprotein	Screening on C2C12 murine myoblasts; muscles (intramuscular injection)	saRNA encoding rabies virus glycoprotein; hybrid nanoparticles with lipids	[80]
17.			Poly(I:C)	Macrophages	Antitumoral – activation of TLR3 and MDA-5 receptors	[81]

^a*in vivo* efficacy has been demonstrated, ^bPBAE-lipid hybrid nanoparticle

The numerous commercially available primary amines and bis-acrylates allow us to explore a wide landscape of chemical functionalities of PBAEs to tailor their structure for a specific application. For instance, one may synthesize a library of PBAEs with varying hydrophobicity, hydrophilicity, and an overall hydrophilic-lipophilic balance, to balance toxicity and efficacy. Usually, the introduction of strongly hydrophobic groups to polycations stabilizes particles but also increases their toxicity [62, 82-84]. Furthermore, functional monomers containing different bases like tertiary amines, imidazole, or morpholine may be used to tune pK_a of PBAEs, thus their behaviour at low pH. For example, the pK_a of the delivery vector has been shown to play an important role in nucleic acid delivery, potentially due to the proton-sponge effect after transition to endosomes enhancing escape into the cytosol [85, 86]. Another broadly applied functionality is a disulfide bond in the polymer main chain, which can be cleaved intracellularly by a high concentration of glutathione, leading to a reduction in chain length, an effective drop in polycation concentration, and cargo release inside the cell [87-90]. To generate more chemical variety in PBAEs, the degree of branching may be tailored by tuning the ratio between bis-functional monomers and multi-functional ones [91-94], which can also lead to hydrogel formation [95, 96].

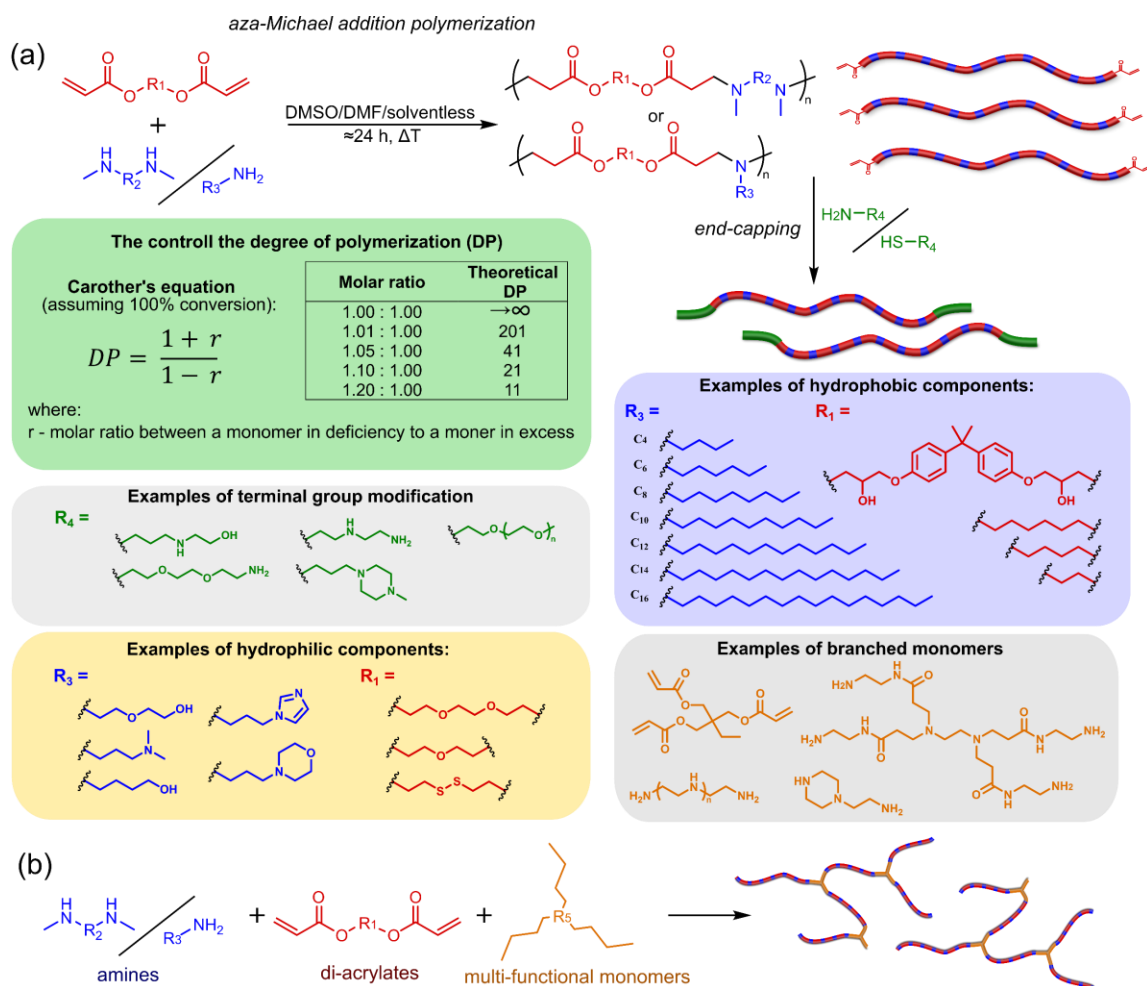


Figure 1.2: Synthesis of PBAEs (a) linear (b) branched polymer synthesis using different types of amines and diacrylates.

1.3 Delivery of nucleic acids

Nucleic acids, crucial for life's functions, primarily serve in storing genetic information or aiding in protein production. They have long been targets for medicine, with drugs disrupting DNA structure or chemistry, vital in treating cancers and immune-related diseases. Recently, nucleic acids themselves are recognized as therapies, like mRNA and plasmid DNA directing protein expression, or miRNA and antisense oligonucleotides suppressing disease-related proteins. However, naked nucleic acids are vulnerable to degradation

and lack efficient cell membrane association, which is essential for the targeted protein expression. LNPs, particularly popular, condense nucleic acids into nanoparticles, protecting and aiding cell membrane translocation. Despite the success of LNPs, alternatives are sought due to limited diversity and tight intellectual property space.

As noted above PBAEs can be versatile, biodegradable polymers offering precise control over chemistry. They form polyelectrolyte complexes with nucleic acids, serving as gene delivery vehicles. Numerous studies have already demonstrated the effectiveness of PBAEs in nucleic acid delivery. In summary, while nucleic acids hold promise for therapeutic interventions, efficient delivery to intracellular targets is essential. PBAEs offer diverse chemistry and precise control, expanding possibilities in gene therapy.

This chapter will outline PBAE chemistries and formulation approaches used for different types of nucleic acid class, including DNA, messenger RNA, self-amplifying RNA and oligonucleotides (Figure 1.3). In each section the mechanism of therapeutic action and challenges associated with delivery, alongside solutions that PBAEs offer will be discussed.

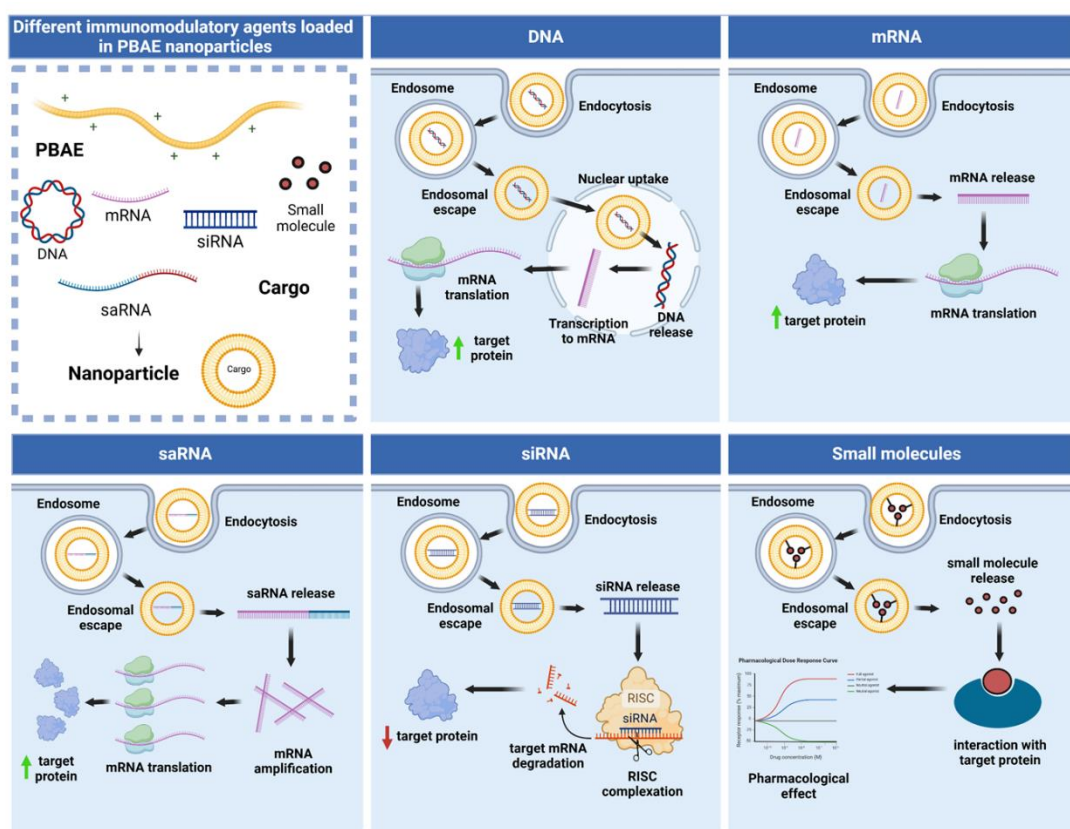


Figure 1.3. Schematic diagram illustrating the diverse cargos carried by PBAE polyplexes (DNA, mRNA, saRNA, siRNA, small molecule drug) and their intracellular fates: DNA polyplexes deliver DNA to the nucleus, where it is transcribed into mRNA by RNA polymerase and then translated by ribosomes to produce the target protein; mRNA polyplexes release mRNA into the cytoplasm for direct translation by ribosomes; saRNA polyplexes utilize a self-amplifying replicon to generate multiple mRNA copies, leading to increased protein production; siRNA polyplexes induce RNA interference through its complexation with RNA-induced silencing complex (RISC), silencing specific genes and reducing target protein production; small molecule drug polyplexes target specific proteins/receptors to elicit pharmacological effects.

1.3.1 DNA

DNA therapies most commonly consist of circular plasmid DNA to induce expression of a specified protein. Depending on the DNA sequence, expression of the desired protein can be achieved either transiently for applications including protein replacement therapies, vaccines for infectious disease, or instead permanently to replace faulty genes in hereditary disorders and to train various immune cells (e.g. dendritic cell or T-cells) to eliminate cancers. Due to

the necessity to deliver DNA to the nucleus, either episomally or for integration into the genome, the most prevalent delivery approach has been through adeno-associated viral (AAV) vectors [97, 98]. However, packing limitations on the nucleic acid size in AAVs, difficulties surrounding large scale biological manufacture, and concerns around vector mediated reactogenicity have therefore shifted attention to non-viral delivery approaches, including PBAEs [99-101]. In this section some of the advancements in PBAE design for DNA are discussed.

One of the key advantages of PBAEs is their tuneable chemistry, both in the pendant chain and end-groups. This enables researchers to be able to decorate resulting DNA polyplex nanoparticles with the desired surface chemistries, which could enhance trafficking and localisation on to immune cells to improve delivery specificity. Pfeifer and co-workers [68] used this approach to great effect by employing a two-step approach to introduce mannose residues at the PBAE end groups (Figure 1.4). They first generated a small library of acrylate terminated PBAEs using different alkyl aminoalcohols and aliphatic diacrylates. The acrylate end groups were then first transformed into amino functionality then further with allyl- α -D-mannopyranoside to enhance potential uptake in antigen presenting cells and macrophages which overexpress mannose binding receptors. In vitro gene delivery assays using DNA polyplexes derived from the mannose PBAEs revealed a significant improvement in uptake and transfection efficiency within RAW264.7 macrophage cells compared to non-mannosylated controls. Furthermore, in vivo immunisation studies utilising ovalbumin encoding pDNA vaccine, administered intraperitoneally or subcutaneously demonstrated that mannosylated PBAEs elicited a robust,

efficient, and safe humoral immune response without the need for adjuvants when compared to control antigens delivered through genetic or protein means. Encouragingly there were no signs of adverse reactions or mortality. For both mannosylated and non-mannosylated polyplexes the total IgG titers were notably higher than those from the OVA pDNA positive control 14 days post inoculation. By day 21, (7 days after the second dose), only the mannosylated polyplexes showed significantly elevated antibody titers compared to the pDNA control, highlighting the improvement in antigen-presenting cells (APC) targeting through PBAE design.

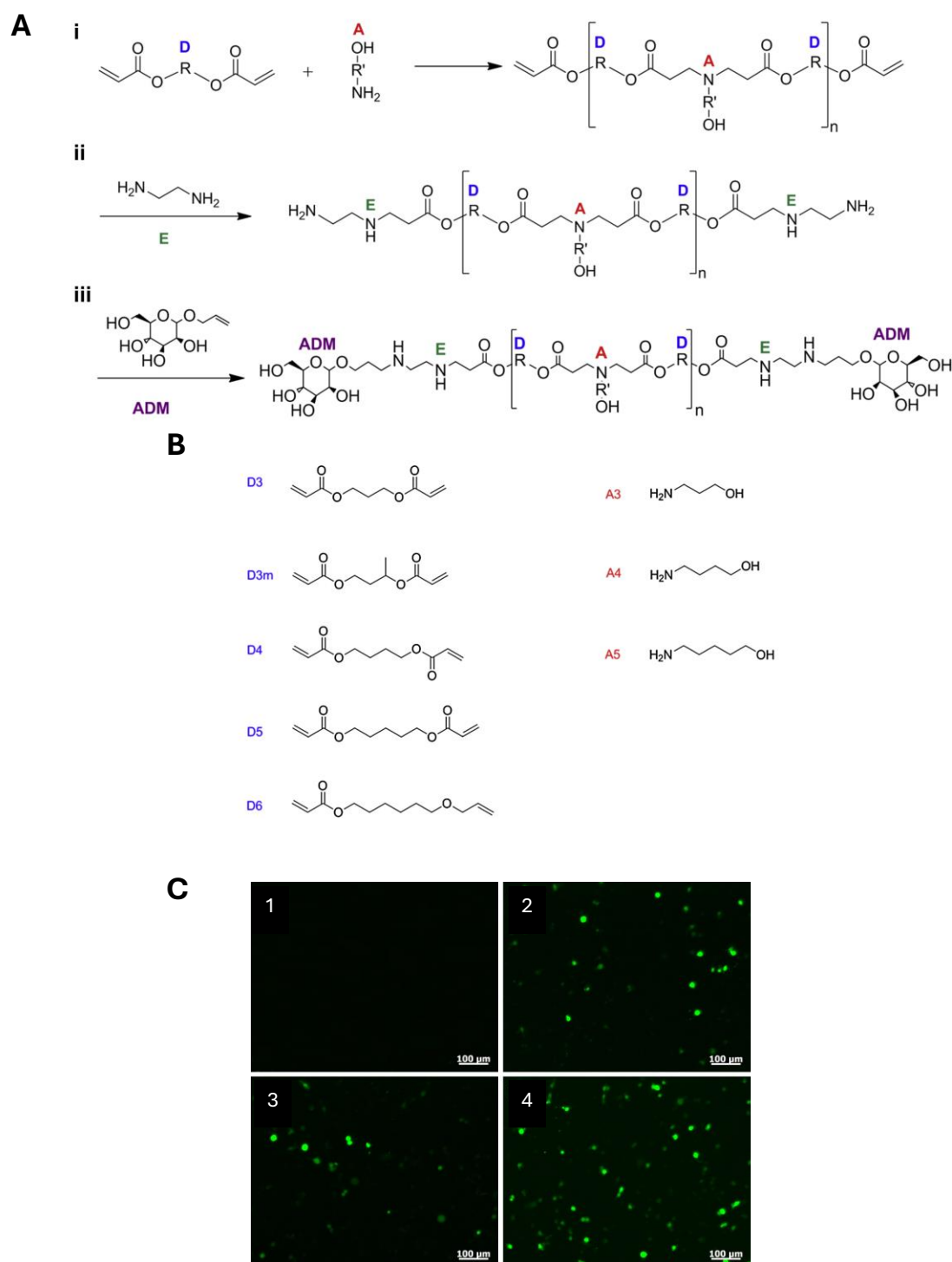


Figure 1.4: (A) Polymer synthesis scheme and (B) associated monomers. Diacrylate-D and amine-A monomers were copolymerized to create acrylate-terminated base polymers (i) which were then amine-capped using excess ethylenediamine (E, ii) and reacted with allyl- α -D-mannopyranoside (ADM) to create mannose-capped polymers (iii). Respective polymers are named by their monomers and mannose content. For example, reacting diacrylate monomer D4 with amine monomer A4 results in the synthesis of polymer 'D4A4.' 'D4A4-Man' represents the mannosylated variant. (C) Fluorescent microscopy of top-performing polymers. Images depict EGFP-positive RAW264.7 cells resulting from transfection using naked pDNA (1), JET-PEI (2), D4A4 (3), and D4A4-Man (4). Reprinted with permission from. Jones, C. H. *et al.* (2015) 'Mannosylated poly (beta-amino esters) for targeted antigen presenting cell immune modulation', *Biomaterials*.

In a follow up study Pfeifer and co-workers [69] then systematically evaluated how PBAE molecular weight and relative mannose content influenced polyplex properties and DNA transfection efficiency, using the same strategy to generate mannose end-group modified PBAEs. Within the library, polymers with the lowest molecular weight and highest relative mannose content exhibited gene delivery transfection efficiencies comparable to or surpassing those of commercial controls (JET-Polyethylenimine (PEI), Xfect and FuGENE HD) in RAW264.7 macrophages when treated with luciferase encoding plasmid DNA. In their library they identified that the most effective polymers formed the smallest polymer-plasmid DNA complexes (approximately 300 nm) with moderately positive zeta potentials (less than 10 mV). Despite a lack of further in vivo investigations, this study highlights the versatility in PBAE design and the opportunity this brings to elucidate structure function relationships.

Immunotherapies are fast becoming a critical tool to treat cancer, typically by stimulating a cytotoxic immune response against tumor associated antigens to selectively destroy malignant cells. However, for these therapies to be effective, coordinated signalling is required for APCs to instruct CD8⁺ T-cells towards the correct target. The most common approaches are to extract APCs and manipulate them *ex vivo* such that they express the patient-specific antigen, however this is a complex process and requires prior knowledge on the antigen itself. Green and co-workers [64] reported a novel 'antigen-agnostic' approach using PBAEs to deliver pDNA encoding for 4-1BBL and interleukin-12, a tumor necrosis factor ligand present on activated T-cells and an APC secreted cytokine respectively to B16-F10 melanoma cells and xenograft tumors. In

principle this should reprogram cancer cells and modify the immunocompetency of the tumor microenvironment in situ. They initially produced a library of PBAEs, similar to that from the studies described by Pfeifer and co-workers above, but with a range of aminated endcaps and assessed their ability to transfect B16-F10 melanoma cells in vitro. The highest performing PBAEs (those with transfection efficiencies above 80% and metabolic activity above 80%) commonly included 1,4-butanediol diacrylate copolymerised with 4-aminobutanol and a piperazine (named 4-4-7 and 4-4-27 in original study) and or primary amine end-group, or 1,5-pentanediol diacrylate copolymerised with 3-aminopentanol and a dimethylamine functional end group (named 5-3-49 in original study). The top 3 performing PBAEs also showed efficient reporter gene expression in equivalent xenografts, with 5-3-49 exhibiting 20-fold luminescence over the negative control and hence was taken forward as the lead PBAE candidate. When complexed with the immunostimulatory pDNA constructs, the 5-3-49 PBAE polyplexes elicited a strong interferon-gamma response, used as a proxy to assess potential T-cell and NK cell activation. Further in vivo studies using this reprogramming PBAE polyplex platform led to a significant reduction in tumor growth rate, in B16-F10 melanoma and MC38 colorectal cancer xenograft in certain instances, complete tumor clearance, especially when co-administered with anti-PD-1 checkpoint blockade therapy. Tumor analyses confirmed that locally administered APC-reprogramming nanoparticles stimulated a cell-mediated cytotoxic immune response, assessed through IFN-gamma expression and upregulation of genes associated with cytotoxic immune response, highlighting the translational potential of PBAEs for immunotherapy applications.

1.3.2 Messenger RNA

Endogenous mRNA transfers genetic information between the permanent storage in the genome and proteins which control cellular and intercellular functions. The transient and therefore non-permanent nature of mRNA has enabled the emergence of new therapeutics such as protein replacement therapies and vaccines where exogenously mRNA constructs can be administered to produce any desired protein. This technology therefore poses many advantages both in terms of manufacture and applicability [102, 103]. As mRNA production is largely sequence agnostic, manufacture of any construct will be very similar, hence enabling a platform technology. This is different from protein therapies which require unique manufacturing routes for each new drug. In principle this should significantly accelerate their development, reducing the time from discovery to accessibility in the clinic. mRNA technology has most recently been used to great effect as vaccines for protection against severe COVID-19 disease, by administering mRNA constructs which encode for the SARS-CoV-2 spike protein [104-106]. The successful large manufacture and clinical approval of Moderna's SpikeVax™ and Pfizer-BioNTech's Comirnaty™ vaccines has initiated a significant surge in new mRNA technologies[106, 107], with over 900 new mRNA therapies under clinical development ranging from vaccines to protein replacement therapies [108-110]. However as with other nucleic acid medicines, the susceptibility of mRNA to endogenous nucleases and poor transport into the cytosol for efficient translation into their encoded proteins relies on the use of a delivery vector, typically LNPs. Given the successes of this technology, PBAE's have been explored for delivery of messenger RNA, typically including fundamental studies to identify structure

function relationships to enhance mRNA delivery with PBAEs and development of non-injectable mRNA medicines [111-113]. This section summarises some literature examples on PBAE delivery systems for mRNA therapeutics.

APCs are a critical part of the adaptive immune system, able to display antigens on their surface and cross-present to other immune cells to eliminate pathogens or produce an antibody response [114, 115]. Delivery of mRNA antigens to APCs could therefore significantly enhance mRNA vaccine and immunotherapy response [116]. Borrós and co-workers report the development of oligopeptide end-modified PBAEs (OM-PBAEs) as APC targeting delivery systems for mRNA [111]. Utilising their established PBAE of 5-aminopentanol copolymerised with 1,4-butanediol diacrylate, they transformed the acrylate end-groups with a further aza-Michael addition of a series of oligopeptides containing cysteine, histidine, and lysine. When formulated with mRNA these OM-PBAEs they were able to produce small polyplex nanoparticles, approximately 140 nm in diameter, which significantly enhanced transfection efficiency of eGFP encoding mRNA in HeLa, ARPE-19, RAW264.7 and JAWSII cell lines. After intravenous administration, the authors reported excellent accumulation in the spleen with OM-PBAEs, and high uptake (through Cy5 labelled PBAEs) in splenic macrophages, dendritic cells and neutrophils. Despite this, relatively modest transfection efficiency was observed in these isolated cells. One potential limitation of their study is that polyplex formulations often contain a high proportion of non-complexed polymer, due to the requirement of high N/P ratios (the ratio between the total number of amines in polymers and total number of phosphates in nucleic acids) to form low particle sizes hence the uptake studies may not be accurately correlating the

internalisation of the nanoparticles with transfection. Previous studies showed that N/P ratio can significantly affect transfection efficiency and cytotoxicity of nanoparticles [117, 118]. This ratio will be dependent on the type of polymer. For instance, PEI can be cytotoxic with even N/P ratio 10; however, in literature PBAE-based nanoparticles were mostly used at an N/P ratio of between 60 – 120 [19, 117].

As described above, therapies based on modulation of immune cells have now reached clinical approval utilising DNA based reprogramming of T-cells, either through CAR-T or T-cell receptor modifications. However, there is now a shift to manipulate these cells using mRNA gene expression. Accordingly, Stephan and co-workers [119] utilised their poly(glutamic acid) (PGA) antibody coating approach described in the DNA section, to decorate mRNA loaded PBAE complexes with a variety of antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD28 and control antibody C1.18.4 (Figure 1.5). The polyanionic PGA coating also serves to reduce non-specific electrostatic interactions between positively charged polyplexes and the electrostatic membrane of non-target cells. To exemplify this PBAE platform, the authors found that the antibody-coated PBAE polyplexes exhibited a tenfold increase in ex vivo T-cell transfection, compared to non-targeted PBAE NPs. Expanding on this success, the versatile platform was utilized for the delivery of two different mRNAs. In one instance, these NPs delivered megaTAL nuclease mRNA to disable endogenous T-cell receptors potentially implicated in graft-versus-host disease. In a separate application, NPs loaded with mRNA encoding the Foxo13A transcription factor were employed to guide CD62L⁺ T-cells away from terminal differentiation and senescence, promoting a central memory phenotype. Collectively, these

outcomes demonstrate the adaptability of the mRNA platform which can use the same PBAE for different therapeutic applications due to the sequence agnostic nature of mRNA.

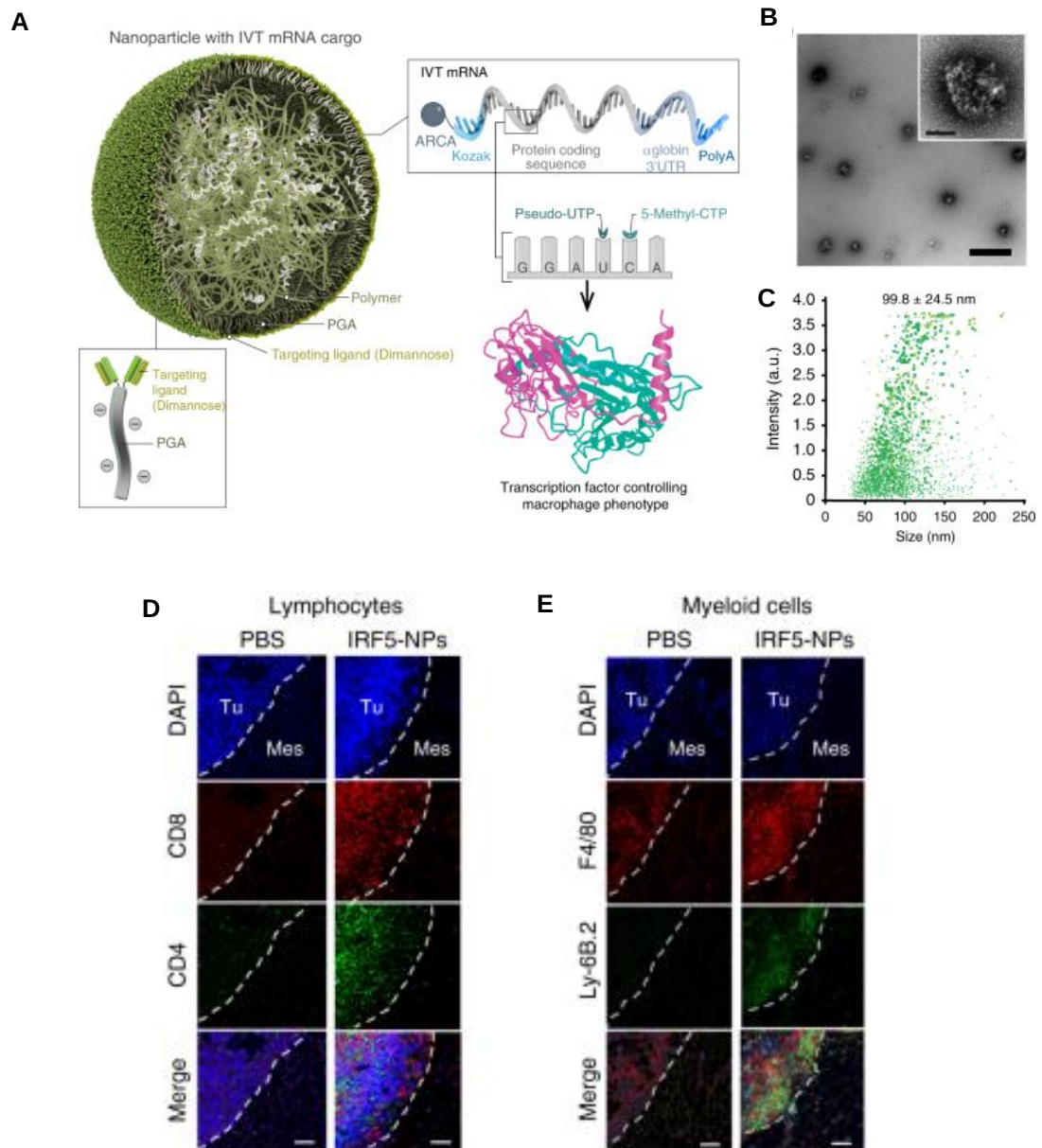


Figure 1.5: (A) Mannose receptor-targeted mRNA nanoparticles efficiently transfect M2 macrophages. Design of macrophage-targeted polymeric NPs formulated with mRNAs encoding key regulators of macrophage polarization. The particles consist of a PBAE-mRNA polyplex core coated with a layer of PGA-Di-mannose, which targets the particles to mannose receptors (CD206) expressed by M2-like macrophages. Also depicted is the synthetic mRNA encapsulated in the NP, which is engineered to encode the reprogramming transcription factors. (B) Transmission electron microscopy of a population of NPs (scale bar 200 nm) and a single NP (inset, scale bar 50 nm). (C) Size distributions measured using a NanoSight NS300 instrument. (D-E) T cells contribute to anti-tumor effects achieved with macrophage-programming nanoparticles. Nanoparticle-mediated macrophage programming increases T cell recruitment into tumor lesions. Shown are representative confocal images of peritoneal metastases of ID8 ovarian cancer cells in the mesentery. Tissues were collected after 6

biweekly i.p. injections of PBS or IRF5/IKK β NPs (50 μ g mRNA/dose), and were stained for the indicated lymphocyte- and myeloid-markers. Tu = Tumor, Mes = Mesentery. Scale bar: 100 μ m. Reprinted with permission from. Zhang, F *et al.* (2019) 'Genetic programming of macrophages to perform anti-tumor functions using targeted mRNA nanocarriers', *Nature communications*.

A critical aspect for the design of a delivery system is the tolerability of the vector. The polycationic nature of PBAEs can however disrupt negatively charged cell membranes to the extent that significant immune responses can be induced. In the case of vaccines and immunotherapies, this inflammatory response may be beneficial, however for therapeutic strategies minimising toxicity is more important. In this light, Irvine and co-workers [73] developed PBAE nanoparticles coated in a phospholipid bilayer to minimise interaction with the cell membrane, following which mRNA was adsorbed to the corona of these hybrid nanoparticles. For their PBAE they built on previous work by Lynn and co-workers and used a 1,4-butanediol diacrylate copolymerised with a bis piperidine monomer, designed to promote endolysosomal escape. Particles were produced either through direct formulation in ethanol, followed by dialysis or a double emulsion technique utilising dichloromethane, with both approaches yielding similar nanoparticle sizes. In further *in vitro* studies the authors demonstrated the ability for these hybrid formulations to induce endosomal escape in DC2.4 dendritic cells. Using Annexin V staining, all lipid enveloped PBAE nanoparticles showed lower toxicities than non-lipid coated formulations, at 50-100 μ g/mL nanoparticle concentrations after 1 h and 12 h of treatment. When formulated with eGFP encoding mRNA, lipid enveloped formulations showed 30% of dendritic cells expressing eGFP, comparable to many commercial non-viral vectors in this cell line. Finally, the lipid coated PBAE formulations displayed substantially improved delivery and gene

expression, compared to non-formulated mRNA when administered intranasally. However, no comparison between lipid enveloped and non-lipid enveloped mRNA was made. These results highlight the versatility of PBAE systems, and their ability to incorporate other 'off the shelf' excipient to boost performance.

The hybrid PBAE-lipid nanoparticle approach has also been investigated for wider delivery applications, including to the lungs, where many immune cells reside, such as alveolar macrophages which form part of our innate immune system. For instance, Anderson and co-workers [77-79] have reported several studies in this area. They highlighted one of their key studies focusing on optimisation of PBAE chemistry for delivery to pulmonary immune cells and lung epithelium. The authors produced a library of PBAEs based on a bisphenol A glycerate diacrylate and 4-(2-amino methyl) morpholine polymer backbone containing different lengths of alkylamine comonomer and various endcaps. Formulations were then co-formulated with mRNA, a range of PEG lipids, phospholipid and cholesterol to produce PBAE lipid hybrids. Given the large formulation space available with these components, a multifactorial experimental design screen was conducted showing the highest transfection efficiency in HeLa cells arising from a C10 alkyl amine comonomer, and diaminopropane end group containing a 7 wt% content of C14-PEG2000 PEGylated lipid. A further partial factorial screen was conducted to identify formulations with higher lung specificity, which showed that hybrid particles containing C18-PEG2000 exhibited high lung specificity. Further investigation demonstrated that when administered intravenously, the lung targeted formulations yielded mRNA expression in 70% of pulmonary epithelial cells.

Analysis of the immune cell reaction showed the highest proportion of transfection within dendritic cells, B cell and alveolar monocytes.

1.3.3 Self-amplifying mRNA

Messenger RNA (mRNA) vaccines and therapeutics have revolutionised our approach to drug and vaccine development, however the short-lived expression of exogenously delivered mRNA represents a significant challenge for therapeutic which require repeat dosing, whilst the high dose requirements also create a cost burden for these new medicines. Innovations in mRNA construct design, such as self-amplifying mRNA (saRNA) have shown to alleviate some of these early challenges [120, 121]. Self-amplifying mRNAs operate by incorporating non-structural proteins which comprise the replication complex derived from alphaviruses into the coding sequence of the mRNA constructs [122, 123]. These are expressed alongside the desired protein/antigen, which recognise the residual saRNA and amplify the sequence, producing further replicases [122]. This cycle repeats itself exponentially, enhancing the expression lifetime of saRNA to over 60 days compared to the 2-7 d lifetime of traditional mRNA [124, 125]. These advantages also enable substantially lower doses, with studies indicating that saRNA vaccines can yield similar levels of protective immunity to mRNA vaccines at greater than 100-fold lower doses also reducing the cost burden of this technology [120, 124]. Despite initially poor results for saRNA in several clinical trials, Japan became the first country to approve an saRNA vaccine (ARCT-154) for protection against severe COVID-19 disease, developed by Arcturus Therapeutics. However, saRNA constructs can be 5-10-fold greater in length compared to conventional mRNA, reaching up to 12000 nt, increasing their vulnerability to endonuclease degradation,

therefore requiring unique formulation approaches [124]. Furthermore, the difficulty in incorporating modified bases while retaining the amplifying function also potentiates their formulation requirements. Whilst there are now many examples in the literature using lipid nanoparticles and other polymeric non-viral vectors for saRNA, there remain relatively few examples of PBAEs used for saRNA [80, 126].

Green and co-workers [80] used self-amplifying mRNA to great effect by showcasing their huge potential as low dose vaccines, delivered using PBAEs. First a semi- high throughput approach was conducted to produce 196 unique polymers with varying backbone monomers, pendant amines and end caps, which were further tests for transfection efficiency of self-amplifying mRNA *in vitro* (Figure 1.6). As with previous studies, they identified that inclusion of higher mol fractions of lipophilic monomers and end caps containing ionisable amines significantly enhanced expression efficiency even as low as 1 ng/well, 100-200-fold lower than the conventional dose of mRNA. Interestingly, the authors showed that coformulation with DMG PEG2000, a common PEGylated lipid was essential for mRNA expression when administered intramuscularly, attributed to improved extracellular transport. Their optimised PBAEs for were also potent delivery systems for rabies virus glycoprotein saRNA vaccine constructs. Mice vaccinated with 100 ng saRNA exhibited high neutralising antibody titers after challenge with live rabies virus, significantly above 0.5 IU mL⁻¹ which is considered as the level required for protective immunity.

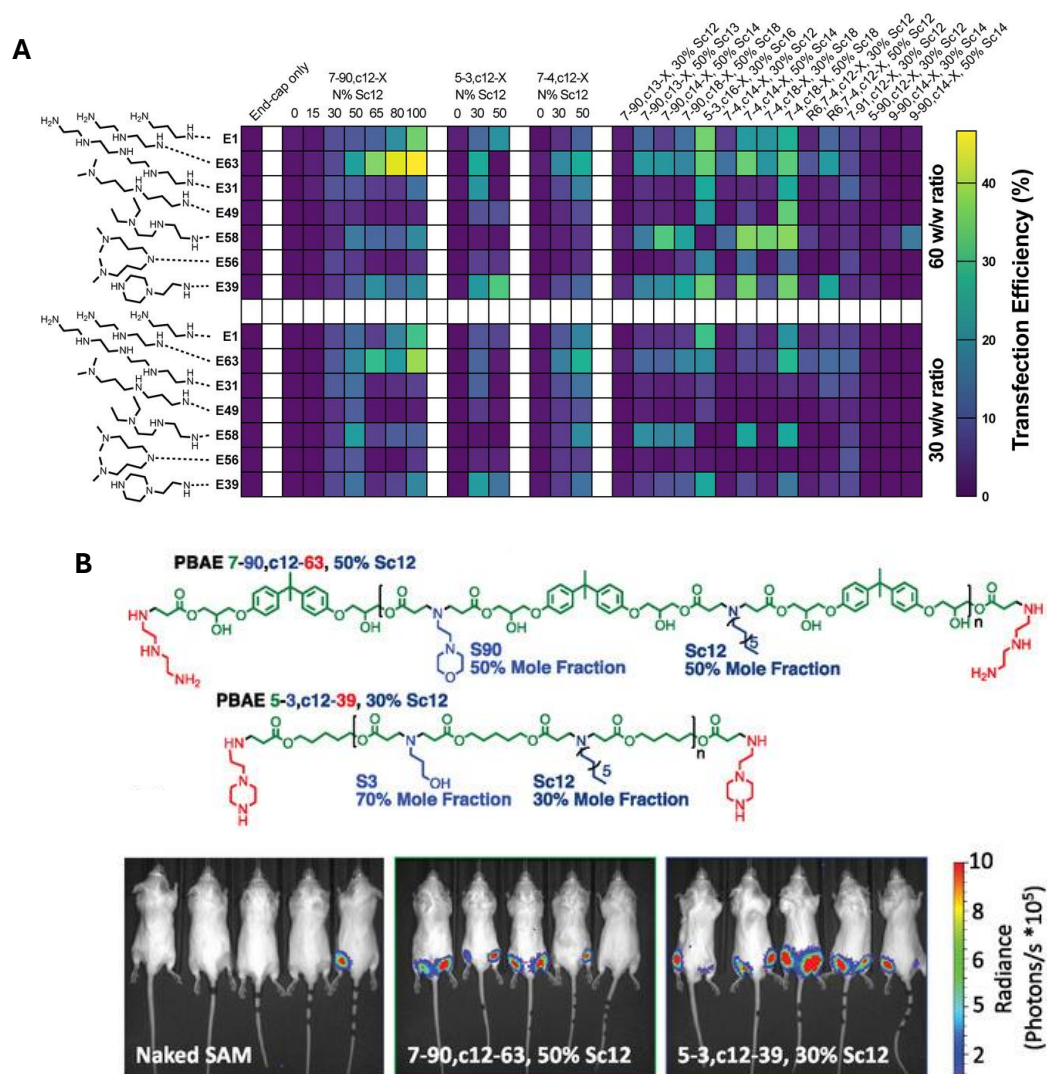


Figure 1.6: (A) A library of 196 PBAEs were synthesised combinatorially from 28 base acrylate terminated polymers and seven end-cap monomers. Polymers were screened in 384-well plates in C2C12 cells for transfection at a dose of 1 ng of eGFP SAM per well at two w/w ratios. Each cell of the heatmap shows mean of two wells of a 384-well plate. Transfection of myoblasts using eGFP SAM was strongly improved compared to 5mou eGFP mRNA used at the same dose. (B) Structures of lead polymers for intramuscular administration and representative IVIS images with the top two nanoparticle formulations compared to naked SAM. Reprinted with permission from. Wilson, D. R.; *et al.* (2018) 'Biodegradable STING agonist nanoparticles for enhanced cancer immunotherapy. *Nanomedicine: Nanotechnology, Biology and Medicine*.

We also recently reported the use of PBAE complexes for delivery of saRNA [126]. As discussed above one of the critical challenges for polycation use as non-viral vectors is to minimise disruption of the cell membrane through charge mediated interaction. We therefore investigated this by incorporating a

polyanion, polyglutamic acid, into saRNA PBAE complexes in two different ways, coating preformed complexes or mixing all three components in one step [126]. PBAE-based polymers were prepared from 1,6-hexanedioldiacrylate and 4-aminobutanol. These results demonstrate that incorporating γ -PGA into polyplexes enhanced transfection effectiveness in HEK293T and A431 cells, while maintaining polyplex size. This inclusion resulted in a significant decrease in zeta potential, reducing the toxicity of the ternary complexes in moDC, NIH3T3, and A431 cells. Furthermore, the presence of γ -PGA contributed to colloidal stability, reducing the aggregation of ternary complexes, as evidenced by insignificant changes in the polydispersity index (PDI) after freeze-thaw cycles.

1.3.4 Therapeutic oligonucleotides

Oligonucleotides are short nucleic acid sequences which have been primarily investigated as regulators of gene expression. These operate by selectively binding to complementary mRNA sequences known as RNA interference (RNAi) to limit the expression of disease related proteins and classically 'undruggable' targets. Oligonucleotides exist in different classes, such as small interfering RNAs (siRNA), microRNAs (miRNA) and antisense oligonucleotides (ASO) for RNA interference each displaying unique mechanisms for RNAi [127, 128]. Furthermore, other oligonucleotides such as short guide RNAs are critical for the function of CRISPR-Cas gene editing technologies. In an immunology context, endogenous microRNAs modulate inflammation and immunity in response to pathogens, due to their role in cell-cell communication[129], hence have garnered interest as a route to modulate the immune system. Furthermore, the solid phase synthesis methodologies for these oligomeric

nucleic acids also means that base, backbone, and sugar modifications can be made with ease to tune stability and affinity [130].

One such approach is RNA interference, which utilizes siRNA to target and silence specific genes within the genome upon introduction into cells. Despite its potential, clinical applications of RNAi have been hindered by challenges in efficiently delivering siRNA across biological barriers. Existing delivery vehicles typically face limitations in effectively delivering siRNA, necessitating chemical modifications for siRNA encapsulation and delivery. An ideal nanomaterial for siRNA delivery must possess the capability to bind and self-assemble with shorter and stiffer siRNA molecules, in comparison to plasmid DNA, to form stable nanoparticles. Additionally, it should facilitate the efficient release of siRNA upon entering the cytoplasm.

In response to these challenges, Jordon Green and co-workers in 2013 performed studies on siRNA delivery using various types of PBAEs. In one study they synthesized and characterized a novel bioreducible PBAE polymer, R647 (2,2'-disulfanediylbis(ethane-2,1-diyl) diacrylate was polymerised with 4-amino-1-butanol and then end-capped with 1-(3-aminopropyl)-4-methylpiperazine) demonstrating high efficacy and low cytotoxicity [112]. R647 exhibited comparable or slightly better siRNA binding than its non-reducible counterpart, 647, and successfully condensed siRNA into nanoparticles with similar properties. Notably, R647 showed rapid siRNA release in a reducing environment, mimicking the cytosol. In human brain cancer cells, R647 nanoparticles achieved near-complete gene knockdown without toxicity, in contrast to 647 nanoparticles, which were either toxic or ineffective. Therefore,

they concluded that this new polymer class holds promise as a safe and effective siRNA delivery vehicle.

In addition, to improve their novel carrier, they aimed to tackle the instability of siRNA nanoparticles and the necessity for precise cytoplasmic targeting, and accordingly designed the PBAE-based polymers that could efficiently form siRNA nanoparticles by carefully balancing polymer bioreducibility and hydrophobicity [113]. They showed that the inclusion of hydrophobicity in PBAE was important to improve particle stability, a characteristic known to enhance the delivery of siRNA in human primary glioblastoma. However, in human primary noncancer brain cells, it was less effective, indicating potential cancer specificity. The tuneable hydrophobicities of bioreducible PBAEs suggest exciting prospects as safe and efficient siRNA delivery vehicles for nanomedicine applications.

1.4 Conclusions and Research Challenges

Nucleic acid medicines offer promising therapeutic avenues for a wide array of diseases by regulating gene expression in specific cells. Despite this potential, their practical application is hindered by challenges in effective and safe gene delivery systems. Cationic polymers, particularly PBAEs, have emerged as promising non-viral gene delivery vectors due to their versatile physicochemical properties, allowing for tailored modifications to meet specific requirements. PBAEs offer several advantages, including pH-dependent degradability, excellent nucleic acid condensation capabilities, and biocompatibility. Their ability to form nanoparticles through electrostatic interactions makes them

suitable for delivering various nucleic acid payloads, including plasmid DNA, mRNA, and siRNA. Recent studies have demonstrated the effectiveness of PBAEs in delivering nucleic acids to target cells, showcasing their potential in diverse biomedical applications.

Continued research efforts should focus on optimizing the design of PBAEs to enhance their efficiency and safety as gene delivery vectors. This includes exploring new monomer combinations, end-group modifications, and strategies to improve targeting and controlled release mechanisms. Besides, while PBAEs show promise in delivering nucleic acids, there is a need for comprehensive studies to evaluate their long-term toxicity and biocompatibility. Understanding the interactions of PBAEs with the body over extended periods is crucial for their clinical translation. Furthermore, researchers should explore advanced delivery strategies utilizing PBAEs, such as hydrogels, polyelectrolyte multilayer films, and nanoparticles. These approaches offer unique advantages in terms of localized delivery, sustained release, and improved therapeutic efficacy.

Coating PBAE nanoparticles with ligands can enhance their functionality, reducing interactions with serum proteins, prolonging circulation time, and facilitating cell-specific targeting. Functionalisation with PEG can minimize interactions with serum proteins and blood clearance by the reticuloendothelial system (RES), while also improving tissue mobility [131, 132]. Furthermore, hybrid nanocarriers formed by combining PBAEs with various materials, such as inorganic materials, other polymers, surface-active molecules, and lipids show promise for enhancing delivery efficacy and cell transfection.

Efforts should be directed towards translating PBAE-based gene delivery systems into clinical applications. Each component of these nanocarriers plays a crucial role in their effectiveness, requiring careful consideration and a defined protocol for their development. To move towards clinical applications, researchers must establish clear manufacturing processes tailored to target cells and nucleic acid types, implement quality control methods, develop imaging techniques to monitor distribution in vivo, address potential immunogenicity, and refine approaches for controlling the release of therapeutic genes. Several key issues hinder the clinical development of polymer-based nanocarriers, including challenges in large-scale manufacturing, ensuring biocompatibility and safety, and navigating government regulations. Understanding how disease pathophysiology influences the behaviour of these carriers in vivo is essential for successful translation to clinical use. More research is needed to explain the relationship between nanocarrier behaviour and patient biology.

In summary, PBAEs represent a versatile and promising platform for nucleic acid delivery, with ongoing research aimed at optimising their design, understanding their long-term effects, and translating them into clinically relevant therapies for various diseases. Continued innovation in this field holds the potential to revolutionise gene therapy.

1.5 Aims and Objectives

The primary goal of this project was to investigate innovative methods for enhancing PBAE functionality and their subsequent utilization in gene delivery.

Our aim was to devise new and effective strategies for gene delivery which are recognised as significant contributors to many diseases from infectious diseases to cancer treatment. We hypothesised that employing optimised PBAEs-based carriers in gene delivery could facilitate cellular uptake, thereby improving the efficacy of the gene therapy. In pursuit of our objective, we utilised PBAEs as a core scaffold, enhancing them through synthetic modifications at the end groups.

As mentioned above, a library of polymers can be synthesised easily due to the commercial availability of various primary amines and bisacrylamides that allows the most effective vector to be identified. [133-135]. Previous studies have reported over 2000 different linear PBAEs via high throughput synthesis method. [18, 135] After the formulation with pDNA, they systematically evaluated the biophysical and transfection properties and then identified one as a high-performing candidate contained 1,4-butanediol diacrylate (BDD) and 5-amino-1-pentanol (5-AP) and end-capping with diamines. Until now, this polymer has been applied in a variety of clinically relevant scenarios as an efficient gene delivery vector [136-138]. Due to the promising treatment properties of BDD and 5-AP, these monomers were aimed to be used for its possible further optimisation in this study. Then, acrylate-terminated PBAEs were used to produce polymer triblock copolymers with positively charged PBAE cores for delivering negatively charged nucleic acids. In addition, amine terminated PBAEs were employed to design small molecule conjugation, resulting in targeted gene delivery. Through these strategies, our aim was to showcase the flexibility, versatility, and suitability of PBAEs as carriers for gene delivery in various medicines. One of the key areas this research innovated was

to utilise the end-group chemistry of PBAEs to enhance delivery efficiency of different nucleic acid therapeutics, either through polymer conjugation (Chapters 2 and 3), or by introducing immunostimulatory compounds as potential adjuvanted nucleic acid vaccines (Chapter 4).

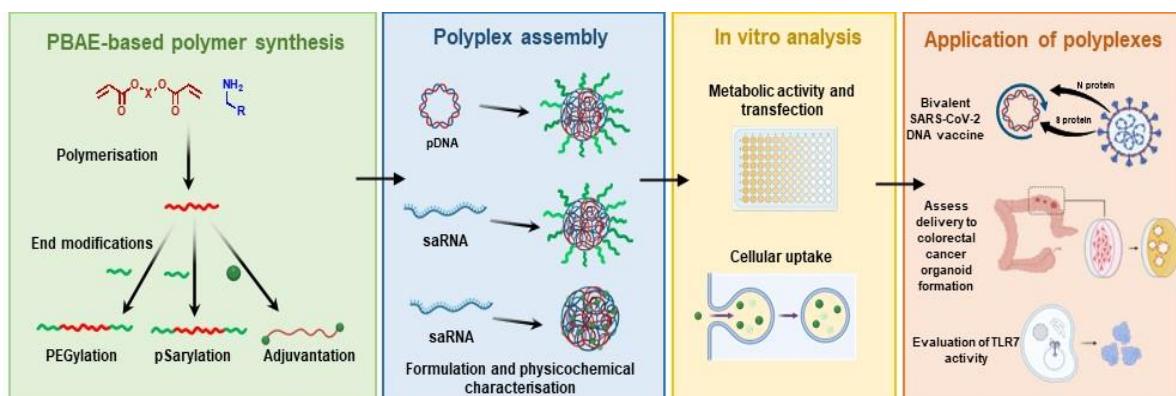


Figure 1.7: Overview of undertaken work. PBAE-based polymer synthesis and end modifications. Polyplex formation utilising pDNA or saRNA. In vitro analysis of synthesised complexes. Application of these formulations.

In chapter 2, we developed PEGylated and non-PEGylated PBAEs for efficient DNA binding and delivery, assessing their performance in terms of safety and effectiveness in cell cultures (Figure 1.7). A primary aim of this chapter was to examine how modulating PEG density on the surface of PBAE polyplexes influences delivery in vitro and in animal models, then further identify how this may influence the immune response generated by polyplex-formulated SARS-CoV-2 DNA vaccines, particularly examining antigen-specific T-cell responses **(Chapter 2)**.

Following the encouraging results of PEGylated PBAEs as gene delivery vehicles, we further searched for alternative hydrophilic polymers (Figure 1.7). This is due to the increasing concern regarding reactogenicity induced by PEG or PEGylated drugs, particularly in light of the heightened antibody response

observed with PEGylated lipids in COVID-19 vaccines [139, 140]. To address this issue and maintain the potency and tolerability of nucleic acid therapeutics, we explored polysarcosine (pSar) as a promising alternative. pSar, a polypeptoid that degrades into a non-toxic and endogenous amino acid, offers advantages over other potential "stealth" polymers due to its physicochemical properties and reduced immunogenicity [141, 142]. Previous studies for the lipid-based nanocarriers showed that pSar-liposomes exhibited reduced levels of immunoglobulin (Ig), specifically IgM and IgG, compared to PEG-liposomes [143, 144]. Additionally, in a repeated dose pharmacokinetics study, the pSar coating on liposomes may help prevent the accelerated blood clearance (ABC) phenomenon [145]. Therefore, we aimed to investigate the conjugation of pSar with PBAEs, a strategy that has not been previously reported, to develop a versatile and tuneable RNA delivery system with enhanced biocompatibility and efficacy. In the LNP-based studies mentioned earlier, the length of pSar was chosen similarly to how PEG length is typically selected for such formulations. In our case, based on the promising results from the PEGylated-PBEA study, we used an 800 Da length for pSar. For an enhanced RNA technology, we utilised self-amplifying messenger RNA (saRNA). The saRNA technology incorporates replicase genes derived from the alphavirus genome within the construct, enabling exponential amplification of the construct within the cytosol upon translation, resulting in enhanced protein expression levels at lower doses. **(Chapter 3).**

Lastly, considering the enhance immune responses of traditional vaccines and immunotherapies by including adjuvants, which are molecules co-formulated with the active ingredient, we conjugated a small molecule to our nanocarrier

(Figure 1.7). Adjuvants can work by either promoting humoral immunity through controlled antigen release, or preferably by directly activating cellular immunity. Since, in contrast to humoral immunity, cell-mediated immunity does not depend on antibodies for its adaptive immune functions [146]. Instead, it is primarily driven by mature T cells, macrophages, and the secretion of cytokines in response to antigens [147]. This activation mimics natural infection processes by binding to pathogen recognition receptors (PRRs) on immune cell surfaces. Toll-like receptor agonists (TLRa's), for instance, stimulate PRRs in antigen-presenting cells (APCs) and macrophages, triggering the expression of pro-inflammatory cytokines and initiating the innate immune response and evaluated extensively in the literature [148, 149]. Specifically, TLR7 has attract attention as a target for vaccine adjuvant [150]. As previous studies showed that showed that dendritic cell activation, is considered to be a key event, is dependent on engagement of TLR7 [151, 152]. Therefore, we aimed to develop a novel adjuvanted formulation for saRNA by conjugating TLR7a, loxoribine, onto a PBAE-based polymer. To the best of our knowledge, this has not been previously addressed in the literature. Loxoribine was selected as the TLR7 agonist due to its commercial availability and its functional group, which can readily react with our thiol end-capped PBAEs. By harnessing the intrinsic chemistry of loxoribine and the well-established delivery capabilities of PBAE polymers for mRNA and saRNA, we aim to formulate adjuvanted polyplexes capable of efficient delivery, enhanced transfectability, and stimulation of the TLR7 pathway in cells. Our objectives include characterising the delivery efficiency and transfection capability of the formulated polyplexes, as well as evaluating their ability to activate the TLR7 pathway **(Chapter 4)**.

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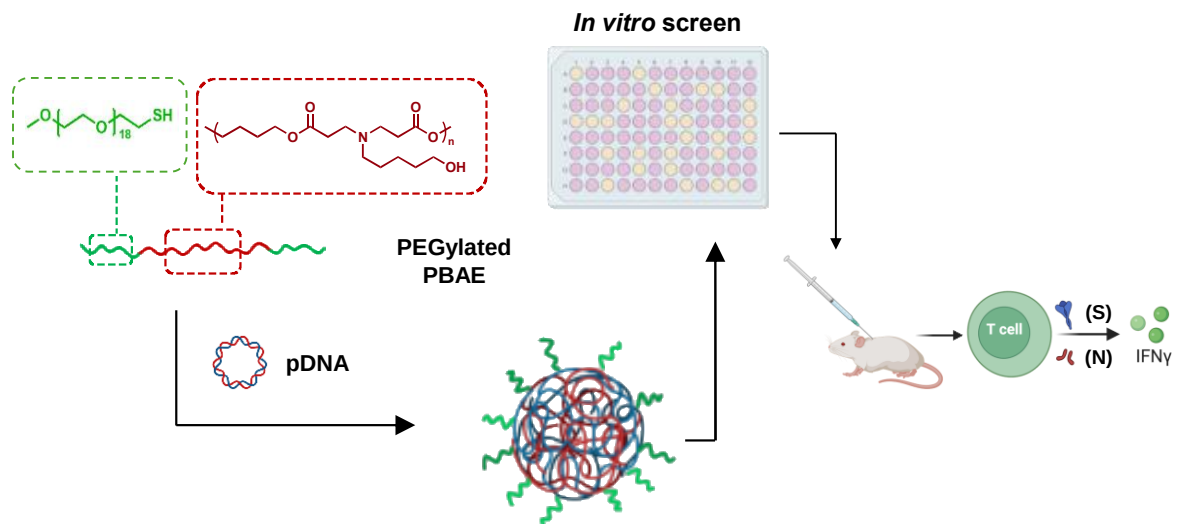
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Chapter 2

Sparsely PEGylated poly(beta-amino ester) polyplexes enhance antigen specific T-cell response of a bivalent SARS-CoV-2 DNA vaccine



Abstract

DNA technology has emerged as a promising route to accelerated manufacture of sequence agnostic vaccines. For activity, DNA vaccines must be protected and delivered to the correct antigen presenting cells. However, the physicochemical properties of the vector must be carefully tuned to enhance interaction with immune cells and generate sufficient immune response for disease protection. In this study, we have engineered a range of polymer-based nanocarriers based on the poly(beta-amino ester) (PBAE) polycation platform to investigate the role that surface poly(ethylene glycol) (PEG) density has on pDNA encapsulation, formulation properties and gene transfectability both in vitro and in vivo. We achieved this by synthesising a non-PEGylated and PEGylated PBAE and produced formulations containing these PBAEs, and mixed polyplexes to tune surface PEG density. All polymers and co-formulations produced small polyplex nanoparticles with almost complete encapsulation of the cargo in all cases. Despite high gene transfection in HEK293T cells, only the fully PEGylated and mixed formulations displayed significantly higher expression of the reporter gene than the negative control in dendritic cells. Further in vivo studies with a bivalent SARS-CoV-2 pDNA vaccine revealed that only the mixed formulation led to strong antigen specific T-cell responses, however this did not translate into the presence of serum antibodies indicating the need for further studies into improving immunisation with polymer delivery systems.

Key words: vaccine, DNA, delivery, nanoparticle, poly(beta-amino) ester, polyplex, COVID-19, SARS-CoV-2

2.1 Introduction

Vaccines are a critical form of prophylactic medicine, which have prevented more deaths than any other medical intervention in history [1]. Despite this, there is still a need to enhance the safety profile, boost efficacy in poorly vaccine-responsive segments of the population and accelerate manufacturing in order to respond rapidly to epidemic and pandemic scenarios [2]. DNA vaccines offer many of these advantages [3]. They operate by administering antigen encoding DNA which instructs local cells at the injection site to express a pathogen related antigen, initiating immunisation and thus protection against disease when challenged by the pathogen itself [3-5]. Although mRNA vaccines have been popularised throughout the COVID-19 pandemic, the overall stability of DNA compared to mRNA may enable DNA technologies to be utilised more widely in low to middle income countries, where access to cold-chain storage is limited [6].

The poor cellular association of DNA when administered 'naked', necessitates the development of safe and effective delivery systems to transport the nucleic acid vaccine construct to dividing cells [7]. The majority of clinical studies for DNA vaccines and therapies have utilised physical delivery systems, such as electroporation [8], jet-injectors [9], or gene gun systems [10], for intradermal administration [9], which is exemplified with the only licensed plasmid DNA vaccine, ZyCoV-D (Cadilla Healthcare) for COVID-19 which was approved for emergency use in India [11]. However, the lack of scalability for this approach and advanced training requirements for healthcare professionals means that these delivery approaches may not be appropriate in rapid response indications. While adeno-associated virus (AAV) vectors overcome some of

these challenges, packaging constraints, expensive cell-based manufacture and potentially harmful immunogenic responses necessitates the need for alternative vectors [12]. For this reason, significant efforts have been placed on developing non-viral nanoparticle-based delivery systems which offer unlimited cargo capacity, biologic free manufacture, and reduced safety concerns [13-16].

Cationic polymers have emerged as a highly versatile nucleic acid delivery platform, which operate by condensing negatively nucleic acids into small, 50-200 nm diameter, nanoparticles typically termed 'polyplexes', assembled through supramolecular electrostatic interactions [17, 18]. Such polyplex nanoparticles stabilise the nucleic acid cargo and facilitate transportation across the physiological and cellular barriers required. Although lipid nanoparticles have been the most accepted delivery approach since their use in the two licensed mRNA COVID-19 vaccines [19-21], polycations offer a significantly broader chemical space to tune formulation stability [22] and specificity towards immune cells, which are vital for vaccination. However, the high charge density on the polyplex surface means that there are potential toxicity concerns arising from cell membrane disruption, non-specific protein absorption, pain at the injection site or generalised reactogenicity towards the vaccine [23, 24]. To date, most researchers have focused on tuning the polymer composition and architecture to circumvent these outstanding challenges.

One especially popular strategy has been to attach poly(ethyleneglycol) (PEG) chains to the surface of polyplexes to attenuate their interaction with the cell membrane [25]. However, there is limited understanding on how PEG grafting density may influence expression and tolerability of a DNA vaccine [26]. This is

particularly important in a vaccine setting where the formulation itself may play a substantial role in activating specific cellular immunity pathways through membrane damage as has been seen with the LNPs for the two licensed COVID-19 vaccines [27-29].

In this study we explore the relationship between PEG density on the surface of polyplexes and their ability to deliver plasmid DNA in vaccine-relevant *in vitro* and *in vivo* models. For this we prepared PEGylated and non-PEGylated PBAEs as hydrolytically degradable, non-toxic, and efficient nucleic acid binding polycations. We demonstrated the high tolerability, potent transfection efficiency and fast intracellular trafficking of pDNA loaded polyplexes derived from each of these materials in HEK293T cells and a dendritic cell line. PEG density was modulated by preparing polyplexes containing mixtures of the polycations. The library of polycations was then further examined *in vivo* for their performance as delivery vectors for pDNA vaccines by formulating them with a bivalent SARS-CoV-2 vaccine encoding for the spike and nucleocapsid proteins, with the mixed polycation formulation eliciting strong antigen specific T-cell response (Figure 2.1).

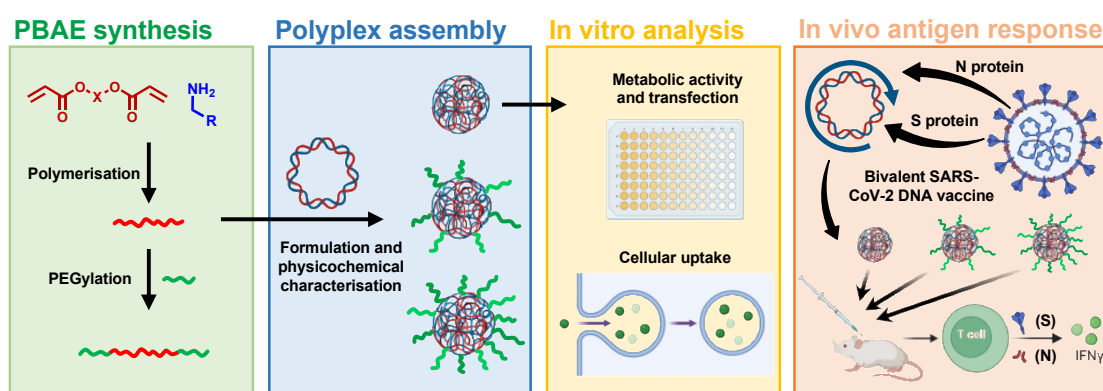


Figure 2.1. Schematic representation of PBAE synthesis, polyplex assembly, in vitro analysis and in vivo antigen response of a bivalent SARS-Cov-2 pDNA vaccine.

2.2 Materials and Methods

2.2.1 Materials

1,4-butanediol diacrylate (BDD), 5-aminopentan-1-ol (5-AP), triethylamine (TEA), PEG-thiol (mPEG-SH), Fluorescein isothiocyanate (FITC), diethyl ether, tetrahydrofuran, sodium acetate (NaOAc) buffer (pH:5.0), dye Gel Red and Goat-anti-mouse IgG, Fc biotin and red CellMask™ Plasma Membrane stain were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO), loading buffer, Tris-acetate-EDTA (TAE) buffer, Lipofectamine 2000, Hoechst 33342, LysoTracker red and 96-well plates were obtained from ThermoFisher. Dulbecco's Modified Eagle Medium (DMEM) cell culture medium, RPMI 1640 cell culture medium, fetal bovine serum (FBS) and Opti-MEM were bought from Gibco, Invitrogen, Carlsbad, CA, USA. PrestoBlue reagent and ONE-Glo D-luciferin substrate were purchased from Promega, UK. Finally, S1 protein (Z03501) and N protein (Z034880) were obtained from GenScript.

2.2.2 Instrumentation and Analysis

2.2.2.1 NMR Spectroscopy

All ^1H -NMR spectra were recorded in ppm (δ) at 400 MHz in d_6 -DMSO using a Bruker Advance III MHz spectrometer that was maintained at 25°C. To analyse the spectra MestReNova 6.0.2 copyright 2009 (Mestrelab Research S.L.) was applied.

2.2.2.2 Gel permeation Chromatography

The gel permeation chromatography (GPC) analysis was performed using the PL-50 instrument equipped with a dual angle light scatter (LS), viscometry (VS)

and differential refractive index (DRI) detection. The system was equipped with a PLgel 5 μm guard column and a 2 $\times$ PLgel Mixed D columns (300 $\times$ 7.5 mm). Dimethylformamide (DMF) with LiBr was utilised as an eluent with a flow rate of 1 mL/min at 50°C. The instrument was calibrated through Poly(methyl methacrylate) standards (Agilent EasyVials) between 550-955500 g mol⁻¹. Using conventional calibration, Cirrus GPC software, dispersity ($\mathcal{D}$) values, molecular weight (M_w) and experimental molar mass ($M_{n,SEC}$) were detected.

2.2.2.3 DLS and Zeta Potential

The dynamic light scattering (DLS), Zetasizer nano-ZS90 (Malvern, Inc.), instrument was used to characterise the particles in terms of the size, PDI and zeta potential. The instrument was used at 25°C to determine the zeta potential, average hydrodynamic diameters, and polydispersity index (PDI) of polyplexes.

2.2.2.4 Transmission Electron Microscopy

Briefly, glow discharged Formvar/carbon coated TEM grids were used, on which were placed the formulation samples (13 μL), which were left for 15 minutes, then the excess solution was removed and the samples allowed to dry at room temperature. Following this, 10 μL of 2% aqueous uranyl acetate was applied to each grid and left for 10 seconds. After air drying, imaging was performed using a Tecnai G2 Spirit TEM with BioTwin lens configuration (Thermo Fisher Scientific, Eindhoven, The Netherlands) at an accelerating voltage of 100 kV.

2.2.3 Polymer Synthesis and Characterisation

2.2.3.1 PBAE synthesis

PBAEs were synthesised using the following two-step general procedure. BDD and 5-AP were mixed at 1.1:1 (diacrylate:amine) molar ratio into a test tube (20 mL). The components were dissolved in DMSO to reach a concentration of 0.5 mg/mL, then a stir bar was added, and a rubber septum was used to seal the tube. The mixture was heated in the dark at 90°C for 24 h with continuous stirring at 300 rpm. The reaction mixture was then used directly for end-capping as detailed below.

2.2.3.2 End capping for non-PEGylated PBAEs

To produce non-PEGylated PBAEs, an excess amount (molar equivalent 0.5) of 5-amino-1-pentanol was added directly to the PBAE solution for 24 h at room temperature. Polymers were isolated by diluting the reaction mixture into 1-fold volume of tetrahydrofuran (e.g. 5 mL of DMSO and 5 mL of tetrahydrofuran) followed by dropwise precipitation in diethyl ether. The polymer was collected via centrifugation and dried under reduced pressure at 37°C to yield a yellow viscous liquid. Finally, polymers were stored at -20°C as solutions (100 mg/mL) in DMSO.

2.2.3.3 End capping for PEGylated PBAEs

To obtain PEG-PBAE-PEG triblock copolymers, the initial non-endcapped PBAE solution was mixed with 800 Da methoxy-PEG-thiol (mPEG-SH) polymer at a molar ratio of 1:2.5 acrylate:mPEG-SH in DMSO containing TEA (0.1 molar equivalents relative to thiol) at room temperature for 24 h. The PEGylated PBAE was isolated and stored as described above.

2.2.3.4 Synthesis of Fluorescently Labelled PBAEs

Fluorescently labelled PBAEs, OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG were synthesised using the following generation procedure. PBAEs were dissolved in 5 mL DMSO (0.5 mg/mL) in a 20 mL vial. FITC was added to vials at a 1:10 (moles of end amine: FITC) molar ratio and TEA was added to the mixture at a 0.1 molar ratio. The reaction mixture was allowed to stir for 24 h in the dark at room temperature. The reaction mixture was then dialysed using molecular weight cut-off 3,5 kDa against 250 mL NaOAc buffer (pH:5.0), in the dark. Purification was continued for 4 days and the dialysis medium refreshed 2 times in a day. After freeze drying, FITC-labelled polymers were collected as a yellow solid and were stored at -20°C as solutions (100 mg/mL) in DMSO.

2.2.3.5 Polymer Buffering Capacity Assay

The buffering capacity of PEGylated and non-PEGylated polymers was evaluated by acid-base titration over the pH range of 10.0–3.0. Briefly, 2 mg of polymer was dissolved in 30 mL of 0.1 M NaCl aqueous solution, and the solution was adjusted to pH 10.0 using 0.1 M NaOH. Then, precise volumes (between 10-20 μ L) 0.1 M HCl were added until a pH of 3 was achieved. The pH after each addition of HCl was recorded. 0.1 M NaCl was set as negative control.

2.2.4 Synthesis of pDNA

Two different types of pDNA (luciferase and green fluorescent protein (GFP)) (Addgene #693601) was transformed into *Escherichia coli* and cultured in 100 mL of Luria broth (LB) with 100 μ g/mL of ampicillin. The pDNA was isolated using a kit Plasmid Plus MidiPrep (QIAGEN, UK). The concentration and purity

of pDNA was measured on a NanoDrop One (ThermoFisher, UK) and Agarose gel electrophoresis, respectively.

2.2.5 Preparation of Polyplexes

Polyplexes were prepared between positively charged polymers and negatively charged DNA by mixing various w/w ratios (32, 64 and 128). Firstly, the working dilutions of polymers and DNA were prepared in 25 mM NaOAc buffer (pH:5.0). Depending on the desired w/w ratio, differing amounts of polymer stock solutions were mixed with nucleic acids in the buffer, gently mixed using a pipette and incubated at room temperature for 30 minutes. Whilst preparing the PBAE/DNA complexes, taking a w/w 64 ratio as an example, 10 µg/mL of the DNA stock solution was mixed with 2.560 mg/mL PBAE by pipette using a 25 mM NaOAc buffer.

2.2.6 Encapsulation Efficiency Using Gel Electrophoresis

A 1% agarose gel was prepared in 50 mL TAE buffer (40 mM Tris-acetate, 1 mM EDTA) using a microwave (approximately 40 seconds) to dissolve the gel and then was stained with the 2 µL of dye Gel Red for the nucleic acid bands' visualization. Finally, 10 µL of nanoparticle solution were mixed with 2 µL of loading buffer, after pipette, 12 µL of the solutions were loaded in each well. Electrophoresis was conducted for 45 min at 90V. The visualisation of the DNA bands was detected with a UV detector (Bio-Rad) at 312 nm.

2.2.7 In Vitro Experiments

2.2.7.1 Cell Culture

HEK293T cells were a gift from Imperial College London and cultured in 10 % (v/v) FBS containing high glucose DMEM cell culture medium. DC2.4 cells were obtained from ATCC and cultured in RPMI 1640 cell culture medium. Media containing l-glutamine was supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), non-essential amino acids (0.1 mM) and 10 % FBS. Absence of mycoplasma was confirmed using polymerase chain reaction. Cells were grown to 90 % confluence in a humidified incubator at 37°C (5% CO₂) and detached with 1x trypsin/EDTA. Viability was assessed using Trypan Blue staining.

2.2.7.2 Cell Viability Assays

To investigate the cytotoxicity of the formulations, a cell viability assay was performed using HEK293T and DC2.4 cell lines. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells/well. For the treatment, the medium was aspirated, and cells were treated with 200 μ L of Opti-MEM containing various w/w ratios of formulations complexed with 10 μ g/mL fluc pDNA concentration (20 μ L of polyplex solution). After 4 hours, nanoparticle solutions were aspirated and 100 μ L of complete medium was added to wells. At 24 hours posttreatment, each well was treated with 100 μ L of 10% PrestoBlue reagent and allowed to incubate for 1 h. Then, the total volume was transferred to a black 96-well plate and the FLUOstar Omega plate reader (BMG LABTECH, UK) was used to determine the fluorescent intensity (540 nm – 590 nm) of each well and normalized to the medium control.

2.2.7.3 In Vitro Transfection Experiments

In vitro transfection experiments were performed using HEK293T and DC2.4 cell lines and commercially available transfectant agent Lipofectamine 2000 was used as positive control. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells per well. For the treatment, the medium was aspirated, then cells were transfected with 200 μ L of Opti-MEM containing various w/w ratios of formulations complexed with 10 μ g/mL fluc pDNA concentrations (20 μ L of polyplex solution). After 4 hours, nanoparticle solutions were aspirated and 100 μ L of medium was added to wells. At 24 hours posttreatment, 50 μ L of media was removed then 50 μ L of the ONE-Glo D-luciferin substrate was placed into each well and mixed well by pipette. Finally, the total 100 μ L was placed in a 96-well plate and FLUOstar Omega plate reader (BMG LABTECH, UK) was used to determine the luminescence.

2.2.7.4 eGFP Expression of pDNA

To visualise transfection efficiency, GFP encoded pDNA + BDD-mPEG (w/w 64) formulation was prepared and eGFP expression was monitored. Briefly, HEK293T cells were plated in CellView™ 35 mm diameter glass bottom cell culture dishes at a density of 40×10^4 cells per well 24 h prior to transfection. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing BDD-mPEG + pDNA (10 μ g/mL). Cells were incubated for 4 hours at 37°C with 5% CO₂. Then, nanoparticles were aspirated, and cells washed 3 times with PBS and stained with 10 μ g/mL Hoechst 33342 and 10 μ g/mL red CellMask™ Plasma Membrane stain applied in PBS solution. After 30 minutes, the staining solution was aspirated, and cells were washed 3 times with PBS then FluoroBrite DMEM was added to cells. Imaging was performed

using confocal microscopy (Leica, Germany) using LAS X software on DAPI and GFP filters. ImageJ software was used to analyse the microscopy data.

2.2.7.5 Cellular Uptake of pDNA using Confocal Microscopy

DC2.4 cells were seeded in CellView™ 35 mm diameter glass bottom cell culture dishes at a density of 30×10^4 cells per well. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing FITC labelled BDD-mPEG + pDNA (0.01 mg/mL). Cells were incubated for 1 and 4 hours at 37°C with 5% CO₂. Then, nanoparticle solutions were aspirated, and cells washed 3 times with PBS and stained with 10 µg/mL Hoechst 33342 and 75nM LysoTracker red applied in PBS solution. After 30 minutes, the solution was aspirated again, and cells were washed 3 times with PBS then FluoroBrite DMEM was added to cells. Imaging was performed using Leica confocal microscopy with LAS X software on DAPI, FITC and LysoTracker filters. ImageJ software was used to process the images.

2.2.7.6 Imaging Cytometry

DC2.4 cells were seeded in a clear 6-well plate at a density of 40×10^5 cells per well. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing BDD-mPEG + pDNA (0.01 mg/mL) formulation. After 4 hours, the nanoparticle solutions were aspirated and 150 µL of 0.05% trypsin was used for detachment of cells from wells. After incubation for 15 minutes, 300 µL FACS buffer was used to neutralise the trypsin and cells were centrifuged at 1500 rpm for 10 minutes. Following this, cells were stained with Zombie Violet (1:500) dye for 30 minutes at room temperature to help with gating on live cells. Then, cells were fixed with 100 µL of 4% paraformaldehyde in PBS for 20 minutes. Fixed cells were centrifuged,

and pellets resuspended in 50 μ L of PBS. Data were acquired using an Image Stream 100 (Amnis, Seattle, US) and on single cell in focus circumstances was used for analysis. Ideas Software (Amnis, Seattle, WA, USA) was used for data analysis. Total cell fluorescence was calculated by default total cell masks for the measurement of the nanoparticle internalisation and cell interiors were identified using the area erode tool (brightfield channel). Internalisation index was shown as percentage of maximum internalisation (percentage of interior cell fluorescence to total cell fluorescence).

2.2.8 In Vivo Experiments

2.2.8.1 Mice and Immunisations

Animal work was carried out under a Home Office Project Licence (PP2706800). Mice were maintained in the animal unit at Nottingham Trent University according to Home Office guidelines and the Project Licence. Studies were approved by both University of Nottingham and NTU AWERBs (animal welfare and ethical review boards). There were 4 different groups and each group contained three female Balb/C (Charles River) mice aged 8-12 weeks. All mice in the positive control group received DNA (SN17, which was gifted by Scancell Ltd., Nottingham, UK) coated onto 1.0 μ m gold particles (BioRad, Hemel Hempstead, UK) and applied intradermally to the mice via gene gun (BioRad, UK) on days 1, 8 and 15. Mice in other three groups received a single 50 μ L injection of nanoparticle solution containing 5 μ g SN17 with PBAE-PEG-based polymers (BDD, mPEG-BDD and Mix). First, nanoparticles were prepared at concentrations of 40 μ g/mL and then were centrifuged using Amicon® Ultra-15 Centrifugal Filter Unit to obtain the above-mentioned desired concentrations. Then, concentrated nanoparticle solutions were applied as

intramuscular (im) injection on days 1,8 and 15. On day 21 mice were sacrificed.

2.2.8.2 IFN- γ ELISpot Assay

Splenocytes harvested from mice from each group were treated and assayed independently. Ex vivo ELISpot assays were used to detect T cell responses using murine IFN γ capture and detection reagents, S1 and N proteins (SinoBiological and Genscript respectively), according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden). In brief, capture antibodies were coated onto wells of 96-well Immobilin-P plate and triplicate wells were seeded with 5×10^5 splenocytes in complete media (RPMI medium 1640 (GIBCO/BRL) supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Sigma) and sodium bicarbonate buffered with additional 20mM HEPES (Sigma)) containing 50 μ M 2-mercaptoethanol (Thermofisher). Synthetic peptides (as detailed above) were used at a final concentration of 5 μ g/mL in complete media plus 2-mercaptoethanol and added to these wells. Whole proteins were used at a final concentration of 1 μ g/mL. Following incubation, captured antibody was detected by a biotinylated anti-IFN γ antibody and development with a streptavidin alkaline phosphatase and chromogenic substrate. Spots were analysed and counted using an automated plate reader (CTL Europe GmbH, Aalen, Germany).

2.2.8.3 ELISA Assay

Blood was collected from mice and allowed to clot for at least 30 mins at room temperature, centrifuged at 1000g for 10 mins and then sera collected. Naïve mouse serum samples were used as control. Sera were diluted in 2% BSA-PBS and incubated for one hour at room temperature (RT) in coated S1 protein,

N protein, Nunc Immuno plate F96 MaxiSorp ELISA plates, all at 200 ng/well and then blocked with casein. S1 antibody binding was detected using a 3-step approach consisting of Goat-anti-mouse IgG Fc biotin (Sigma Aldrich) followed by Streptavidin-HRPO conjugate (Invitrogen) and finally TMB (3,3', 5, 5'-tetramethylbenzidine) core plus reagent (Bio-Rad BUF062C) for development. N antibody binding was detected using a 2-step approach where Goat pAb to mouse IgG (HRP) (Abcam) was used after the serum incubation step, followed by the development described above. In both instances, once sufficiently developed, the reaction was stopped using 1M H₂SO₄ and the plates were read at 450 nm wavelength on a plate reader.

2.2.8.4 Histopathological analysis

Livers and kidneys were collected from each group for the histological and immunohistochemical studies and then tissues were perfused by 200 mL of paraformaldehyde (4%) in phosphate buffer saline (0.1M) for 48 hours. Then tissues were processed using alcohol series and Xylene. After 24 h, tissues were embedded in paraffin blocks and left at room temperature for overnight. Haematoxylin and eosin (H&E) and Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining were used for the paraformalin fixed tissue sections which were 10 µm. An Olympus C 5050 digital camera attached on an Olympus BX 51 microscope was used to record the sections. For the apoptotic cell percentage quantification of tissues QuPath software version 0.5.1 was used.

2.2.9 Statistical analysis

Graphs and statistics were prepared in GraphPad Prism 9.5.1 version. Statistical differences were analysed using either one-way or two-way ANOVA adjusted for multiple comparison (either Tukey's or Dunnet's), p value lower than 0.05 was considered as statically significant and the levels of statistical significance were set $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

2.3 Results and Discussion

2.3.1 Synthesis and Characterisation of PBAEs

We initially synthesised an acrylate end-group terminated poly(beta-amino) ester derived from an aza-Michael addition polymerisation between 1,4-butanediol diacrylate (BDD) and 5-amino-1-pentanol (5AP) which could be used for post-functionalisation. This PBAE composition was chosen due to several previous pre-clinical studies indicating excellent formulation properties (particle size, stability, and zeta potential) as well as high transfection efficiencies both in vitro and in vivo across a range of nucleic acid cargos [30, 31]. Diacrylate and amine monomers were mixed then heated for 24 h at a ratio of 1.1:1 acrylate:amine (Figure 2.2A) to yield the base PBAE pBDD-5AP. ^1H -NMR spectroscopy indicated pBDD-5AP had the desired co-monomer composition and exhibited terminal acrylate groups required for post modification (Figure 2.2A). GPC analysis revealed a monomodal chromatogram with $\bar{D} = 1.3$ and an average molar mass of 7700 g mol^{-1} . The pBDD-5AP PBAE was end-capped either with a further aza-Michael addition with 5-AP or via thiol-acrylate Michael addition with an 800 g mol^{-1} mono-

thiolated PEG to yield OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG (Figure 2.2B and Figure 2.2C), as this molecular weight of PEG was found as the most effective nanocarrier [32]. Full end-capping was confirmed with the disappearance of acrylate signals in the ^1H NMR spectra (Figure 2.2E). We observed a modest increase in molar masses for 5-AP end-capped polymers, reaching 8500 g mol^{-1} while the PEG terminated PBAEs exhibited a clear shift towards higher molar mass ($M_{n,\text{th}} = 12300 \text{ g mol}^{-1}$) due to PEG conjugation. Overall, all polymer characterisation confirmed the preparation of our targeted structures (Figure 2.2B).

The buffering capacities of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG were evaluated by acid-base titration (over the pH range of 10.0–3.0) as pK_a of polycations has been suggested to be important for facilitating endosomal buffering and endosomal escape of polymer based nucleic acid delivery systems [33, 34]. Both PEGylated and non-PEGylated PBAEs showed similar titration curves suggesting that PEGylation did not affect the buffering capacity (Figure 2.2D) thus any coformulations between the OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG should have identical buffering capabilities.

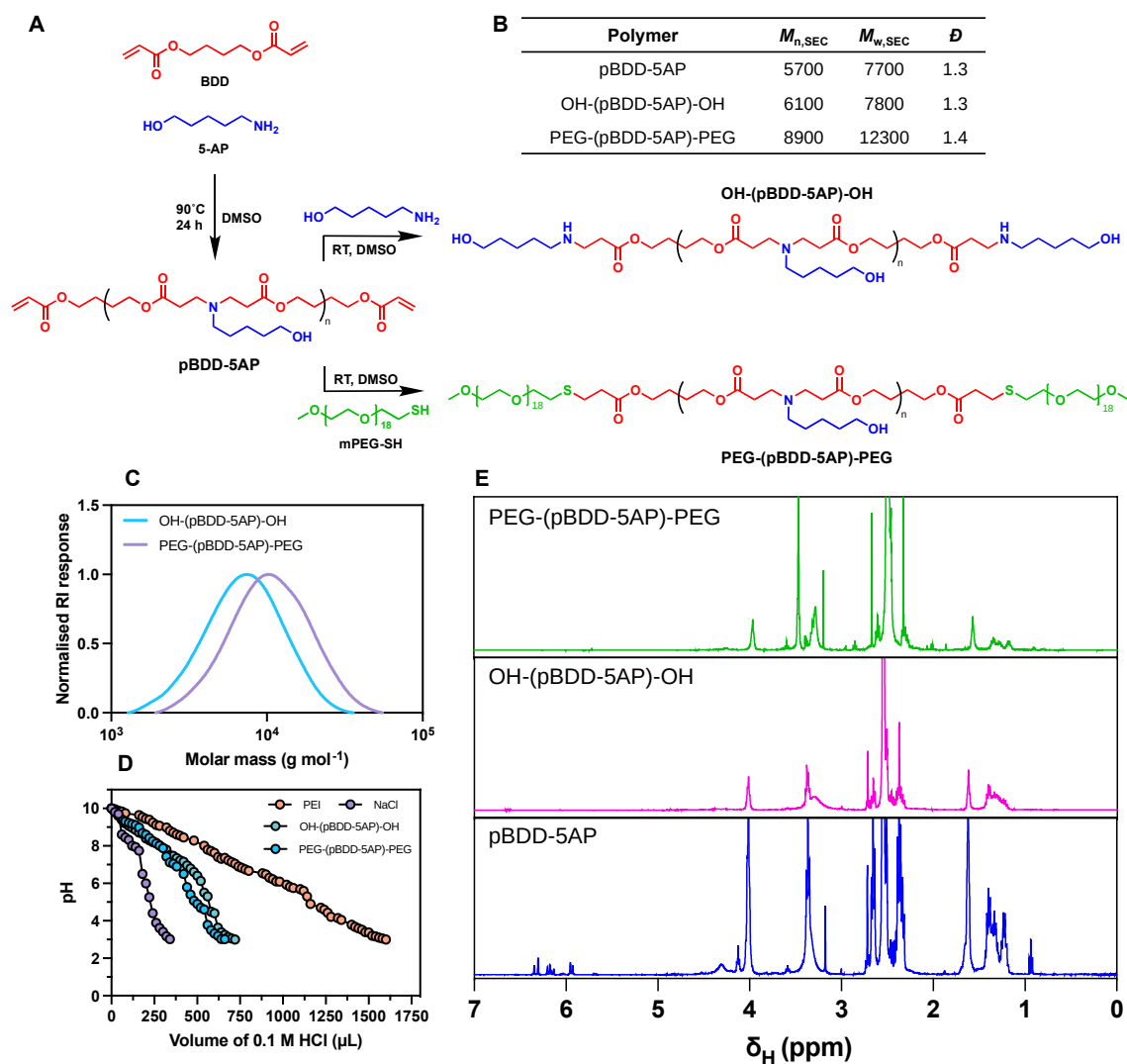


Figure 2.2. Synthesis and characterisation of PBAEs. (A) Synthetic scheme for step-growth Michael-addition polymerisation of BDD, 5-AP and mPEG-SH to produce PEG-PBAE-PEG triblock copolymer. First, acylate-terminated base polymer was synthesised by polymerisation of monomer BDD and 5-AP. In the second step, base polymer was further terminated with 0.8 kDa mPEG-SH. (B) Molecular weights of PBAEs determined using GPC and (C) the molecular weight distribution of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG. (D) Buffering capacity of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG compared to polyethyleneimine (PEI) and negative control of a 0.1 M NaCl_(aq) solution. (E) ^1H NMR spectra of pBDD-5APm OH-(pBDD-5AP)-OH showing disappearance of the acrylate peaks at 6-7 ppm indicating complete capping with either 5-amino-1-butanol for OH-(pBDD-5AP)-OH or PEG-SH for PEG-(pBDD-5AP)-PEG (deuterated DMSO was used).

2.3.2 Effect of PEG Density on Polyplex Physicochemical Properties

Polyplex preparation was performed with three different polymer / fluc pDNA ratios weight-to-weight ratios (w/w ratios of 32, 64 and 128) for OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG PBAEs to optimise size, surface charge, encapsulation efficiency, biocompatibility, and DNA expression. To assess the effect of PEG density on the surface on the nanoparticle surface, we also prepared polyplexes prepared with a 1:1 weight:weight mixture of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG, termed 'mix' (Figure 2.3A). This ratio represents a molar ratio of 2/3 PEG-(pBDD-5AP)-PEG/ OH-(pBDD-5AP)-OH and has been previously shown to be more effective at delivering DNA in glioblastoma than the PEGylated or non-PEGylated formulations alone; however, it has not been utilised in DNA vaccination [32, 39].

Hydrodynamic diameters of all nanoparticles were observed to be between 100-150 nm with low polydispersity index (PDI) values (< 0.2) determined by dynamic light scattering (DLS) (Figure 2.3B), with no major changes in particle size observable across different w/w ratios. Notably, PEG-(pBDD-5AP)-PEG polyplexes produced smaller hydrodynamic diameters compared to OH-(pBDD-5AP)-OH non-PEGylated particles, suggesting that end modification of the polymer with mPEG-SH induced more compact structures in the final kinetically-trapped polyplexes, consistent with previous reports on PEGylated polyplexes [35]. In addition, zeta potentials of the particles were measured to be between 10-15 mV and no significant difference was observed between the groups (Figure 2.3C). This could be due to the low molecular weight of used PEG which was 800 Da. TEM micrographs of PEG-(pBDD-5AP)-PEG/pDNA

polyplexes prepared at a w/w ratio of 64 showed the complexes to be spherical, and between 30 to 180 nm in diameter consistent with DLS analysis, albeit with particle shrinkage in the dry state for electron microscopy (Figure 2.3D, Figure 2.3E and Figure S2.1). Interestingly for the PEGylated and mixed polyplexes we observed a clear core-shell structure across most of the particles imaged, with a dark core and less electron dense corona attributable to the PEG surface. In contrast non-PEGylated polyplexes did not exhibit this core-shell morphology with only a single phase of electron dense core evident. We believe these TEM images and morphological differences evidence the absence or presence of PEG expressed on the surface of these polyplexes, in line with the formulations produced.

Furthermore, as DLS analysis is typically skewed towards larger particles it is likely any smaller species may be underrepresented in the overall sizes reported by light scattering. The presence of some free nucleic acids in agarose gel electrophoresis experiments for OH-(pBDD-5AP)-OH formulations indicated less complete complexation with the non-PEGylated material. In contrast, full incorporation of DNA was observed for the PEG-(pBDD-5AP)-PEG and mixed polyplexes (Figure 2.3F). Overall, these results indicate that all three formulations were capable of encapsulating pDNA with both the PEG-(pBDD-5AP)-PEG and mixed polyplexes offering smaller particle sizes and marginally higher pDNA encapsulation efficiency.

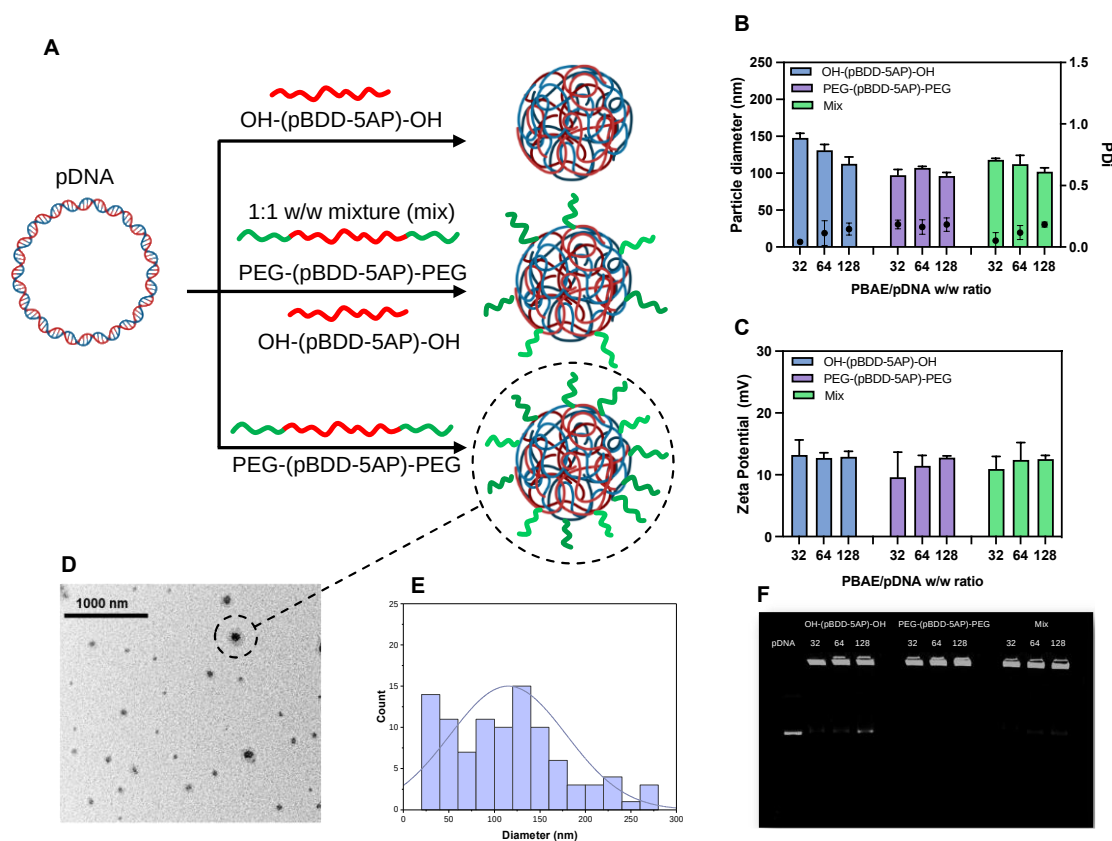


Figure 2.3. Formulation of PBAEs with pDNA to produce polyplex nanoparticles. (A) Schematic representation of pDNA formulation with OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG or a 1:1 w/w mixture of OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG termed 'mix' in pH 5.0 25 mM sodium acetate at a final pDNA concentration of 10 µg/mL. (B) Particle size (bars) and PDI (floating points) and (C) Zeta potential for the OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG and 'mix' polyplexes at a PBAE/pDNA w/w ratio of 32, 64 and 128 determined by dynamic light scattering at 25°C. Bars represent the mean ± SD for 3 independent formulations. (D) Representative transmission electron micrograph of PEG-(pBDD-5AP)-PEG polyplexes and (E) measured particle size distribution from five separate TEM images. (F) Encapsulation efficiency of pDNA within polyplexes evaluated using agarose gel retardation assay.

2.3.3 In Vitro Evaluation of pDNA Delivery

The acute toxicity and pDNA delivery efficiency of the individual OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG was examined in HEK293T and DC2.4 cells as a model and vaccine relevant dendritic cell line respectively. Similar to the formulation characterisation study above, polyplexes were prepared at polymer/pDNA w/w ratios of 32, 64, and 128 utilising a firefly luciferase encoding pDNA and compared against lipofectamine (positive expression

control) and Triton X-100 (positive toxicity control). All the formulations exhibited more than 50% cell viability and, in general, DC2.4 cells displayed high metabolic activity compared to HEK293T cells which may be due to the lower transfectability of the dendritic cells (Figure 3.4A) and Figure 3.4B) [36, 37]. Similar to previous studies, increasing w/w ratio resulted in increased toxicity due to increased polycation concentration which is known to disrupt the negative phospholipid bilayer [38]. Interestingly, a significant difference was observed for the mixed and PEG-(pBDD-5AP)-PEG formulation in the DC2.4 cell line with cell viability around 50% and 94% for w/w 128 and w/w 64, respectively (Figure 3.4A and Figure 3.4B). Notably, formulations prepared at higher w/w ratios yielded greater transfection efficacy often exceeding the luciferase expression of the lipofectamine positive control (Figure 3.4C and Figure 3.4D). Unlike in HEK293T cells the OH-(pBDD-5AP)-OH formulations did not show statistically significantly higher luciferase expression than naked pDNA in DC2.4 cells, yet both the mixed and PEG-(pBDD-5AP)-PEG derived polyplexes yielded increased pDNA expression in the DC2.4 cells. This could be due to the above-mentioned compact structure of PEGylated formulations where the diameter of nanoparticles was observed to be smaller than that of non-PEGylated ones, and thus, above shown increased nucleic acid encapsulation. This phenomenon may also be the result of PEG shielding the dsDNA from cytosolic DNA sensors which are present in the DC2.4 cells but not HEK293 cells thus initiating a type-1 interferon response and inhibiting downstream RNA translation and thus expression of the luciferase reporter [39]; however, this would require further investigation.

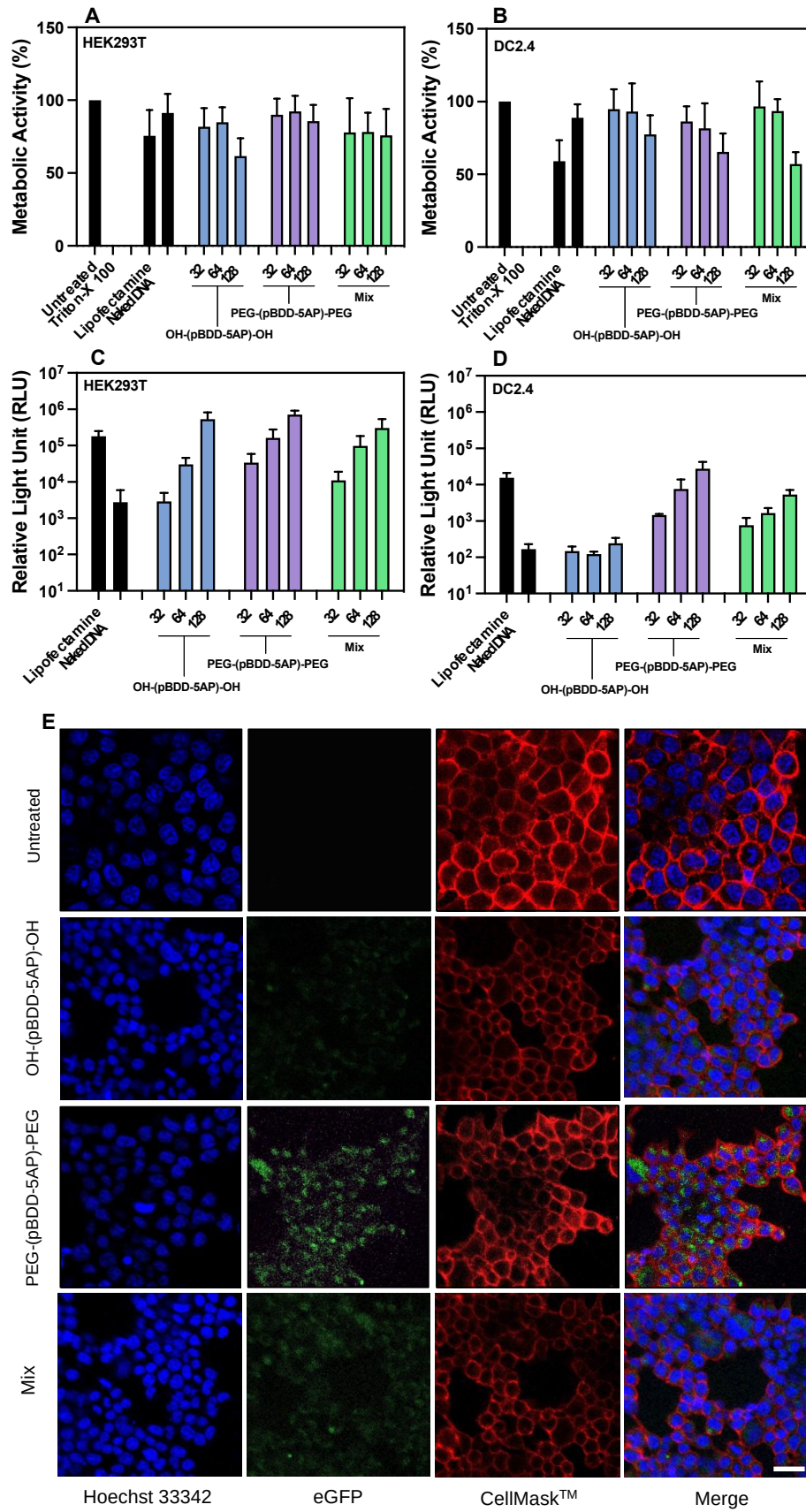


Figure 2.4. Metabolic activity and transfection efficiency of PBAE derived pDNA polyplexes. Effect of PBAE polyplexes formulated with firefly luciferase encoding pDNA at PBAE/pDNA w/w ratios of 32, 64 and 128 on metabolic activity (A and B) and transfection efficiency (C and D) on HEK293T cells (A and C) and DC2.4 cells (B and D). Cells were treated with polyplexes containing pDNA at a concentration of 500 ng/mL (100 ng/well) in serum-free Opti-MEM. Metabolic activity was compared against Triton X (positive control) and untreated cells (negative control) and calculated by normalizing metabolic activity to untreated cells. In vitro transfection efficiency was analysed after 24 h post-transfection using the Promega ONE-GLO luciferase assay and compared against Lipofectamine 2000 (positive control) and naked pDNA (negative control) expressed as relative light units (RLU) for luciferase expression. Bars represent the mean $\pm$ SD (n=3). (E) Representative live cell confocal microscopy images of HEK293T cells 4 h post-transfection with an eGFP encoding pDNA formulated with OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG or a 1:1 w/w mixture of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG termed 'mix' at a PBAE/pDNA w/w ratio of 64. Cells were stained with Hoechst 33342 CellMask™ Plasma Membrane Stain Deep Red to visualise the nucleus and cell membrane respectively. Images are displayed as individual channels and a merged signal. Scale bar corresponds to 20 μ m (x40). Note: the contrast of the images has been enhanced for clarity, original images can be found in the supporting information (Figure S2.2).

Due to the encouraging results with all three formulations at w/w 64 exhibiting high luciferase expression and minimal reduction in metabolic activity, we further investigated these formulations for pDNA expression with live cell confocal microscopy. All polymers were formulated with GFP-encoding pDNA and exposed to HEK293T cells for four hours. Following incubation, green fluorescence attributed to GFP expression could be observed within the cytosol indicating successful transfection (Figure 3.4E). To evaluate the cellular association of PEG-(pBDD-5AP)-PEG (w/w 64) polyplexes in DC2.4 cells, we first generated a FITC labelled PEG-(pBDD-5AP)-PEG by conjugating FITC on to the pendant hydroxyl groups of the core PBAE. Cellular uptake was assessed through imaging flow cytometry, a single cell microscopy technique at 30 min, 1 h, 2 h, and 4 h after treatment (Figure 2.5A). It was observed that 97.8% of DC2.4 cells were FITC-positive indicating successful cell association within 1 hour of incubation. There was a further increase to 99.5% FITC-positive at both 2 h and 4 h incubation times overall highlighting rapid association of the administered polyplexes. Notably the median fluorescence intensity rose from

4500 arbitrary units up to 18400 between 30 min and 4 h incubation, indicating an initial rapid uptake by the DC2.4 cells which continued throughout the course of the experiment. Compared to traditional flow cytometry the images from each cell can be analysed to compare internalisation vs membrane association. The median of internalisation score of the polyplexes was approximately 1.4 indicating a high proportion of nanoparticles were internalised inside the cells since having a score less than 0.3 can be considered to be surface bound (Figure 2.5A, B and C) [40].

To further assess the intracellular internalisation mechanism of the PEG-(pBDD-5AP)-PEG in DC2.4 cells we employed confocal microscopy to evaluate colocalization with a lysosomal (Lysotracker™ red) and nuclear (Hoecht 33342) stain (Figure 2.5D). After 1 and 4 h post administration, confocal micrographs showed that polyplexes successfully internalised within dendritic cells and merged images demonstrated with minimal co-localisation with the acidic lysosomes with image analysis indicating Pearson's Correlation Coefficient between FITC labelled polyplexes and Lysotracker™ stained lysosomes were below 0.5 (Table S2.1). Given the ~100 nm particle diameters it is likely that these formulations will be uptaken by endocytosis, however the minimal colocalization with the acidic lysosomes may indicate successful escape at the before endosomal maturation in lysosomes compartments. Although further endosomal escape studies would be needed to fully investigate this phenomenon, it is likely this feature heavily explains the high expression efficiencies observed, as endosomal escape is one of the key bottlenecks in cytosolic gene and drug delivery.

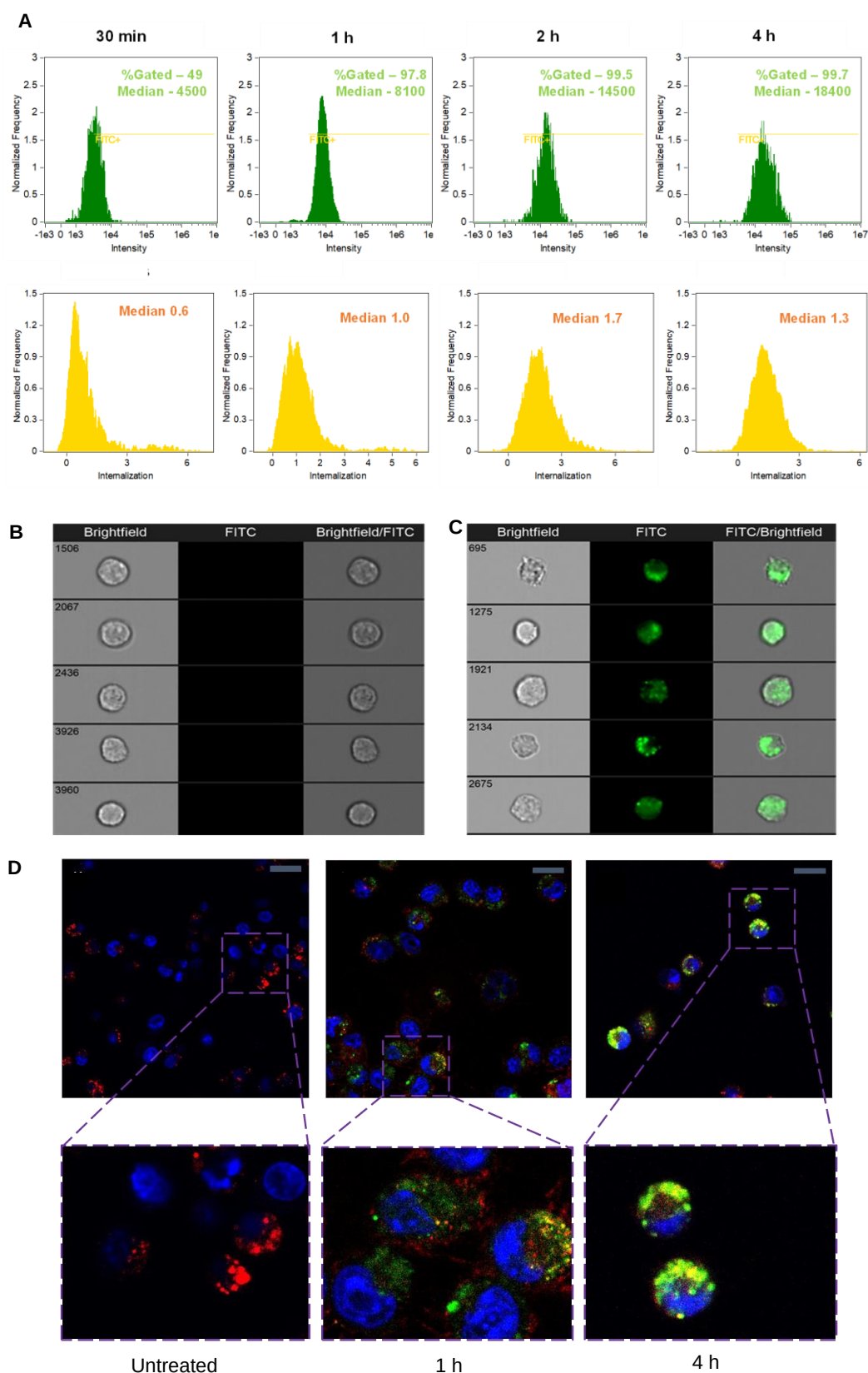


Figure 2.5. Cellular uptake of PBAE derived pDNA polyplexes. (A-C) Imaging cytometry (A) histograms of fluorescence intensity and internalisation for DC2.4 cells after 30 mins, 1 h, 2 h and 4 h following treatment with FITC-labelled PEG-(pBDD-5AP)-PEG polyplexes at a PBAE/pDNA w/w ratio of 64 at 500 ng/mL pDNA. Untreated fluorescence intensity histogram

can be found in the supporting information (Figure S2.3) Data were analysed using IDEAS software. (B and C) Representative images for imaging cytometry for untreated and treated group after 4 h. (D) Representative merged live cell confocal microscopy images of DC2.4 cells treated pDNA polyplexes derived from FITC-labelled PEG-(pBDD-5AP)-PEG at a PBAE/pDNA w/w ratio of 64 after 1 h and 4 h at 100 ng/well (500 ng/mL) pDNA. Scale bar 10 μ m (40x). Note: the contrast of the images has been enhanced for clarity, original images can be found in the supporting information (Figure S2.4)

2.3.4 In Vivo Delivery of a SARS-CoV-2 Bivalent pDNA Vaccine

Due to the encouraging in vitro pDNA expression and polyplex uptake in DC2.4 cells we hypothesised that these materials may be effective in delivering a pDNA vaccine against infectious disease. We therefore evaluated antigen specific T-cell responses of our formulations encapsulating a bivalent pDNA vaccine, encoding for both the nucleocapsid and spike protein of SARS-CoV-2 [41]. This construct has previously shown positive neutralising antibody titres in vivo following jet injector administration, however there is a need for more accessible delivery technologies for this vaccine candidate. Genomic studies on SARS-CoV-2 evolution have indicated that mutation in the spike (S1) protein is the predisposing factor to waning vaccine immunity against new variants. Therefore, a vaccine inducing expression of the nucleocapsid (N) protein may have advantages for future protection against SARS-CoV-2 infections (a “variant-agnostic”) vaccine as the nucleocapsid features are relatively well conserved between variants [42].

We therefore produced polyplexes derived from OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG and the 1:1 w/w mixture between these polymers, termed ‘mix’ at a PBAE/pDNA w/w ratio of 64. 3 groups of female Balb/C mice were injected intramuscularly with a 5 μ g pDNA dose and compared against a positive control of 1 μ g pDNA vaccine delivered via gene gun. Doses were

administered in a prime-boost regime, with 3 identical im injections spaced 7 days apart (Figure 2.6A). Serum was collected at day 21 post-immunisation and then the mice were euthanised for spleen harvest, to evaluate whether any bivalent SARS-CoV-2 pDNA PBAE-formulated vaccines could induce spike and nucleocapsid T cell immune responses after booster doses. No adverse effects were observed during the course of the animal experiment, with body weight of the animals remaining relatively constant and no animals requiring to be removed (Figure 2.6D).

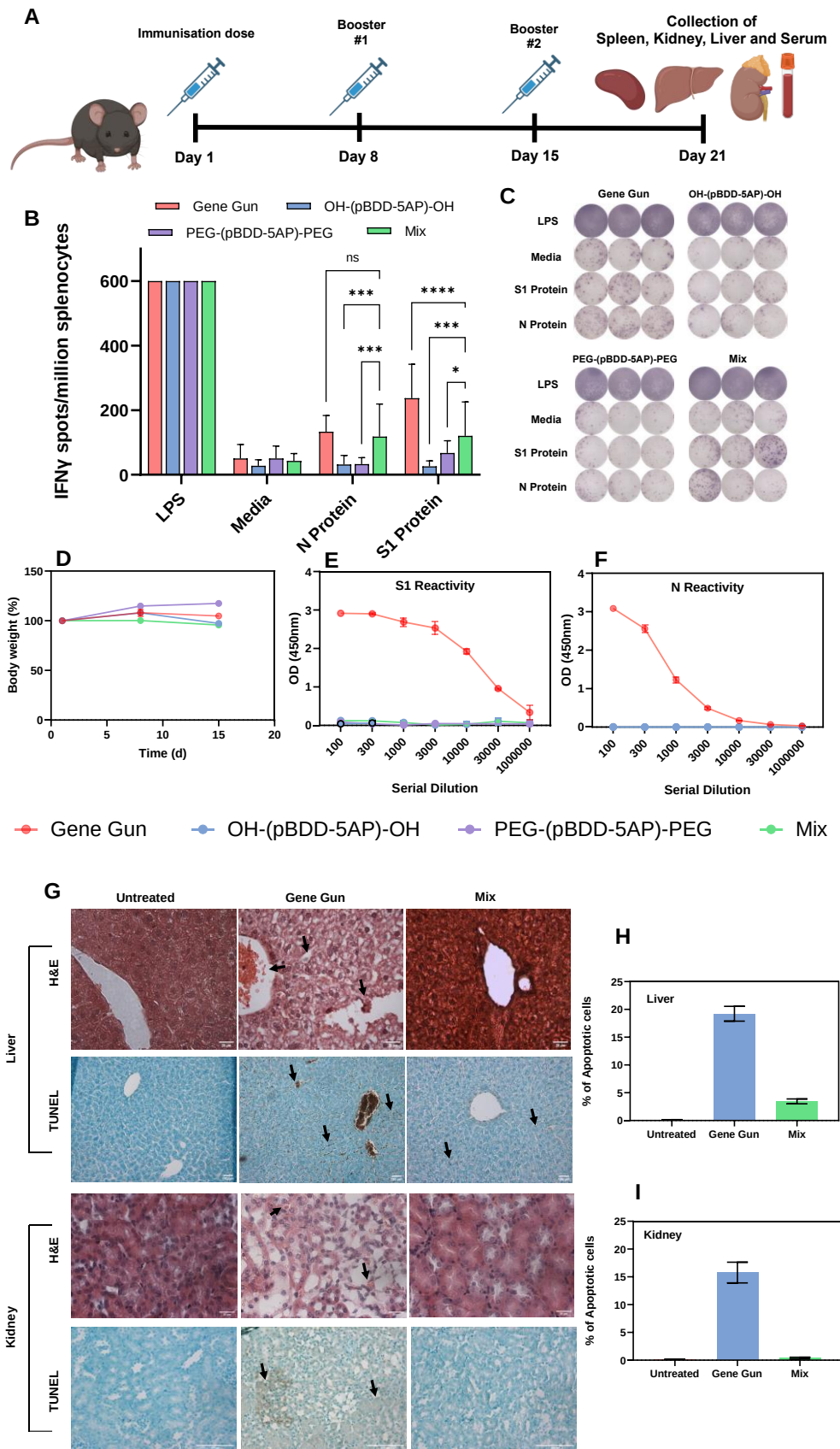


Figure 2.6. In vivo transfection, immunisation performance and tolerability following intramuscular administration of PBAE polyplexes containing a SARS-CoV-2 bivalent pDNA

vaccine. (A) Schematic of the dosing schedule. (B) Antigen-specific T cell responses were quantified by IFN- γ ELISpot with splenocytes isolated from vaccinated mice 21 days following intramuscular administered 5 μ g pDNA bivalent SARS-CoV-2 vaccine (encoding for both spike (S1) and nucleocapsid (N) proteins) complexed with OH-(pBDD-5AP)-OH, PEG-(pBDD-5AP)-PEG or a 1:1 w/w mixture of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG termed 'mix' at a PBAE/pDNA w/w ratio of 64. These were compared against a positive control of gold particle associated gene gun delivered vaccine. Doses were administered in a prime-boost regime, with 3 identical injections spaced 7 days apart. (C) Representative images of IFN- γ spot-forming cells (D) Animal body weight data over the time course of the experiment. (E) Serum N and (F) S1 antibody response 21 d after vaccination as determined by an ELISA assay. (G) Histopathological analysis of liver and kidney to investigate tissue damage for the non-treated, positive control and 'mix' polyplexes. H&E and TUNEL staining was utilised for the analyses of general tissue structure and apoptotic cells respectively. Scale bar 20 μ m and 100 μ m for H&E and TUNEL images respectively the percentage of apoptotic cells in the (H) liver and (I) kidney histopathology was quantified using QuPath software. Bars represent the mean $\pm$ standard deviation (n=3). Statistical analyses were performed with a two-way ANOVA with Tukey multiple comparisons post-hoc test with level of statistical significance represented as $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

The levels of antigen specific T-cells were investigated by evaluating the IFN γ expression via an ELISpot assay performed on splenocytes harvested from vaccinated mice. Gene gun vaccinated mice elicited the highest IFN γ secretion when stimulated with both N and S1 proteins ($p < 0.0001$ against media control). In contrast there was no statistically significant ($p > 0.05$ against media control) T-cell IFN γ expression after stimulation with the N and S1 proteins for the OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG polyplexes (Figure 2.6B and Figure 2.6C) compared to non-stimulated splenocytes, despite the PEGylated PBAE yielding the highest in vitro transfection performance in DC2.4s. Surprisingly we observed excellent IFN γ secretion for the sparsely PEGylated, mixed polyplexes, with equivalent and 70% spot formation compared to the gene gun positive control for the S1 protein and N protein respectively (Figure 2.6B, Figure 2.6C). The reasons for this enhanced efficacy, and importantly poor correlation between the in vitro and in vivo experiments are not immediately clear, however it is possible that modulation of PEG density

may attenuate immunogenic response or improve diffusion of the formulations beyond the injection site.

ELISA assays were performed with collected serum samples at day 21 to detect N and S1 antibody reactivity. Serial dilutions of positive control gene gun serum indicated potent reactivities suggesting the positive control immunisation stimulates strong antibody responses to both the nucleocapsid and spike proteins, consistent with previous data [43]. However, none of the PBAE formulations stimulated detectable antibody responses despite evidence of N and S1 protein expression in the mixed PBAE formulation (Figure 2.6E, Figure 2.6F). This may be due to the reduced DNA dose compared to previous DNA vaccine studies and may not be an adequate dose to stimulate antibody responses despite initiating protein expression or poor adjuvanticity of the formulation [43]. The poor antibody response may also be related to quantity of DNA taken up by antigen presenting cells or trafficked to lymph nodes being higher from gene gun, than from the PBAE formulations. Recent findings have suggested that inclusion of lipophilic side chain monomers into PBAEs may improve transfection of dendritic cells, hence could be an area of exploration for DNA vaccine polymer formulations in future [44].

The improved safety profile of new vaccine excipients and technologies is of utmost importance, particularly given recent reports on autoimmune hepatic injury following SARS-CoV-2 vaccination [45]. Accordingly, histological analysis of the liver was conducted from mice at the end of the vaccination study. We observed that the liver architecture and components of the mixed formulation were similar to those of the non-treated group, whereas enlarged interhepatic plate spaces and endothelial cells were distinct in the liver tissue in the gene

gun treated group (Figure 2.6G). The highly loaded blood vessels may also indicate severe inflammation for the positive control group, which again was not observed for the mixed formulations. Similarly, the findings for the H&E-stained kidney tissue architecture and the components were close to non-treated group compared to Mix. Finally, the vaccine induced inflammatory response may initiate apoptotic mechanisms in these cells, hence apoptosis was investigated via a TUNEL assay and then the data was analysed using QuPath software to quantify this. As expected, liver and kidney sections from untreated mice displayed minimal apoptosis, and a similar low level of apoptosis was observed for the mixed formulation. In contrast the positive gene gun control displayed several apoptotic regions and thus high apoptotic cell percentage quantified by QuPath (Figure 2.6H and Figure 2.6I). The higher inflammatory and apoptotic features observed for the positive control, compared to the PBAE formulations may be correlated with their greater antibody responses.

Taken together, the results from this work indicate that PEGylation of PBAEs plays a strong role in the in vitro and in vivo delivery efficiency of pDNA vaccines. Given the relatively similar physicochemical properties between PEGylated, non-PEGylated and mixed polyplexes it is unlikely this is the driving force in the enhanced activity seen for the PEGylated derivatives in the DC2.4 cell line. Previous studies on PBAEs have shown that high transfections efficiencies are typically achievable with polyplex nanoparticles of diameters below 200 nm [46], however the size alone does not guarantee efficacy, with monomer choice for the base PBAE critical for activity. Furthermore, the 3-4 orders of magnitude decrease in luciferase expression between the HEK293Ts and DC2.4 for the OH-(pBDD-5AP)-OH polyplexes, which was not as extensive

for PEGylated derivatives, hints that shielding the dense positive charge on the polyplex surface may enhance the downstream intracellular pathways that are important in pDNA delivery or expression. For instance, it has previously been observed that the membrane disruptive nature of polycations can stimulate intracellular inflammatory pathways which may inhibit DNA transcription or mRNA translation as a defence to viral replication [47]. Such innate responses are more functional in immune cells than in HEK293Ts potentially explaining the differences observed between cell lines [48]. The screening of positive charge may therefore explain why PEGylated PBAE polyplexes improve DNA expression, despite previous literature indicating that PEGylation drastically reduces uptake and therefore expression of nucleic acid formulations [49]. In contrast, the greater efficacy of the sparsely PEGylated 'mix' formulation in vivo may be explained by the known ability for PEGylated materials to diffuse further from the injection site, whilst potentially stimulating a moderate immunogenic response through weaker membrane interactions. As our markers for efficacy in the in vivo assay were related to vaccine response rather than antigen expression alone, this could result in enhanced activity for the 'mixed' formulation, while the PEGylated material does not stimulate a strong enough immune response, while the non-PEGylated derivative may yield an inflammatory response thus inhibiting the expression of the antigen.

2.4 Conclusions

In conclusion, in this work we demonstrate the role of PEGylation density in the formulation and delivery of pDNA in a vaccine relevant setting. Polyplexes derived from solely the PEG-(pBDD-5AP)-PEG or the 1:1 w/w mixture between PEG-(pBDD-5AP)-PEG and OH-(pBDD-5AP)-OH displayed smaller particle

sizes, high encapsulation efficiencies and high transfectability of reporter pDNA constructs in HEK293T cells and importantly dendritic cells displaying their potential in vaccination. In further in vivo studies with a bivalent SARS-CoV-2 pDNA vaccine, only the mixed polyplex formulations demonstrated IFN γ similar to the gene gun positive control; however, did not exhibit any antibody response. The findings in this study suggest that polymer delivery systems are a promising avenue for DNA vaccines with the surface properties of polyplexes eliciting a strong role in the performance of these delivery systems. However future research should focus on routes to improve the quality of immune response achieved from these vaccines when administered with polymer vectors. There is also significant scope to identify which polymer properties can tailor towards antibody or T-cell response, the main effectors in infectious disease vaccines and immunotherapies respectively.

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2.6 Author Contributions

Acknowledgement of research work done by co-authors:

- H. Bayraktutan: Performed all the experiments unless stated otherwise. Data curation, formal analysis, investigation, methodology, validation, visualization, writing of the original draft of the manuscript.
- P. Symonds, V. Brentville, C. Galley, C.L. Bennett: Planned and performed in vivo experiments and critically reviewed and edited the manuscript.
- C. Moloney: helped with histopathology experiments and critically reviewed and edited the manuscript.
- A. Mata, L. Durrant, C. Alexander, and P. Gurnani: Supervisors of H. Bayraktutan, supervised the student while conducting the work and critically reviewed and edited the manuscript.

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2.8 Supporting Information

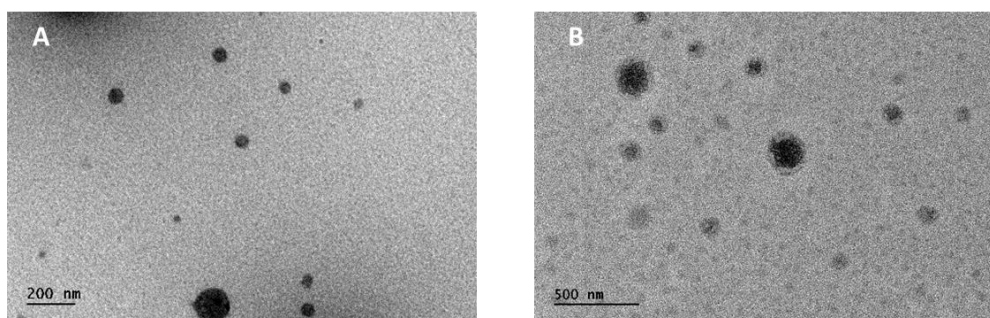


Figure S2.1. Representative transmission electron micrograph of (A) OH-(pBDD-5AP)-OH polyplexes - the scale bar corresponds to 200 nm and (B) Mix polyplexes - scale bar corresponds to 500 nm.

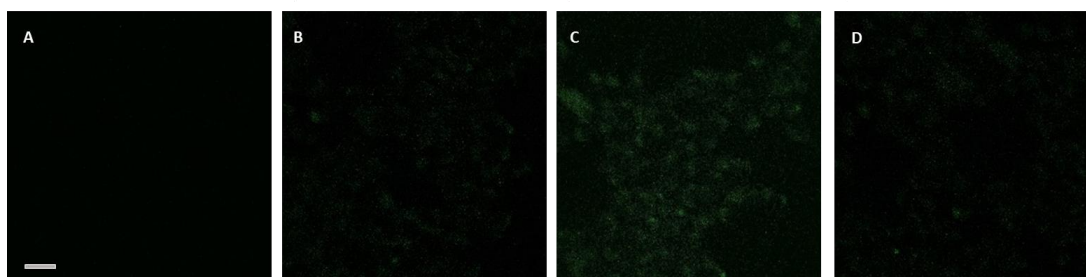


Figure S2.2. Live cell confocal microscopy original images of HEK293T cells 4 h post-transfection with an eGFP encoding pDNA formulated with (A) Nontreated (B) OH-(pBDD-5AP)-OH, (C) PEG-(pBDD-5AP)-PEG or (D) a 1:1 w/w mixture of OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG termed 'mix' at a PBAE/pDNA w/w ratio of 64. Scale bar corresponds to 20 μ m (x40).

Table S2.1. Co-localisation with the acidic lysosomes with image analysis indicating Pearson's Correlation Coefficient between FITC labelled polyplexes and LysoTracker™ stained lysosomes were around 0.38.

Image	Pearson's Correlation Coefficient
1	0.37
2	0.29
3	0.42
4	0.36
5	0.46

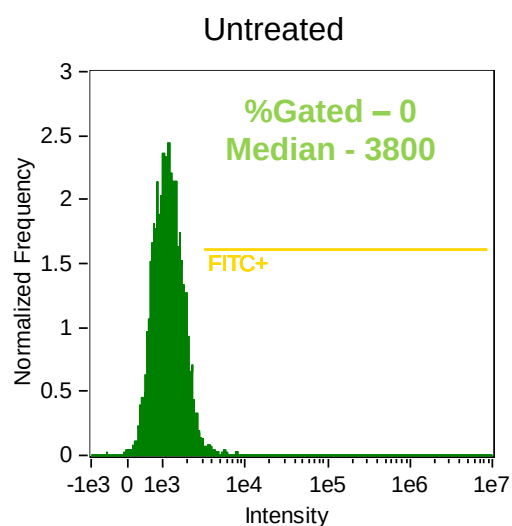


Figure S2.3: Imaging cytometry. Fluorescence intensity histogram of untreated group for DC2.4 cells. Data were analysed using IDEAS software.

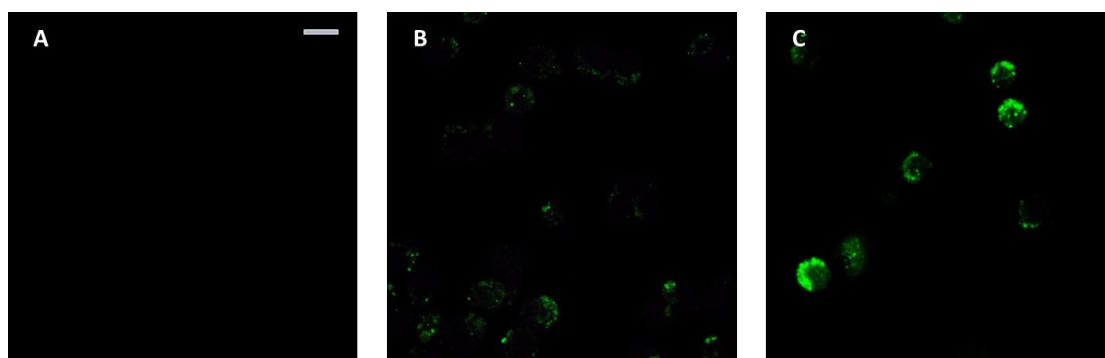
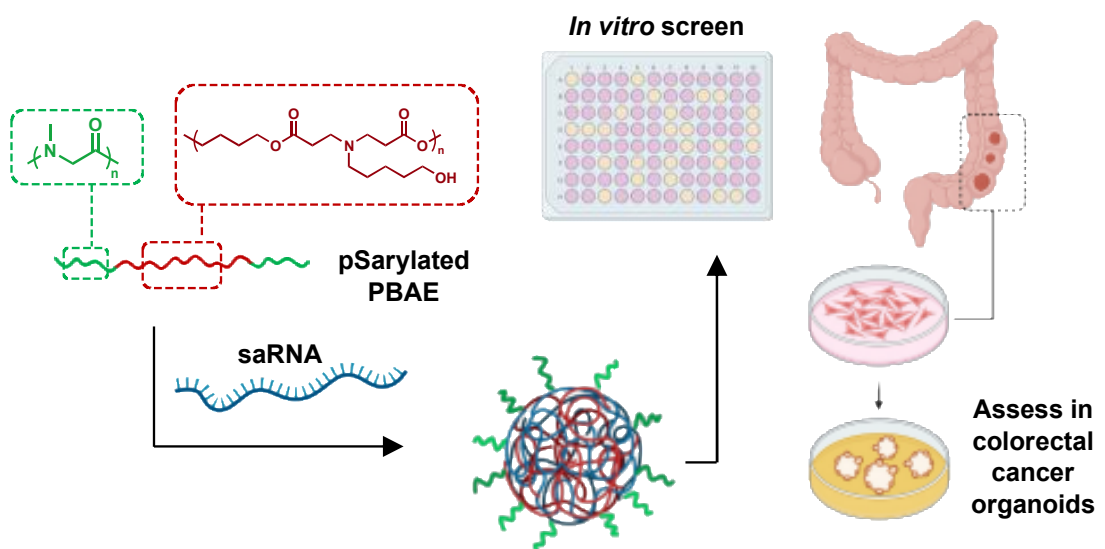


Figure S2.4. Live cell confocal microscopy original images of DC2.4 cells treated pDNA polyplexes derived from FITC-labelled PEG-(pBDD-5AP)-PEG at a PBAE/pDNA w/w ratio of 64 (A) Nontreated (B) after 1 h and (C) after 4 h at 100 ng/well (500 ng/mL) pDNA. Scale bar 10 μ m (40x).

Chapter 3

Polysarcosine functionalised cationic polyesters efficiently deliver self-amplifying mRNA



Abstract

Messenger RNA (mRNA) technology is a potent platform for new vaccines and therapeutics. To deliver RNA medicines effectively, safe delivery vectors are crucial. Among these, poly(β -amino ester)s (PBAE)s complexes with nucleic acids have been shown as promising alternatives to conventional lipid nanoparticles, but toxicity and colloidal stability concerns still remain. Conjugation of poly(ethylene glycol) (PEG) has been used to mitigate these issues, but challenges associated with PEG-induced reactogenicity have emerged. Polysarcosine (pSar) has shown promise as a PEG alternative, demonstrating reduced reactogenicity when incorporated into lipid nanoparticles. We therefore developed a synthetic route to conjugate pSar on to PBAE end-groups, resulting in a pSar-PBAE-pSar triblock copolymer. Polyplexes containing various ratios (0, 20, 50, 80, 100% pSar) of the pSarylated and a non-pSarylated PBAE were prepared with self-amplifying messenger RNA, to examine the effect of surface pSar density. RNA encapsulation efficiencies, formulation properties and gene transfectability in vitro were then assessed indicating complete encapsulation of the cargo and sub-200 nm particle diameters in all cases. Despite high gene transfection in HEK293T cells, only the 50%-pSar displayed significantly higher expression of the reporter gene than the negative control in Caco-2. Further evaluation of 50%-pSar was conducted using 3D human colorectal cancer organoids, which displayed high transfection ability within the core of the organoid indicating high penetration of these nanoparticles. As a result, these findings indicate that

pSarylated PBAEs have the potential to serve as a promising component material for enhancing delivery of nucleic acid therapeutics.

Key words: saRNA, PBAE, polysarcosine, polyplex, HEK293T and Caco-2 cells, colorectal cancer, organoid

3.1 Introduction

Messenger RNA (mRNA) technology is now recognised as a versatile and potent medicinal platform that enables the transient expression of selective proteins either as vaccines against infectious disease, or therapeutics for a range of indications, including genetic disorders, and cancer [1, 2]. The successful demonstration of safety and efficacy for Moderna (SpikeVax) and BioNTech-Pfizer's (Comirnaty) mRNA-based vaccines against COVID-19, has significantly accelerated this technology, with many more mRNA vaccines and therapeutics now in clinical trials [3].

One of the most important components of an effective RNA medicine is a safe and efficient delivery system to protect the genetic sequence from endogenous nucleases and facilitate translocation to the cytosol of the target cells for expression [4]. A variety of nonviral delivery strategies have therefore been investigated to deliver nucleic acids, including lipid nanoparticles (e.g. liposomes, cubosomes) and polyelectrolyte complexes (polyplexes) [3, 5, 6]. Polyplexes are self-assemblies of positively charged polymers which condense negatively charged nucleic acids into small (50-200 nm) nanoparticles driven by opposing electrostatic interactions. One of the promising classes of polycations broadly tested as RNA delivery vectors are poly(β -amino ester)s (PBAE)s [7]. Due to ester bonds within the PBAEs' main chain, these polymers are readily biodegradable via hydrolysis, decreasing their potential long-term accumulation and enhancing RNA release. These features, together with high flexibility of chemical functionality allowing for simple structural manipulation for a variety of nucleic acid types [8, 9] render PBAE-based polyplexes prospective candidates for RNA formulations. However, the polycationic and hydrophobic

nature of some specific sub-classes of PBAEs can result in cytotoxicity via membrane disruption, which has prevented their clinical application. Previous reports have shown that PBAEs coupled with poly(ethylene glycol) (PEG) exhibit decreased surface cationic charges when complexed with nucleic acids, and this can mitigate toxicity as well as decrease interactions with serum proteins [10, 11]. Such an approach has been proven to prolong systemic circulation time of nanoparticles by shielding their surface from aggregation and phagocytosis and increase in-serum *in vitro* and *in vivo* transfection efficacy [12-15].

Despite the popularity of PEG to confer “stealth” properties to nanoparticles in serum and the fact that PEG is classified as Generally Regarded as Safe (GRAS) [10] there is a growing number of reports on strong reactogenicity induced by PEG or PEGylated drugs [16]. This issue has become more prevalent following the widespread use of PEGylated lipids within SpikeVax and Comirnaty COVID-19 vaccines, which have boosted pre-existing PEG antibody titers by up to 60-fold [17]. This creates an urgent need to develop PEG alternatives, as future mRNA therapeutics which include PEG components may be highly immunogenic, thereby reducing potency and tolerability [18-20]. One emerging PEG alternative is Polysarcosine (pSar), a polypeptoid which degrades into sarcosine (*N*-methylated glycine) – a non-toxic, endogenous and non-proteinogenic amino acid which therefore endows it with key advantages over other potential “stealth” polymers such as poly(vinyl pyrrolidone)s [21], polyphosphoesters [22], polyglycerols [23], polypeptides [24], and poly(2-alkyloxazoline)s [25]. Despite the similar physicochemical properties to PEG, recent examples of polysarcosinyllated lipid nanoparticles have shown colloidal

stability and, importantly, substantially reduced immunogenicity compared to PEGylated derivatives [26]. However, to our knowledge pSar-PBAE conjugation has not been reported and may offer a versatile approach to a tuneable RNA delivery system combined with the advantages offered by pSar.

In this report, we demonstrate successful conjugation of pSar with a PBAE to yield a pSar-PBAE-pSar triblock copolymer (Figure 3.1). To test application in an RNA delivery setting, the pSarylated PBAE was formulated with self-amplifying RNA, an alternative mRNA technology which includes replicase genes derived from the alphavirus genome within the construct. When saRNAs are translated, they not only express the encoded protein, but also cause replication of the RNA within the cytosol, enhancing protein expression levels at significantly lower doses, lengthening expression half-life and reducing the cost compared to mRNA [6, 27-29]. The saRNA constructs were formulated into polyplexes with varying content of pSarylated and non-pSarylated PBAEs to evaluate the effect of pSar density. The *in vitro* tolerability and transfection efficiency of these blended pSarylated-PBAE polyplexes was then examined in two immortalised cell lines, (HEK293T and Caco-2). Finally, the cellular uptake and transfection efficiency in 3D human colorectal cancer organoids were investigated.

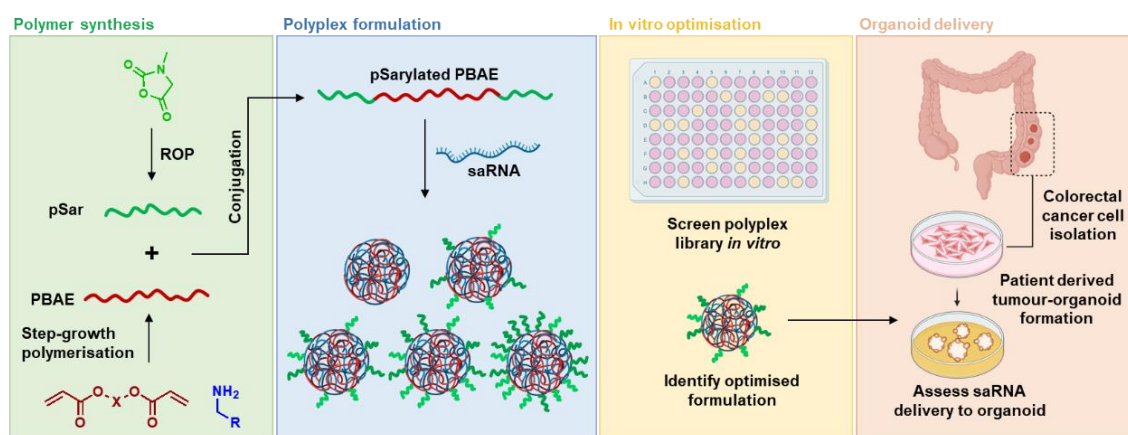


Figure 3.1. Schematic representation of PBAE synthesis, polyplex assembly, in vitro analysis and the cellular uptake and transfection efficiency in 3D colorectal cancer organoids.

3.2 Materials and Methods

3.2.1 Materials

1,4-butanediol diacrylate (BDD), 5-aminopentan-1-ol (5-AP), triethylamine (TEA), *N*-Boc-Sarcosine, Fluorescein isothiocyanate (FITC), diethyl ether, tetrahydrofuran, sodium acetate (NaOAc) buffer (pH:5.0), dye Gel Red and Goat-anti-mouse IgG, Fc biotin and red CellMask™ Plasma Membrane stain were purchased from Sigma-Aldrich. Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), acetonitrile (ACN), tetrahydrofuran (THF), hexane, diethyl ether, dimethyl sulfoxide (DMSO), loading buffer, Tris-acetate-EDTA (TAE) buffer, tris-(2-hydroxyethyl) phosphine (THP), Lipofectamine Messenger MAX™, Hoechst 33342, LysoTracker red and 96-well plates were obtained from ThermoFisher. Dulbecco's Modified Eagle Medium (DMEM) cell culture medium, RPMI 1640 cell culture medium, fetal bovine serum (FBS) and Opti-MEM were bought from Gibco, Invitrogen, Carlsbad, CA, USA. PrestoBlue reagent and ONE-Glo D-luciferin substrate were purchased from Promega, UK.

3.2.2 Instrumentation and Analysis

3.2.2.1 NMR Spectroscopy

All ^1H -NMR spectra were recorded in ppm (δ) at 400 MHz in d_6 -DMSO using a Bruker Advance III MHz spectrometer that was maintained at 25°C. To analyse the spectra MestReNova 6.0.2 copyright 2009 (Mestrelab Research S.L.) was applied.

3.2.2.2 Gel Permeation Chromatography

The gel permeation chromatography (GPC) analysis was performed using the PL-50 instrument equipped with a dual angle light scattering (LS), viscometry (VS) and differential refractive index (DRI) detection system. The chromatography components were a PLgel 5 μm guard column and a 2 $\times$ PLgel Mixed D columns (300 $\times$ 7.5 mm). Dimethylformamide (DMF) with LiBr (0.1% wt/wt) was utilised as an eluent with a flow rate of 1 mL/min at 50°C. The instrument was calibrated through Poly(methyl methacrylate) standards (Agilent EasyVials) between 955500–550 g mol^{-1} . Using conventional calibration, Cirrus GPC software, dispersity ($\mathcal{D}$) values, molecular weight (M_w) and experimental molar mass (M_n , SEC) were measured.

3.2.2.3 DLS and Zeta Potential

A dynamic light scattering (DLS), Zetasizer nano-ZS90 (Malvern, Inc.), instrument was used to characterise the particles in terms of the size, PDI and zeta potential. The instrument was used at 25°C to determine the zeta potential, average hydrodynamic diameters, and polydispersity index (PDI) of polyelectrolyte complexes.

3.2.2.4 Transmission Electron Microscopy

Briefly, glow discharged Formvar/carbon coated TEM grids were used, on which were placed the formulation samples (13 μ L), which were left for 15 minutes, then the excess solution was removed, and the samples allowed to dry at room temperature. Following this, 10 μ L of 2% aqueous uranyl acetate was applied to each grid and left for 10 seconds. After air drying, imaging was performed using a Tecnai G2 Spirit TEM with BioTwin lens configuration (Thermo Fisher Scientific, Eindhoven, The Netherlands) at an accelerating voltage of 100 kV.

3.2.3 Polymer Synthesis and Characterisation

3.2.3.1 Sarcosine N-carboxyanhydride (SarNCA)

A magnetically stirred solution of Boc-sarcosine (5.00 g, 26.5 mmol) and methyloxirane (18.5 mL, 265 mmol) in CH₃CN (100 mL) was cooled down in an ice bath and solid triphosgene (3.93 g, 13.5 mmol) was added in stepwise. Then, the reaction flask was half-closed to allow carbon dioxide to escape from the vessel. After 1.5 hours stirring at the ice bath, 50 mL of cold water was carefully added stepwise to quench the reaction. The mixture was extracted with ethyl acetate (2 x 100 mL), and the combined organic layers were washed with brine (2 x 100 mL), dried over anhydrous MgSO₄, and the solvent was removed using a rotatory evaporator. The obtained crude product was purified by recrystallization from hexane/THF at a freezer (-20°C) yielding 2.10 g (69%) of white crystals. ¹H NMR (400 MHz, DMSO-d₆) δ 4.22 (s, 2H, -CH₂-), 2.87 (s, 3H, -CH₃); ¹³C NMR (400 MHz, DMSO-d₆) δ 167.36, 152.59, 51.12, 29.79.

3.2.3.2 Cystamine

To a solution of cystamine hydrochloride (8.0 g, 35.8 mmol) in water (20 mL) a 10% w/w solution of NaOH_{aq} (20 mL) was added and the mixture was stirred at room temperature for 15 minutes. Afterwards, it was extracted 3-times with DCM (3 x 30 mL), the organic phase was dried over magnesium sulfate (MgSO₄) and concentrated on a rotatory evaporator yielding 4.2 g (78%) of a colourless oil.

¹H NMR (400 MHz, DMSO-d₆) δ 2.81 (t, *J* = 6.6 Hz, 4H, -CH₂-NH₂), 2.73 (t, *J* = 6.6 Hz, 4H, S-CH₂-), 2.20 (s, 4H, -NH₂); ¹³C NMR (400 MHz, DMSO-d₆) δ 42.09, 41.28

3.2.3.3 *N,N*-Bis(Poly(sarcosine))-cystamine (pSar-Cystamine)

To a solution of SarNCA (1.00 g, 8.7 mmol) in anhydrous DMF (3.2 mL) a solution of cystamine (36.7 mg, 0.24 mmol) in anhydrous DMF (2 mL) was added under argon atmosphere. After stirring overnight at room temperature, the product was isolated by precipitation in diethyl ether (80 mL) and purified by dissolving in MeOH (5 mL) and precipitation from diethyl ether (80 mL) two more times. The product was dried under vacuum overnight yielding 520 mg (80%) of white powder. Molecular weight determined by SEC: *M_n* = 4.3 kDa, *M_w* = 4.5, *Đ* = 1.06; for ¹H NMR see Spectrum S2 in Supplementary Information.

3.2.3.4 pBDD-5AP

To 1,4-butanediol diacrylate (BDD, 1 g, 5.0 mmol) in DMSO (0.95 mL) 5-aminopentan-1-ol (5-AP, 470 mg, 4.59 mmol) in DMSO (2 mL) was added and the reaction was stirred at 90°C overnight. Then, the mixture was diluted with THF (2.94 mL), the product was isolated by precipitation from diethyl ether (40

mL), and further purified by re-dissolving in THF (2 mL) and precipitation in diethyl ether (40 mL) thrice. The product was dried under vacuum overnight yielding 1250 mg (85%) of a yellowish oil. Molecular weight determined by SEC: $M_n = 6.2$ kDa, $M_w = 8.0$ kDa, $\bar{D} = 1.3$; for ^1H NMR see Spectrum S1 in Supplementary Information.

3.2.3.5 OH-(pBDD-5AP)-OH

To produce non-pSarylated PBAEs, an excess amount of 5-AP (260 mg, 0.26 mL, 2.52 mmol) was added to the pBDD-5AP (1000 mg, 5.04 mmol) in DMSO (5 mL) and the mixture was stirred for 24 hours at room temperature. Polymers were isolated by diluting the reaction mixture into THF (5 mL) followed by dropwise precipitation in diethyl ether (40 mL) thrice. The polymer was collected via centrifugation and dried under reduced pressure at 37°C to yield 800 mg (80%) a yellow viscous liquid. Finally, polymers were stored at -20°C as 100 mg/mL solution in DMSO. Molecular weight determined by SEC: $M_n = 6.9$ kDa, $M_w = 8.0$ kDa, $\bar{D} = 1.2$; for ^1H NMR see Spectrum S4 in Supplementary Information.

3.2.3.6 pSar-(pBDD-5AP)-pSar

pBDD-5AP (100 mg, 0.02 mmol), pSAR-Cystamine (108.1 mg, 0.07 mmol), triethylamine (2.27 mg, 3.13 μL , 0.02 mmol) and tris-(2-hydroxyethyl) phosphine (6.84 mg, 0.03 mmol) were added in DMF (4 mL) and the reaction was stirred at room temperature overnight. Then, the mixture was dialysed against DMF (1 L) for 30 hours and DMF solution was changed once. Afterwards, the mixture was precipitated in diethyl ether (40 mL) and to remove remaining DMF re-dissolved in methanol (2 mL) and was precipitated in diethyl ether (40 mL) twice. The product was dried under vacuum overnight yielding 85

mg (78 %) of a sticky yellowish oil. Molecular weight determined by SEC: $M_n = 9500$, $M_w = 11000$, $\bar{D} = 1.2$; for ^1H NMR see Spectrum S3 in Supplementary Information.

3.2.4 Synthesis of Fluorescently Labelled Polymers

BDD and BDD-pSAR polymers were dissolved in 5 mL DMSO (0.5 mg/mL) in a 20 mL vial. The dye FITC was added to the vials at a 1:10 (moles of end amine: FITC) molar ratio and triethylamine was added to the mixture at a 0.1 molar ratio to polymer. The reaction mixture was allowed to stir for 24 hours in the dark at room temperature. The reaction mixture was then dialysed in the dark using molecular weight cut-off 3,5 kDa against 250 mL NaOAc buffer (pH:5.0). Purification was continued for 4 days and the dialysis medium refreshed 2 times in a day. After freeze drying, FITC-labelled polymers were collected as a yellow solid and were stored at -20°C as 100 mg/mL solution in DMSO.

3.2.5 Polymer Buffering Capacity Assay

The buffering capacity of pSarylated and non-pSarylated polymers was evaluated by acid-base titration over the pH range of 10.0–3.0. Briefly, 2 mg of polymer was dissolved in 30 mL of 0.1 M NaCl aqueous solution, and the solution was adjusted to pH 10.0 using 0.1 M NaOH. Then, precise volumes (between 10-20 μL) 0.1 M HCl were added until a pH of 3 was achieved. The pH after each addition of HCl was recorded. 0.1 M NaCl was set as negative control.

3.2.6 Synthesis of saRNA

For firefly luciferase saRNA synthesis, luciferase encoded pDNA was linearised with MluI-HF (NEB, UK) for 3 hours at room temperature. Uncapped RNA was synthesised in a MEGAscript reaction (Ambion, UK) using 1 µg of linearised DNA as a template. The RNA was then purified by LiCl precipitation (1:1 volume ratio) for 24 hours at -20°C. Then, transcripts were centrifuged at 14000 rpm at 4°C for 20 minutes, washed once with 70% ethanol, centrifuged for 5 minutes, the ethanol wash was removed, and the pellet was dried at room temperature for 5 minutes. The purified RNA was then capped using ScriptCap Cap 1 capping system kit (CellScript, Madison, WI, USA) and used according to the manufacturer's protocol. Capped transcripts were again purified by LiCl precipitation. The purified capped saRNA was resuspended in RNAase/DNAase free water and stored at -80°C at 800 µg/mL.

3.2.7 Preparation of Polyplexes

Polyplexes were prepared by electrostatic interactions between positively charged polymers and negatively charged saRNA by mixing various w/w ratios (32, 64 and 128). Firstly, the working dilutions of polymers and saRNA were prepared in 25mM NaOAc buffer (pH:5.0). Depending on the desired w/w ratio, differing amounts of polymer stock solutions (100 mg/mL in DMSO) were mixed with nucleic acids in the buffer, gently mixed using a pipette and incubated at room temperature for 30 minutes. Whilst preparing the PBAE/saRNA complexes, taking a w:w 64 ratio as an example, 10 µg/mL of the RNA stock solution was mixed with 2.560 mg/mL PBAE by pipette using a 25mM NaOAc buffer.

3.2.8 Encapsulation Efficiency Determination Using Ribogreen Assay

A fluorescence-based Quant-iT RiboGreen assay (ThermoFisher, UK) was utilised to be able to detect free RNA in solution after polymer encapsulation. Samples were diluted in 1× TE buffer (v/v 1:1) and the assay was performed according to the manufacturer's protocol. Samples were then loaded on a black, 96-well plate, and analysed for fluorescence on a microplate reader at an excitation of 485 nm and emission at 528 nm.

3.2.9 In vitro experiments

3.2.9.1 Cell Culture

Cell line HEK293T was cultured in 10 % (v/v) FBS containing high glucose DMEM cell culture medium. The human colon colorectal Caco-2 cell line was cultured in RPMI 1640 cell culture medium. Media used for cell culture studies contained L-glutamine and 10 % FBS, and the absence of mycoplasma was confirmed using standard polymerase chain reaction assays. Cells were grown to 90 % confluence in a humidified incubator at 37°C, 5 % CO₂ and detached with 1x trypsin/EDTA. Viability was assessed using Trypan Blue staining.

3.2.9.2 Cell Viability Assays

To investigate the cytotoxicity of the formulations, a cell viability assay was performed in the HEK293T and Caco-2 cell lines. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells/well. For the treatment, the medium was aspirated, and cells were treated with 200 µL of Opti-MEM containing various w/w ratios of formulations complexed with 10 µg/ml fluc saRNA concentration (20 µL of polyplex solution). After 4 hours,

nanoparticles were aspirated and 100 μ L of complete medium was added to wells. At 24 hours posttreatment, each well was treated with 100 μ L of 10 % PrestoBlue reagent and allowed to incubate for 1 hour. Then, the total volume was transferred to a black 96-well plate and the FLUOstar Omega plate reader (BMG LABTECH, UK) was used to determine the fluorescent intensity (540 nm – 590 nm) of each well and normalized to the medium control.

3.2.9.3 In Vitro Transfection Experiments

In vitro transfection experiments were performed in HEK293T and Caco-2 cell lines, and the commercially available transfection reagent Lipofectamine Messenger MAXTM was used as positive control. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells per well. For the treatment, the medium was aspirated, then cells were transfected with 200 μ L of Opti-MEM containing various w/w ratios of formulations complexed with 10 μ g/mL fluc saRNA concentrations (20 μ L of polyplex solution). After 4 hours, nanoparticles were aspirated and 100 μ L of medium was added to wells. At 24 hours post-treatment, 50 μ L of media was removed then 50 μ L of the ONE-Glo D-luciferin substrate was placed into each well and mixed well by pipette. Finally, the total 100 μ L was placed in a 96-well plate and FLUOstar Omega plate reader (BMG LABTECH, UK) was used to determine the luminescence.

3.2.9.4 Cellular Uptake of saRNA using Confocal Microscopy

Caco-2 cells were seeded in CellViewTM 35 mm diameter glass bottom cell culture dishes at a density of 30×10^4 cells per well. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing FITC labelled BDD-pSAR + saRNA (0.01 mg/mL). Cells were incubated for 1

and 4 hours at 37°C with 5% CO₂. Then, nanoparticles were aspirated, and cells washed 3 times with PBS and stained with 10 µg/mL Hoechst 33342 and 75nM LysoTracker red applied in PBS solution. After 30 minutes, solution was aspirated, and cells were washed 3 times with PBS then FluoroBrite DMEM was added to cells. Imaging was performed using Leica confocal microscopy with LAS X software on DAPI, FITC and LysoTracker filters. ImageJ software was used to process the pictures.

3.2.9.5 Imaging Flow Cytometry

Caco-2 cells were seeded in a clear 6-well plate at a density of 40×10^5 cells per well. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing BDD-pSAR + saRNA (0.01 mg/mL) formulation. After 4 hours, nanoparticles were aspirated and 150 µL of 0.05% trypsin was used for detachment of cells from wells. After incubation for 15 minutes, 300 µL FACS buffer was used to neutralise the trypsin and cells were centrifuged at 1500 rpm for 10 minutes. Following this, cells were stained with Zombie Violet (1:500) dye for 30 minutes at room temperature in order to help with gating on live cells. Then, cells were fixed with 100 µL of 4% paraformaldehyde in PBS for 20 minutes. Fixed cells were centrifuged, and pellets resuspended in 50 µL of PBS. Data were acquired using an Image Stream 100 (Amnis, Seattle, US) and on single cell in focus events was used for analysis. Ideas Software (Amnis, Seattle, WA, USA) was used for data analysis. Total cell fluorescence was calculated by default total cell masks for the measurement of the nanoparticle internalisation and cell interiors were identified using the area erode tool (brightfield channel). Internalisation index

was shown as percentage of maximum internalisation (percentage of interior cell fluorescence to total cell fluorescence).

3.2.9.6 Isolation of tissue-derived colorectal cancer organoids

Fresh colorectal tumor tissue harvested from surgical human specimens was used to obtain patient-derived colorectal cancer organoids (Ethics: 17/EM/0126). The harvested tissues were fragmented using a scalpel blade and scissors to mechanically dissociate intestinal glands. Additionally, DNase I and Liberase were added for 1 hour at 37 °C to prevent cell clumping and to increase single-cell yield. After incubation, the sample was diluted with Advanced DMEM and FBS to inactivate the enzymes and filtered using a 70 µm cell strainer to remove clumps. The filtered mixture was centrifuged, and the cell pellet was resuspended with cold Matrigel. In a pre-warmed 48 well plate, Matrigel with cells was drop casted and transferred to the incubator 10 minutes allowing the gel to form before the addition of IntestiCult™ Organoid Growth Medium (OGM) (Stem Cell Technologies).

3.2.9.7 Colorectal patient-derived organoids culture

The culture media for colorectal patient-derived organoids was a complete cell culture medium for intestinal 3D organoids, IntestiCult™ OGM. To split organoids and dissolve Matrigel mechanical dissociation (vigorous pipetting) with cold Advanced DMEM was used. The diluted sample of Matrigel with organoids was centrifuged and the organoid pellet was resuspended with TrypLE cell dissociation reagent for 10 minutes at 37°C. After incubation, the cell suspension was diluted with Advanced DMEM, centrifuged and the supernatant was discarded. Single cells and small clusters derived from fragmented organoids were encapsulated with fresh cold Matrigel and drop

casted in a pre-warmed 48 well plate. After 10 minutes of incubation at 37 °C to allow the gel formation, IntestiCult™ OGM was added. The passage of colorectal patient-derived organoids in Matrigel was performed every 5–10 days.

3.2.9.8 Nanoparticles transfection in patient-derived organoids

Once colorectal patient-derived organoids had reached the desired compaction level (5 days after splitting), a formulation of 50%-pSar (w/w 128) (1.28 mg/mL) containing GFP-saRNA (10 µg/mL) was mixed with the above-mentioned organoid media, using 300 µL of the polyplex solution and 2700 µL from the organoid media. Then this solution was added onto the colorectal patient-derived organoids. After 4 hours incubation, nanoparticles were removed, and fresh media was replaced onto the organoids. GFP expression was imaged after 24 and 48 hours using a Leica confocal microscope with LAS X software and GFP filters. ImageJ software was used to process the images.

3.2.10 Statistical analysis

Graphs and statistics were prepared in GraphPad Prism 9.5.1 version. Statistical differences were analysed using either one-way or two-way ANOVA adjusted for multiple comparison (either Tukey's or Dunnet's), *p* value lower than 0.05 was considered as statically significant and the levels of statistical significance were set $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

3.3 Results and discussion

3.3.1 Synthesis and Characterisation of PBAEs

In the first step a poly(β -amino ester) was prepared from 1,4-butanediol diacrylate (BDD) and 5-aminopentanol (5AP), *via* a Michael-addition based step-growth polymerization with 10 mol% excess of BDD in relation to 5AP, to ensure termination of the polymer with acrylate groups (pBDD-5AP, Figure 3.2A, Scheme S3.1 and Spectrum S3.1). The selection of this PBAE composition was based on prior pre-clinical investigations, which demonstrated that pBDD-5AP PBAEs can display good formulation characteristics (such as small particle size, high colloidal stability, and high zeta potentials) and potent transfection performance, both *in vitro* and *in vivo*, with various types of nucleic acid cargoes [30]. Before producing our polysarcosine conjugated PBAE, we first generated a sarcosine N-carboxyanhydride (NCA-Sar) from N-Boc-sarcosine (Boc-Sar) using a moisture-tolerant protocol developed by Z. Tian et al. (Scheme S3.1 and Figure 3.2A) [31]. This was then conjugated to the acrylate functional pBDD-5AP PBAE via a base catalysed thia-Michael addition reaction, first using tris(hydroxypropyl)phosphine (THP) to reduce the disulphide bridge into reactive thiols. The degree of polymerization (DP) of the pBDD-5AP polymer was calculated from the ^1H NMR spectrum (Figure 3.2A and Spectrum S3.1) by comparing integrals of acrylate signals at 6.31 ppm and methylene groups at 1.62 ppm within the main-chain, whereas the DP of pSar-Cystamine (Figure 3.2A and Spectrum S3.2) was calculated using integrals of cystamine signals at 3.70 ppm at methyl signals for the sarcosine repeating units at 2.80 ppm.

^1H NMR spectroscopy confirmed the successful conjugation of pSar to the PBAE, generating pSar-(pBDD-5AP)-pSar (Figure 3.2A and Spectrum S3.3), revealing quantitative conversion of acryloyl groups (no visible signals at 5.9 – 6.4 ppm) and the expected ratio between pBDD-5AP and pSar repeating units, calculated from the pBDD-5AP methylene signals at 1.62 ppm and 2.80 ppm from methyl groups from pSar. As a control polymer without pSar, we also synthesised a hydroxyl terminated PBAE, OH-(pBDD-5AP)-OH, by endcapping the acrylate functional pBDD-5AP with further 5-AP, with ^1H NMR spectroscopy confirming full disappearance of acrylate end groups (Figure 3.2A and Spectrum S3.4). Size exclusion chromatographs of the synthesised polymers revealed the pre-functionalised PBAE, pBDD-5AP, exhibited $M_{n,\text{SEC}}$ of 6200 g mol $^{-1}$ and $\bar{D} = 1.3$, similar to other reported PBAEs (Figure 3.2B and 3.2C). Upon functionalisation with 5-AP to produce OH-(pBDD-5AP)-OH, there was no significant increase in molar mass ($M_{n,\text{SEC}} = 6900$ g mol $^{-1}$, and $\bar{D} = 1.2$) meanwhile conjugation of pSar to generate pSar-(pBDD-5AP)-pSar, produced a clear shift towards higher molar mass ($M_{n,\text{SEC}} = 9500$ g mol $^{-1}$ and $\bar{D} = 1.2$) indicating successful conjugation of pSar to the pBDD-5AP PBAE. Overall, our characterisation data confirmed the successful synthesis of our targeted pSar conjugated PBAE.

The buffering capacities were assessed for OH-(pBDD-5AP)-OH and pSAR-(pBDD-5AP)-pSAR as the pK_a of polycations has been recognised as crucial for enhancing endosomal buffering and endo/lysosomal escape of polymer-based nucleic acid delivery systems (Figure 3.2D). Acid-base titration in the pH range of 10.0-3.0 for PBAEs with and without pSarylation displayed similar titration curves, implying that pSarylation did not impact their buffering capacity

as shown in Figure 3.2D. Notably the PBAEs displayed weaker buffering capacities compared to a branched PEI control, likely due to PEI's structure containing a high density of primary, secondary and tertiary amines compared to our PBAEs only exhibiting tertiary amines at a reduced density across the polymer chain.

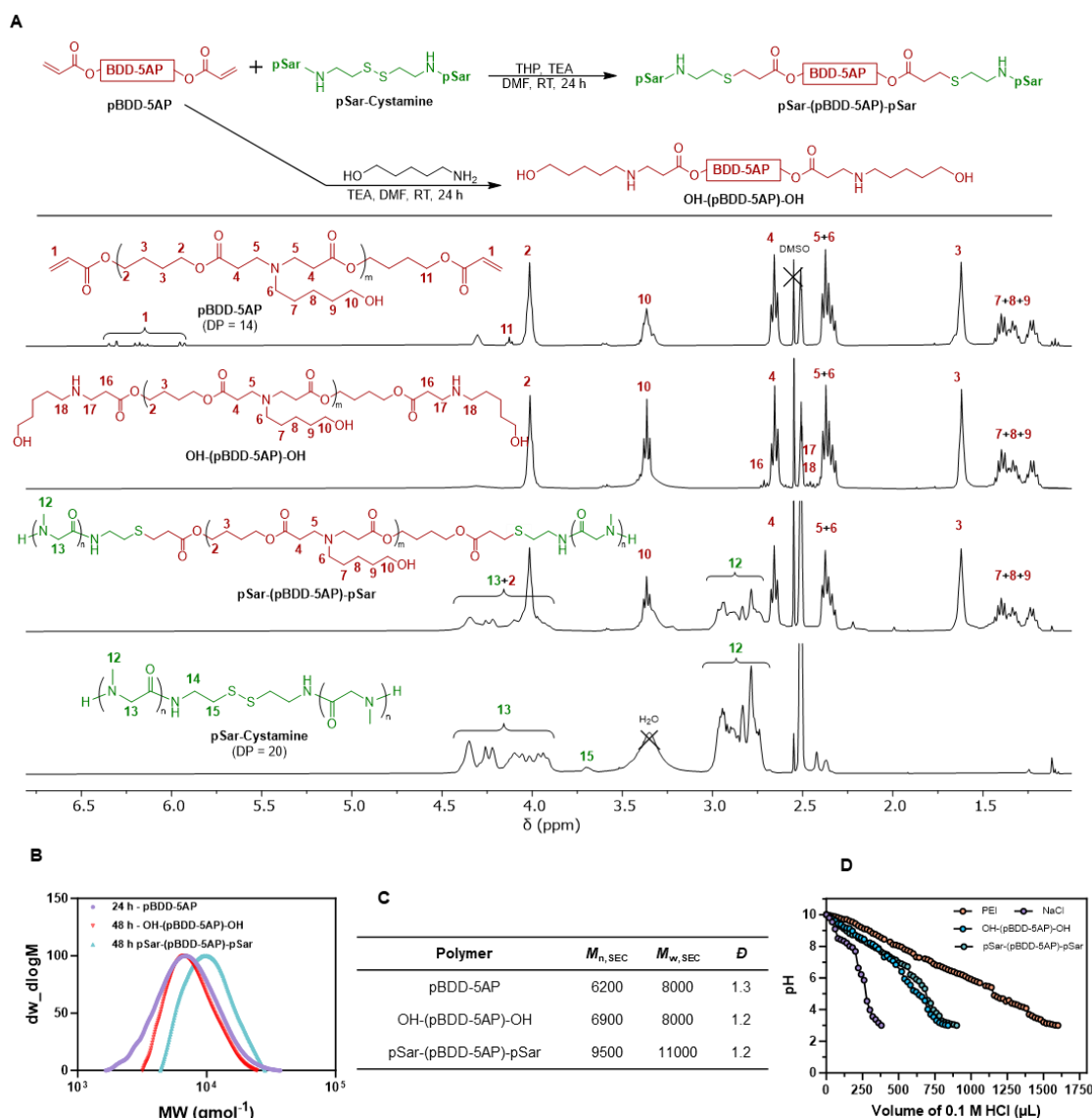


Figure 3.2. Synthesis and characterisation of PBAEs. (A) Synthetic scheme for step-growth Michael-addition polymerisation of BDD, 5-AP and pSar-Cystamine to produce pSar-PBAE-pSar triblock copolymer. ^1H NMR (400 MHz, DMSO-d_6) spectra of pBDD-5AP, pSar-Cystamine, and pBDD-5AP-pSar. *DPs calculated from ^1H NMR spectra as described in the main text. (B) Molecular weights of PBAEs determined using GPC and (C) the molecular weight distribution of OH-(pBDD-5AP)-OH and pSAR-(pBDD-5AP)-pSAR. (D) Buffering capacity of OH-(pBDD-5AP)-OH and pSar-(pBDD-5AP)-pSar compared to polyethylene imine (PEI) and negative control of a 0.1 M $\text{NaCl}_{(aq)}$ solution.

3.3.2 Effect of pSAR Density on Polyplex Physicochemical Properties

Polyplex preparation was performed at three different polymer / fluc saRNA weight-to-weight ratios (w/w ratios of 32, 64 and 128) for OH-(pBDD-5AP)-OH and pSar-(pBDD-5AP)-pSar PBAEs to optimise the physicochemical properties of the polyplex. These include particle size, surface charge, encapsulation efficiency, biocompatibility, and RNA expression. To assess the effect of pSar density on the nanoparticle surface, we also prepared polyplexes prepared with 20%-pSar, 50%-pSar and 80%-pSar which are mixtures of pSar-(pBDD-5AP)-pSar and OH-(pBDD-5AP)-OH as a weight% (Figure 3.3A).

Hydrodynamic diameters of all nanoparticles were observed to be between 100-150 nm with low polydispersity index (PDI) values (< 0.2), regardless of pSar content, determined by dynamic light scattering (DLS) (Figure 2.3C). Some particles e.g. 50%-pSar and 100%-pSar possessed a significantly decreasing size with w/w ratio. In addition, zeta potential of the particles was measured to be between 20-25 mV and no significant difference was observed between the groups (Figure 2.3B). This could be due to the low molecular weight of pSar which was 800 Da. The encapsulation efficiency of nanoparticles was analysed utilizing Ribogreen assay and revealed full RNA encapsulation for nanoparticles produced at all pSar-(pBDD-5AP)-pSar/OH-(pBDD-5AP)-OH ratios (Figure 3.3D). Finally, TEM micrographs of all polyplexes prepared at a w/w ratio of 128 displayed spherical complexes between 35 to 220 nm in diameter consistent with DLS analysis and taking into account some particle

shrinking in the dry state (Figure 3.3E). Furthermore, as DLS analysis can over represent larger particles present in heterogeneous particle distributions, it is likely that any smaller species visible by TEM may not have been included in the particle size averages [32]. Overall, these results indicate that all five formulations were capable of encapsulating saRNA and producing particle sizes below the typical 200 nm diameter threshold for efficient gene delivery.

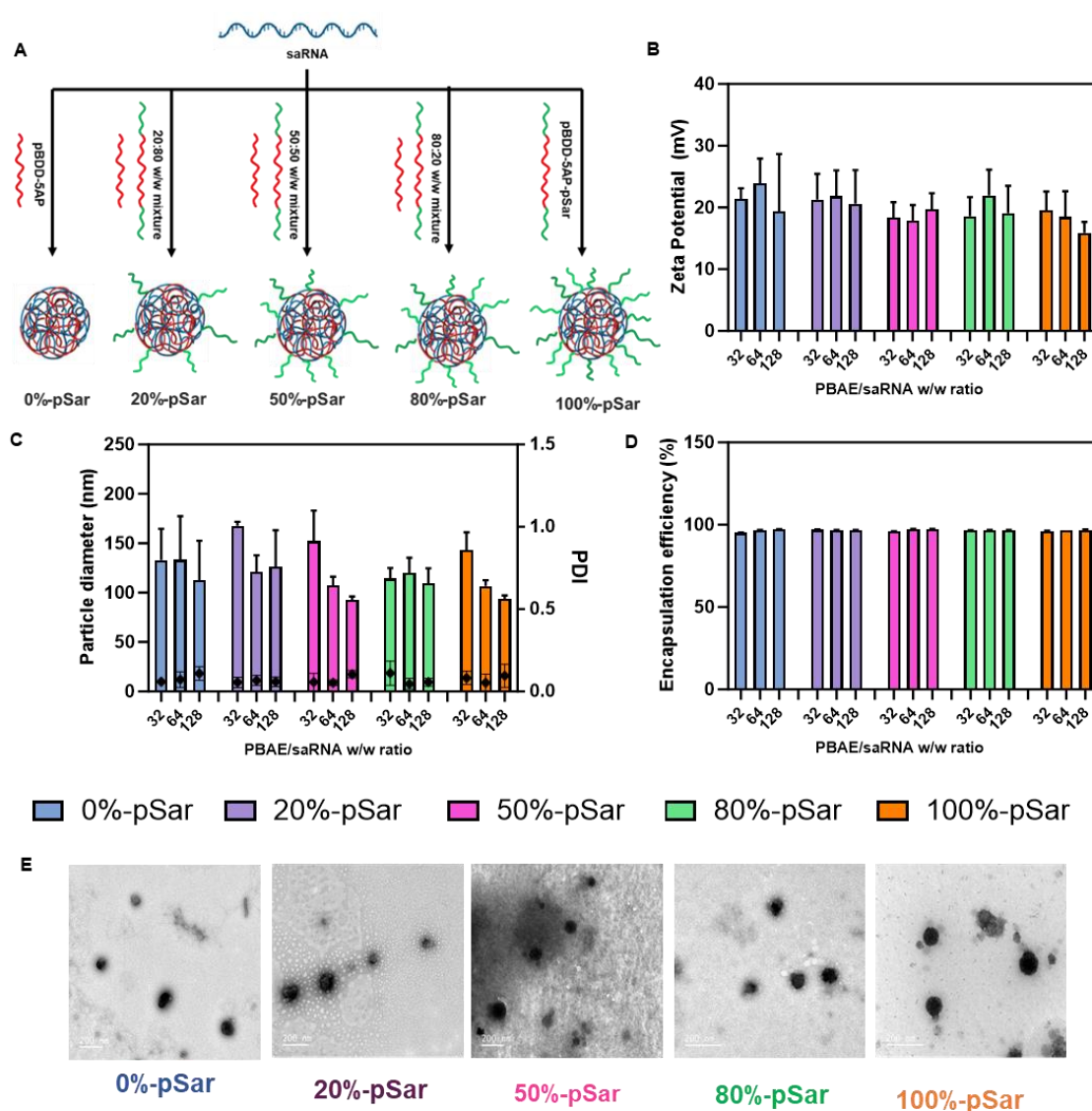


Figure 3.3. Formulation of PBAEs with saRNA to produce polyplex nanoparticles. (A) Schematic representation of saRNA formulation with OH-(pBDD-5AP)-OH, pSar-(pBDD-5AP)-pSar or different mixtures, called 'mix' of pSar-(pBDD-5AP)-pSar and OH-(pBDD-5AP)-OH as

a weight% which is termed as 0%-pSar, 20%-pSar, 50%-pSar, 80%-pSar and 100%-pSar. The nanoparticles were prepared in pH 5.0 25 mM sodium acetate at a final saRNA concentration of 10 µg/mL. (B) Zeta potential and (C) particle size for the OH-(pBDD-5AP)-OH, pSar-(pBDD-5AP)-pSar and 'mix' polyplexes at a PBAE/saRNA w/w ratio of 32, 64 and 128 determined by dynamic light scattering at 25°C. Bars represent the mean $\pm$ SD (n=3). (D) Encapsulation efficiency of saRNA within polyplexes evaluated using Ribogreen assay. (E) Representative transmission electron micrograph of all polyplexes (Scale bars correspondence to 200 nm).

3.3.3 In Vitro Evaluation of saRNA Delivery

3.3.3.1 Transfection and metabolic activity of pSarylated polyplexes

The toxicity and saRNA delivery/expression efficiency of the individual OH-(pBDD-5AP)-OH, pSar-(pBDD-5AP)-pSar and co-formulated polyplexes were examined in HEK293T and Caco-2 cell lines. These cell lines were chosen as HEK293T human kidney cell line is the main cell line used for pilot transfection studies as they are easy to transfect, and culture, whilst Caco-2 colon cancer cell line is a model of intestinal barrier integrity and can spontaneously differentiate into a heterogeneous mixture of intestinal epithelial cells as occurs in the intestine. Similar to the formulation characterisation study above, polyplexes were prepared at polymer/saRNA w/w ratios of 32, 64, and 128, utilising a firefly luciferase encoding saRNA and compared against Lipofectamine MessengerMAX™ (positive expression control) and Triton X-100 (positive toxicity control). All the formulations exhibited more than 50% cell viability, as determined from the metabolic activities. Similar to previous studies, increasing w/w ratio, specifically above w/w 32, resulted in increased toxicity due to increased polycation concentration which is known to disrupt the negative phospholipid bilayer (Figure 3.4A) [33]. In general, Caco-2 cells displayed high metabolic activity compared to HEK293T cells which may be due to the lower transfectability of the intestinal epithelial cells (Figure 3.4B)

[34]. Notably, using HEK293T cells, formulations prepared at higher w/w ratios yielded greater transfection efficacy explicitly for co-formulated groups (20%-pSar, 50%-pSar and 80%-pSar) (Figure 3.4B). For the w/w ratio 128 the transfection efficiency was close in extent to the positive control, Lipofectamine MessengerMAX™. Unlike in HEK293T cells most of the formulations did not show statistically significantly higher luciferase expression than naked saRNA in Caco-2 cells; however, only the polyplexes 50%-pSar and 80%-pSar yielded increased saRNA expression in this cell line. Particularly, no substantial difference was detected between the 50%-pSar and positive control lipofectamine. Similarly, a previous study showed that mixed of PEGylated and non-PEGylated polymers (50:50) displayed the most effective gene delivery vector for glioblastoma cells [35].

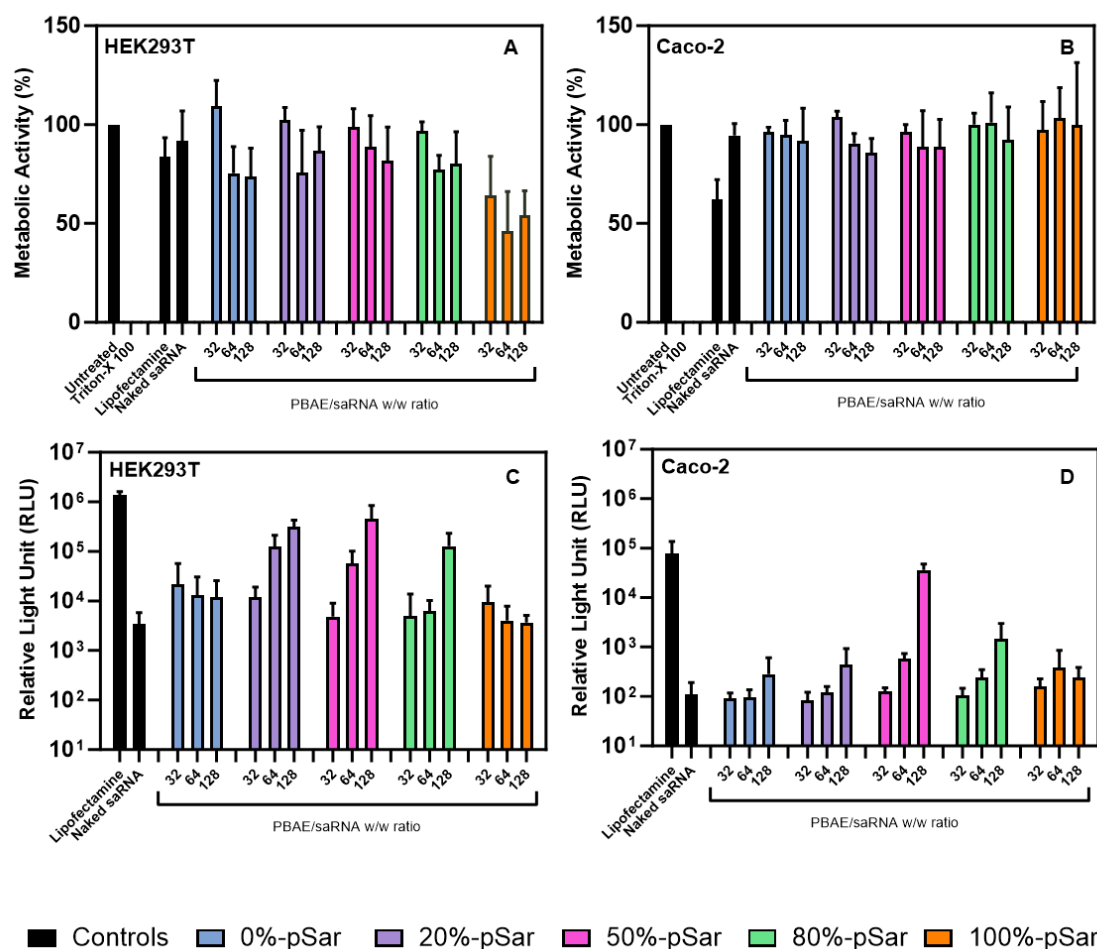


Figure 3.4. Metabolic activity and transfection efficiency of PBAE derived saRNA polyplexes. Effect of PBAE polyplexes formulated with firefly luciferase encoding saRNA at PBAE/saRNA w/w ratios of 32, 64 and 128 on metabolic activity (A and B) and transfection efficiency (C and D) on HEK293T cells (A and C) and Caco-2 cells (B and D). Cells were treated with polyplexes containing saRNA at a concentration of 500 ng/mL (100 ng/well) in serum-free Opti-MEM. Metabolic activity was compared against Triton X (positive control) and untreated cells (negative control) and calculated by normalizing metabolic activity to untreated cells. In vitro transfection efficiency was analysed after 24 h post-transfection using the Promega ONE-GLO luciferase assay and compared against Lipofectamine Messenger MAXTM (positive control) and naked saRNA (negative control) expressed as relative light units (RLU) for luciferase expression. Bars represent the mean $\pm$ SD (n=3).

3.3.3.2 Cellular uptake of pSarylated polyplexes

Due to the encouraging results with 50%-pSar polyplex at w/w 128, we further investigated cellular uptake of this polyplex formulation. To evaluate the cellular association of 50%-pSar polyplexes in Caco-2 cells, we first generated a FITC labelled pSar-(pBDD-5AP)-pSar through alcohol isothiocyanate coupling on the

pendant hydroxyl groups of the 5-AP monomers within the core PBAE. Cellular uptake was assessed through imaging flow cytometry 2 h and 4 h after treatment (Figure 3.5A). It was observed that 76% of Caco-2 cells were FITC-positive, indicating successful cell association within 2 hours of incubation. There was a further increase to 80% FITC-positive at 4 h incubation time, overall highlighting rapid association of the administered polyplexes and release of the saRNA into the cytosol. The median fluorescence intensity rose from 7900 arbitrary units up to 8100 between 2 h and 4 h incubation, indicating an initial rapid uptake by the Caco-2 cells and release into the cytosol occurred within 2 hours in this experiment. Compared to traditional flow cytometry the images from each cell can be analysed to compare internalisation vs membrane association. The median of internalisation score of the polyplexes was approximately 1.8 indicating a high proportion of nanoparticles were internalised inside the cells since having a score less than 0.3 can be considered to be surface bound (Figure 3.5B) [36].

To assess further the intracellular internalisation mechanism of the pSar-(pBDD-5AP)-pSar in Caco-2 cells we employed confocal microscopy to evaluate colocalization with a lysosomal (LysoTracker™ red) and nuclear (Hoechst 33342) stain (Figure 2.5C). After 4 h post administration, confocal micrographs showed that polyplexes successfully internalised within Caco-2 and merged images demonstrated with minimal co-localisation with the acidic lysosomes with image analysis indicating Pearson's Correlation Coefficient between FITC labelled polyplexes and LysoTracker™ stained lysosomes were below 0.5. Given the ~100 nm particle diameters it is likely that these formulations were internalised by an endocytosis mechanism, however the

apparent low colocalization with the acidic lysosomes may indicate that the particles escaped before endosomal maturation to lysosomal compartments. Although further endosomal escape studies would be needed to investigate this phenomenon in more detail, it is possible that enhanced endosomal escape was a factor in the high expression efficiencies observed for these complexes. However, it should also be noted that fluorescein is a pH responsive dye hence may exhibit reduced fluorescence within the acidic lysosomal compartments, adding an uncertainty to measures of dye co-localisation, nonetheless the high transfection efficiencies observed for this formulation indicates clear propensity of endosomal escape which may have all escaped within the first 2 hours.

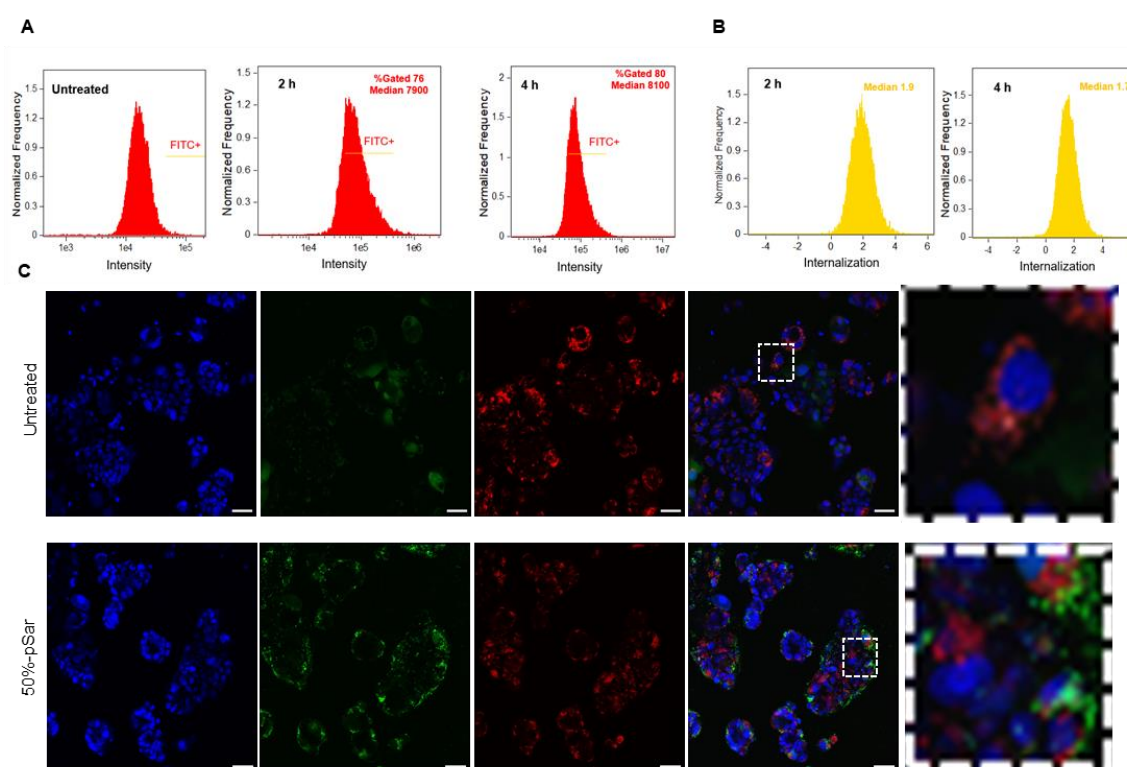


Figure 3.5. Cellular uptake of PBAE derived saRNA polyplexes. (A-B) Imaging cytometry histograms of fluorescence intensity and internalisation of Caco-2 cells after 2h and 4 h following treatment with FITC-labelled 50%-pSar polyplexes at a PBAE/saRNA w/w ratio of 128 at a concentration of 500 ng/mL saRNA. Data were analysed using IDEAS software. (C) Representative confocal microscopy images of Caco-2 cells treated saRNA polyplexes derived from FITC-labelled pSar-(pBDD-5AP)-pSar at a PBAE/saRNA w/w ratio of 128 for 50%-pSar polyplexes after 4 h at 100 ng/well (500 ng/mL) saRNA. Scale bar 10 μ m (63x).

3.3.4 Assessment of pSarylated polyplexes in colorectal cancer organoids

Following successful in vitro saRNA transfection and polyplex uptake of 50%-pSar (w/w 128) in Caco-2 cells, we hypothesised that this material may be an effective in delivering saRNA to intestinal organoids. Organoids are 3D organ-like structures that are cultivated from human tissue-derived stem cells in a laboratory setting. For instance, colon organoids comprise stem cells, goblet cells, enterocytes, endocrine cells, paneth cells and transit amplifying cells. These organoids naturally form complex structures, mimicking some aspects of real organ physiology, which holds promise for translating preclinical research into clinical applications [37].

We therefore evaluated our chosen formulation 50%-pSar (w/w 128) encapsulating a GFP-saRNA in human colon colorectal organoids to monitor saRNA expression via confocal microscopy (Figure 3.6). The 50%-pSar saRNA formulation was incubated with organoids once they had reached the desired compaction level. After 4 h, nanoparticles were removed, and fresh media was replaced onto the organoids. GFP expression was determined after 24 and 48 h. Confocal microscopy revealed GFP expression within the central regions of the organoids at 24 h, with increasing fluorescence intensity observable after 48 h (Figure 3.6). Previous studies have shown expression of exogenously delivered nucleic acids at the surface of cancer organoids, however the 50%-pSar PBAE formulations were substantially improved compared to the prior systems in terms of generating high expression levels deep within the core of

the organoid, indicating high penetration of these nanoparticles within the dense matrix of cells [38, 39]. These results indicate that pSarylated PBAEs may represent promising new materials for tumor directed delivery of nucleic acid therapeutics. Additionally, the full preservation of the organoid structure over the 48 h transfection experiment may suggest that these materials could be used to aid delivery of mRNA and saRNA to further organoid models to examine how expression of exogenously administered RNA varies within the surface and core of these tumor models.

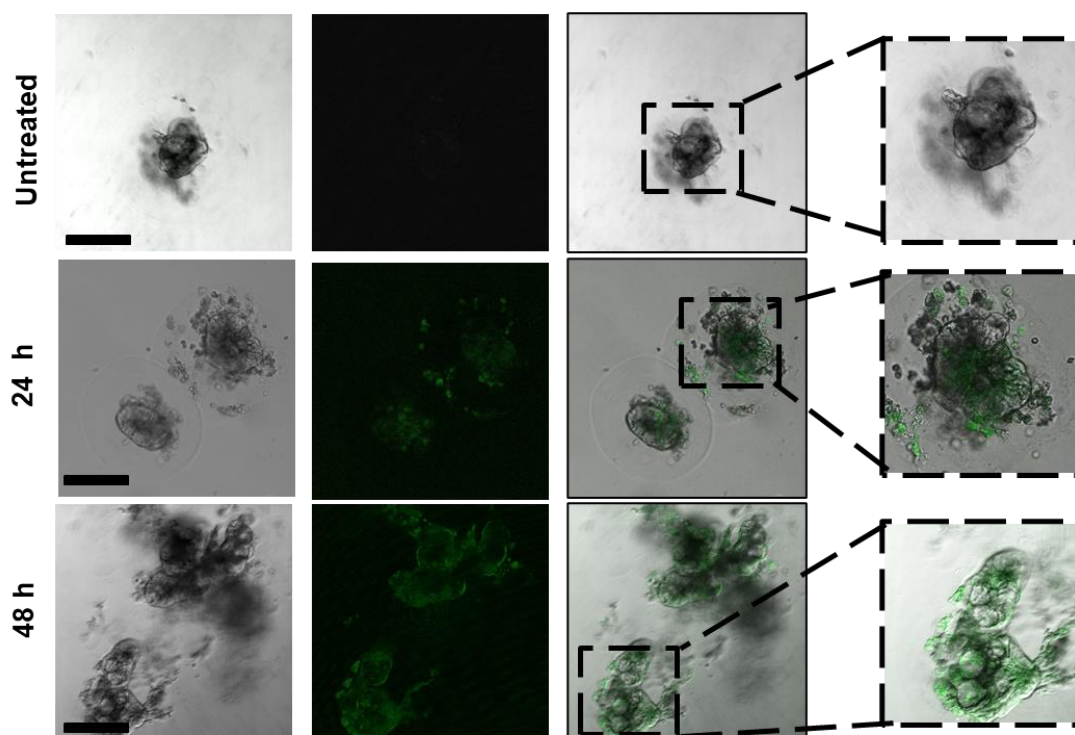


Figure 3.6. Analysis of GFP expression of 50%-pSar in human colon colorectal cancer organoids using confocal microscopy after 24 and 48 h (40X – scale bar 100 μ m).

3.4 Conclusions

In this work we have developed a new methodology to produce polysarcosinyolated poly(beta amino esters) as an alternative steric stabilising

group to PEG. We used this new pSar-PBAE conjugate to produce a library of polyplexes with different pSar densities on the nanoparticle surface by mixing pSarylated PBAEs with analogous unfunctionalized PBAEs in different ratios. We observed that pSarylation had minimal effect on polyplex physicochemical properties, while all pSar densities yielded higher transfection efficiencies than the positive control lipofectamine in HEK293T cells. In contrast, only the 50%-pSar polyplexes produced saRNA expression comparable to lipofectamine in colorectal cancer cells (Caco-2), with uptake studies indicating fast cellular uptake and limited colocalization in lysosomes indicating potential for endosomal escape. Finally, the 50%-pSar polyplexes showed potential translation for tumor delivery, exhibiting high expression of saRNA within the core colorectal cancer organoids within 24 h, likely due to the enhanced permeability of these polyplexes endowed with the pSar coating. The efficient diffusion to the organoid core may make these formulations amenable to intratumoral delivery of genetic material encoding for pro-inflammatory cytokines which are required for stimulation of the immune system around the local tumor environment in immuno-oncology applications. Overall, these results indicate that pSarylation is a suitable alternative for PEGylation of PBAEs, yielding potent gene delivery vehicles. Further studies can be conducted to test steric stability of the pSarylated nanoparticles. In addition, as pSar is an established PEG alternative, future research should focus on studying the differences in immunogenicity between PEG-PBAE and pSar-PBAE conjugates, which is especially important for vaccine and immunotherapy nucleic acid formulations.

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3.6 Author Contributions

Acknowledgement of research work done by co-authors:

- H. Bayraktutan: Performed all the experiments unless stated otherwise. Data curation, formal analysis, investigation, methodology, validation, visualization, writing of the original draft of the manuscript.
- R.J. Kopiasz and U. C. Oz: helped with polymer synthesis, NMR analysis and critically reviewed and edited the manuscript.

- A. Elsherbeny and N. Gumus helped with in vitro studies. A. Elsherbeny performed confocal imaging and N. Gumus performed cellular uptake experiments. Critically reviewed and edited the manuscript.
- K. Polra, P. F. McKay and R. J. Shattock: synthesised and purified saRNA and critically reviewed the manuscript.
- M.M Espuga, P. Ordóñez-Morán and A. Mata: planned and performed organoid-based experiments. Critically reviewed and edited the manuscript.
- A. Mata, C. Alexander, and P. Gurnani: Supervisors of H. Bayraktutan, supervised the student while conducting the work. Critically reviewed and edited the manuscript.

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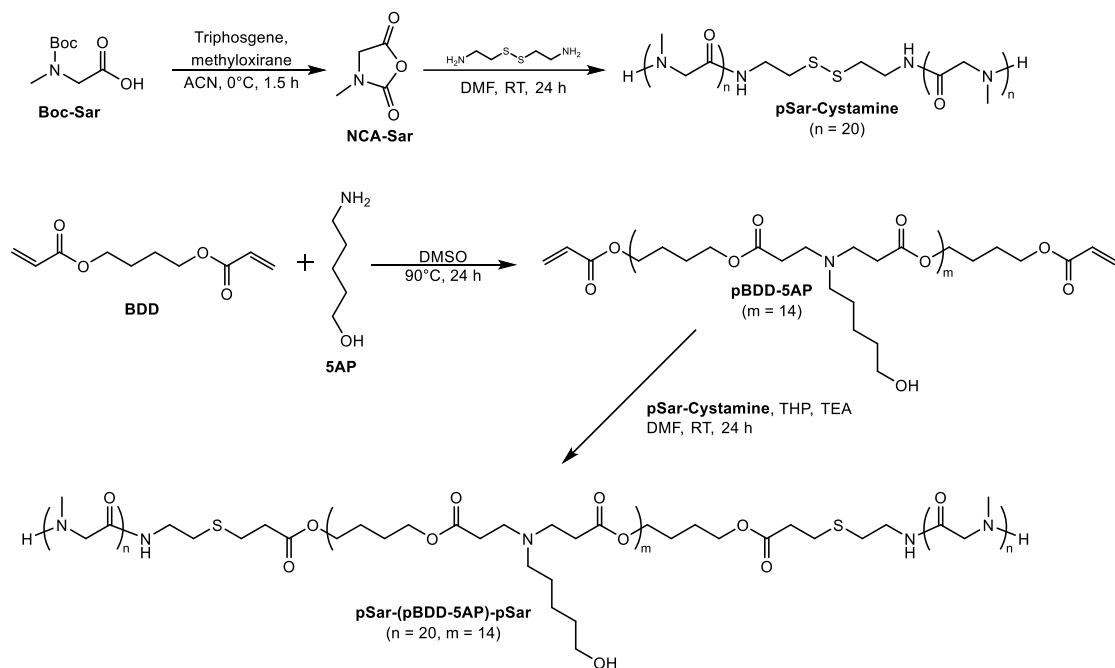
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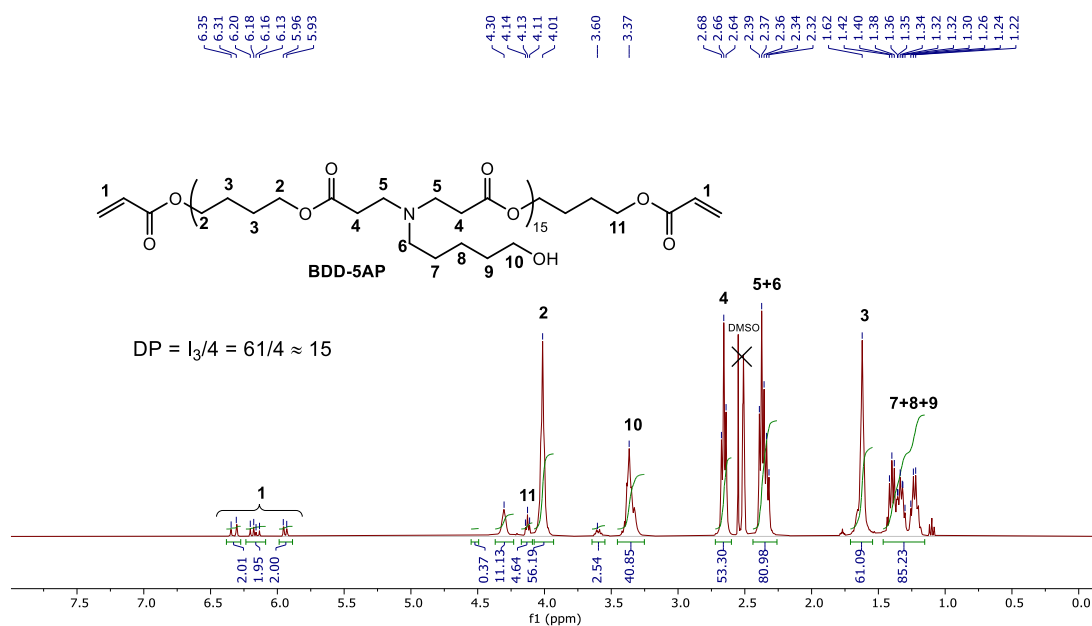
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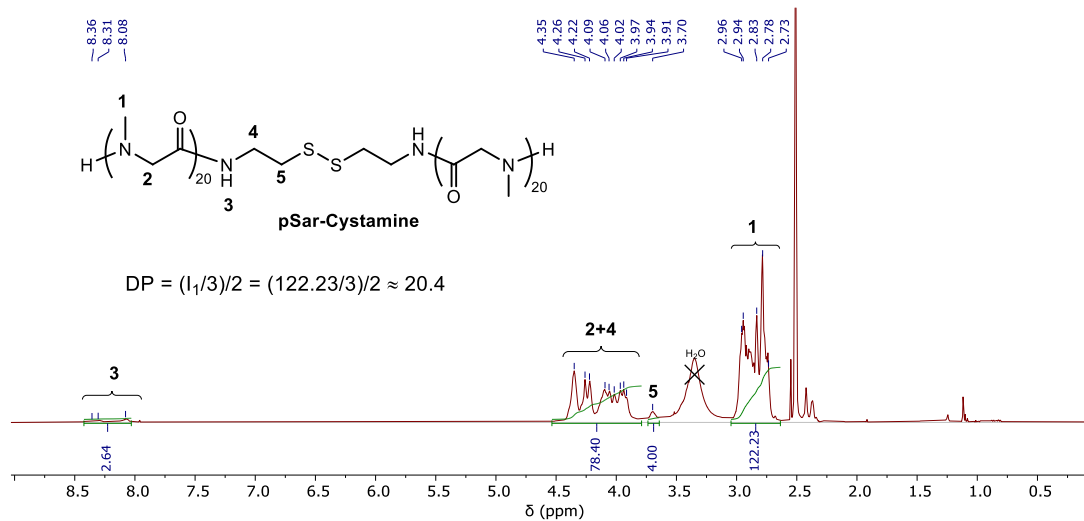
3.8 Supporting Information



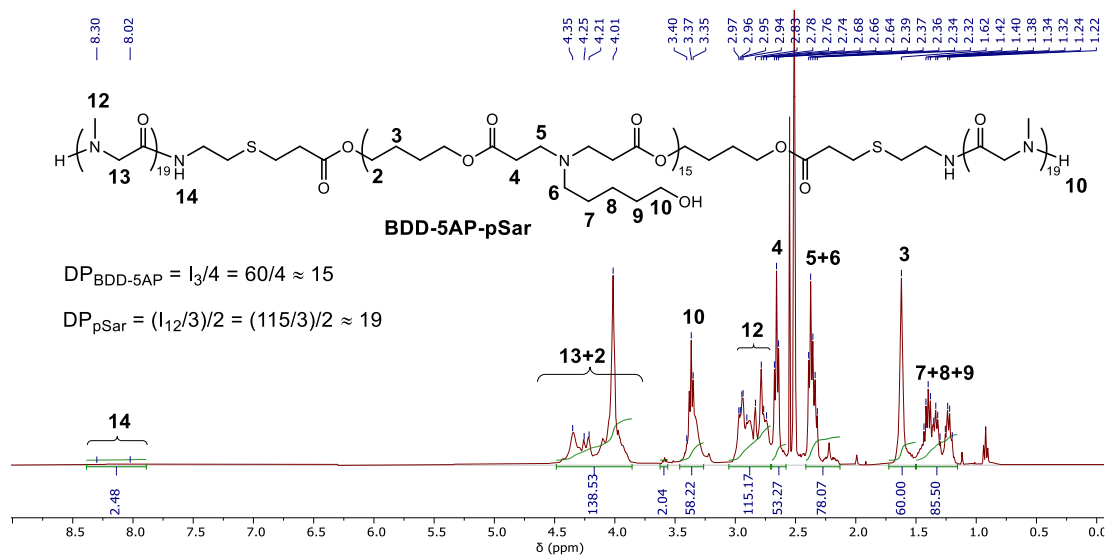
Scheme S3.1: Synthesis scheme of the final block copolymer pSar-(pBDD-5AP)-pSar.



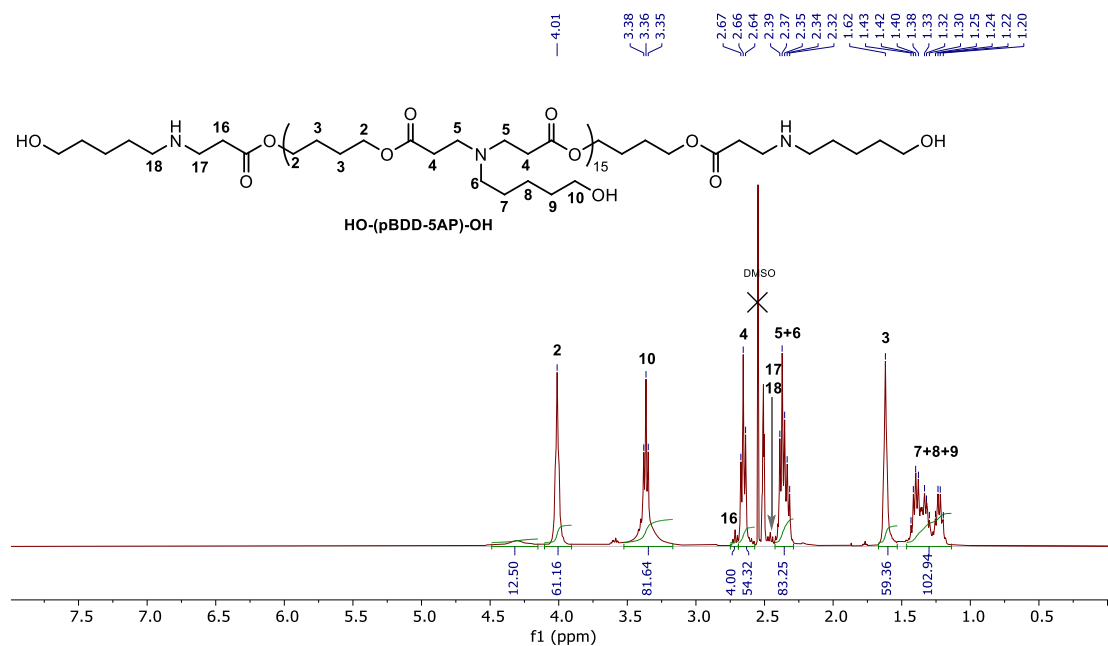
Spectrum S3.1. ^1H NMR (400 MHz, DMSO-d_6) of pBDD-5AP.



Spectrum S3.2. ^1H NMR (400 MHz, DMSO-d_6) of pSar-Cystamine.



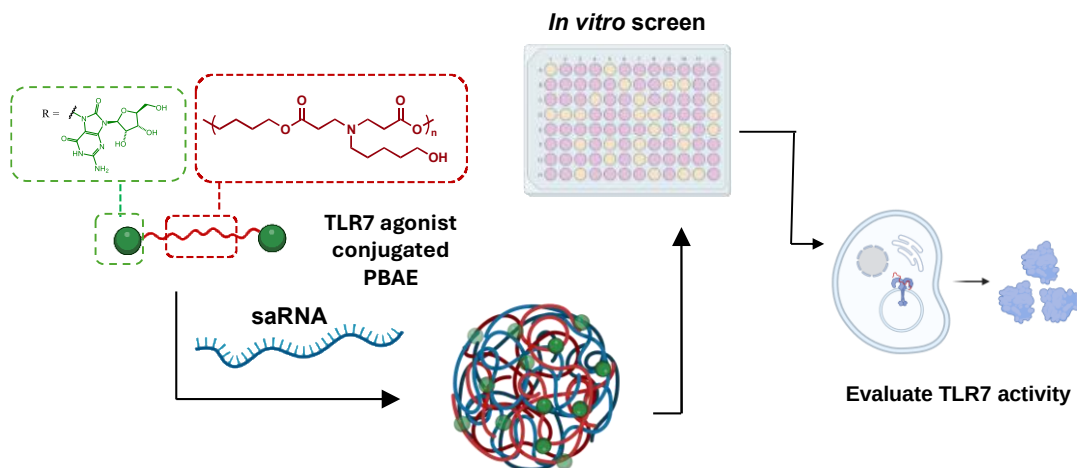
Spectrum S3.3. ^1H NMR (400 MHz, DMSO-d_6) of pSar-(pBDD-5AP)-pSar.



Spectrum S3.4. ^1H NMR (400 MHz, DMSO-d_6) of HO-(pBDD-5AP)-OH.

Chapter 4

TLR7 conjugated poly(beta amino ester) polyplexes towards adjuvanted mRNA vaccines



Abstract

Messenger RNA (mRNA) technology serves as a powerful foundation for novel vaccines and treatments. Ensuring effective delivery of RNA medicines requires safe vectors, due to the poor cellular association of naked mRNA and susceptibility to degradation by endogenous nucleases. Recently, nanoparticle-based carries have attracted significant attention, particularly poly(beta-amino esters) which have showed promise as mRNA delivery systems. It is clear following the first generation of mRNA vaccines to protect against severe COVID-19 disease, that the durability of immune response remains short and does not protect against infection. Hence there is a need for delivery systems which enhance the robustness of immune response. In this study, we report the development of a TLR7 adjuvanting PBAE through direct conjugation of loxoribine, a potent TLR7 agonist. We observed that the loxoribine conjugated PBAE was able to condense self-amplifying RNA efficiently into small (100 – 200 nm) polyplex nanoparticles with complete encapsulation efficiency. Formulations specifically at w/w ratio 128 showed high transfection in HEK293T and DC2.4 cell lines with good cytotoxicity profiles. In addition, TLR activation assays using TLR7 reporter HEK-Blue cells showed that loxoribine conjugated formulations can efficiently agonise TLR7 with some synergy observed between loxoribine and the delivered saRNA. Consequently, these findings suggest the potential of adjuvanted polyplex for enhanced mRNA vaccines.

Key words: saRNA, PBAE, Loxoribine, TLR7 agonist, polyplex, HEK293T, DC2.4 and HEK-BLUE cells

4.1 Introduction

Messenger RNA technology represents a safe, potent and manufacture-friendly route to express transiently endogenous or engineered proteins in a target cell or tissue. mRNA has therefore been widely studied for the development of novel vaccines against infectious disease and immunotherapies for a variety of malignancies [1, 2]. These operate by administering mRNA encoding for pathogen or malignant cell surface protein epitopes to train the immune system to recognise these and eliminate them, either on infection by the pathogen, or as against cancer cells expressing those epitopes [3, 4]. In both applications, the quality and robustness of the immune response is critical in the eventual adaptive immune response against the target antigen and therefore therapeutic/prophylactic performance.

Naked mRNA displays poor cellular association and susceptibility to degradation from endogenous nucleases, meaning that a suitable delivery vector is required to protect the nucleic acid cargo and facilitate its transportation to its target in the cytosol [5]. These delivery systems are typically nanoparticle-based materials, such as lipid nanoparticles (LNP), which have been clinically used for three approved nucleic acid products (Onpattro, Spikevax and Comirnaty) [6]. However, their chemical versatility and limited intellectual property space creates an urgent need for alternative delivery systems. Among these, polycations are now recognised a potent but clinically immature delivery approach [7, 8]. These operate by condensing negatively charged mRNA into small (50-200 nm) polyplex nanoparticles with positively charged cationic polymers through opposing electrostatic interactions [9]. The

significantly broader chemical versatility of polymeric materials makes these ideal systems for improving the immune response of mRNA vaccines.

In principle, messenger RNA vaccines and immunotherapies display self-adjuvanting behaviour, due to the agonist properties of exogenous RNA on endosomal (Toll-like receptor 7/8) and cytosolic (RIG-I, MDA-5) single stranded RNA sensors which mimic the inflammatory process of an infective RNA virus [10, 11]. However, the condensation of mRNA into nanoparticles and use of modified nucleotides, such as 1-methylpseudouridine (m¹ψ) hinders the activation of these processes in antigen-presenting cells (APCs) which weakens the initial steps of the adaptive immune response [12]. However, excessive activation of these inflammatory processes simultaneously puts APCs into an 'anti-proliferative' state, thus shutting down the cells' protein synthesis machinery, and reducing the translation efficiency of the delivered mRNA construct [12, 13].

Traditional vaccines and immunotherapies achieve a robust immune response by utilising adjuvants, immunostimulatory molecules which are co-formulated with the active ingredient [14]. Adjuvanticity can be introduced either from a humoral immunity perspective, through controlled release of the antigen (e.g. alum, hydrogels), or preferably direct activation of cellular immunity through the co-formulation of molecules which simulates the natural infection process by binding to pathogen recognition receptors (PRRs) at the immune cell surface [15]. Key example of these are agonists for Toll-like receptors (TLRa's), a specific PRR class found in APCs, macrophages and other immune cells which, when stimulated, elicit downstream expression of pro-inflammatory cytokines which form the innate immune response [16].

Development of adjuvanted mRNA formulations, which co-deliver the antigen encoding nucleic acid and adjuvant to the same cells could therefore represent a powerful strategy to strengthen and broaden the immune response of mRNA vaccines, whilst providing a flexible platform to mitigate any inflammatory induced inhibition of mRNA translation [17 – 19]. There is significant evidence that LNP formulations display intrinsic adjuvanticity, but these effects are not always predictable and are dependent on the cationic/ionisable lipid chemistries. In contrast, there has been significant progress development in targeted adjuvant formulations for mRNA vaccines and therapies, often through co-formulation or conjugation of TLRa's, such as polyinosine-cytosine (polyI:C, TLR3a), imidazoquinolines (TLR7/8a) and CG rich oligonucleotides (CpG-ODN, TLR9a) from an LNP perspective [17, 20, 21]. A guanosine analogue would trigger a TLR7 response because Toll-like receptor 7 (TLR7) is known to recognize single-stranded RNA (ssRNA) and synthetic small molecules, including guanosine analogues [22]. TLR7 is part of the innate immune system and is located in the endosomes of certain immune cells, such as plasmacytoid dendritic cells and B cells. When a guanosine analogue binds to TLR7, it mimics the presence of viral ssRNA, leading to the activation of the receptor [22]. This triggers a signaling cascade that results in the production of type I interferons and other pro-inflammatory cytokines, thus initiating an immune response. The ability of guanosine analogues to activate TLR7 is leveraged in therapeutic applications to enhance immune responses [23]. For example, Appel lab created poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) nanoparticles and conjugated a potent TLR7/8 agonist moiety on their surface [24]. They hypothesised that the pharmacokinetic profile of the nanoparticles can reduce

systemic toxicity by localizing TLR7/8 agonist presentation to the tumor bed and tumor-draining lymph nodes. They showed that when combined with anti-programmed death-ligand 1 checkpoint blockade, peritumoral injection of TLR7/8 agonist NPs slows tumor growth, extends survival, and decreases systemic toxicity compared to free TLR7/8 agonists in a murine colon adenocarcinoma model. These nanoparticles represent a modular platform for controlling the pharmacokinetics of immunostimulatory molecules, enhancing potency and reducing toxicity [24]. Meanwhile the few limited reports of adjuvanted polycation delivery systems focus on co-formulation of polyI:C and CpG-ODNs, however direct conjugation of a TLR agonist adjuvant has not been reported for polymer-based mRNA formulations to our knowledge [25, 26].

Herein, we report the development of a TLR7a conjugated polycation as an adjuvanted formulation for self-amplifying messenger RNA, an mRNA technology which includes replicase genes derived from the alphavirus genome within the construct (Figure 4.1). This means that when saRNAs are translated they can exponentially amplify the construct within the cytosol whilst also expressing the encoded protein. saRNAs can therefore enhance protein expression levels at significantly lower doses, lengthening expression half-life and reducing the cost compared to mRNA. We utilised the intrinsic chemistry of loxoribine (a guanosine analogue and known as a TLR7 agonist) to conjugate this on the end-groups of a poly(beta amino ester) cationic polymer, which are now well-established polymeric delivery systems for mRNA and saRNA modalities. The loxoribine-PBAE conjugate was then formulated with saRNA to form adjuvanted polyplexes, which were characterised for their delivery efficiency, transfectability and ability to stimulate the TLR7 pathway in cells.

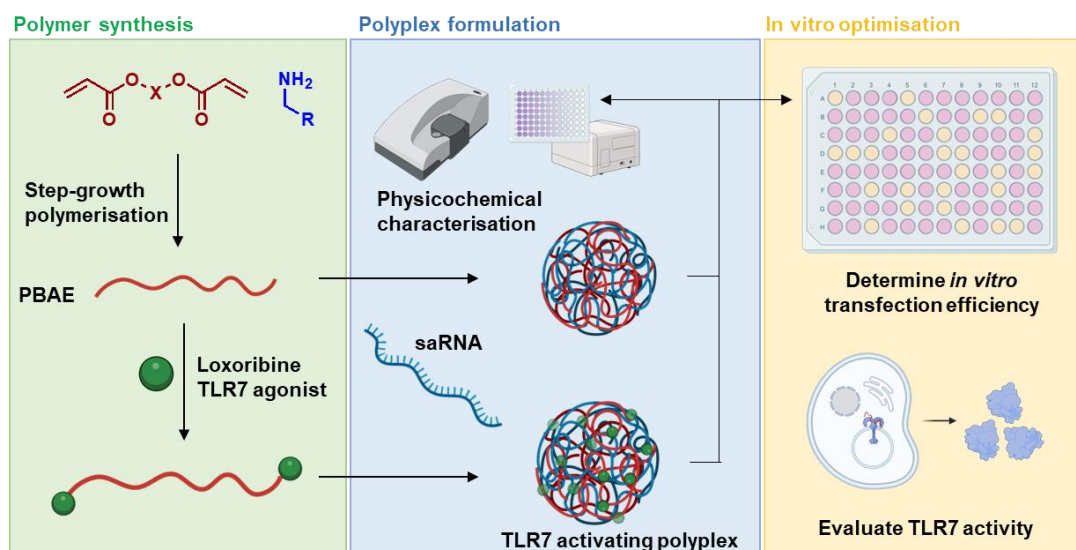


Figure 4.1. Schematic representation of PBAE synthesis, polyplex assembly, analysis of in vitro transfection and toxicity profiles and the evaluation of TLR7 activation.

4.2 Materials and Methods

4.2.1 Materials

1,4-Butanediol diacrylate (BDD), 5-aminopentan-1-ol (5-AP), 2,2'-(ethylenedioxy)bis(ethylamine), 3-mercaptopropionyl-N-hydroxysuccinimide ester, tris(2-carboxyethyl)phosphine (THP), triethylamine (TEA), fluorescein isothiocyanate (FITC), diethyl ether, tetrahydrofuran, sodium acetate (NaOAc) buffer (pH:5.0) and the dye Gel Red were purchased from Sigma-Aldrich. Loxoribine (LOX) was purchased from InvivoGen. Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), acetonitrile (ACN), tetrahydrofuran (THF), hexane, diethyl ether, dimethyl sulfoxide (DMSO), loading buffer, Tris-acetate-EDTA (TAE) buffer, tris-(2-hydroxyethyl) phosphine (THP), Lipofectamine Messenger MAX™, Hoechst 33342, LysoTracker red and 96-well plates were obtained from ThermoFisher. Dulbecco's Modified Eagle Medium (DMEM) cell culture medium, RPMI 1640 cell culture medium, HEK-Blue Detection medium,

fetal bovine serum (FBS) and Opti-MEM were bought from Gibco, Invitrogen, Carlsbad, CA, USA. PrestoBlue reagent and ONE-Glo D-luciferin substrate were purchased from Promega, UK.

4.2.2 Instrumentation and Analysis

4.2.2.1 NMR Spectroscopy

All ^1H -NMR spectra were recorded in ppm (δ) at 400 MHz in d_6 -DMSO using a Bruker Advance III MHz spectrometer that was maintained at 25°C. To analyse the spectra MestReNova 6.0.2 copyright 2009 (Mestrelab Research S.L.) was applied.

4.2.2.2 Gel permeation Chromatography

The gel permeation chromatography (GPC) analysis was performed using the PL-50 instrument equipped with a dual angle light scatter (LS), viscometry (VS) and differential refractive index (DRI). The system was equipped with a PLgel 5 μm guard column and a 2 $\times$ PLgel Mixed D columns (300 $\times$ 7.5 mm). Dimethylformamide (DMF) with LiBr (0.1% wt/wt) was utilised as an eluent with a flow rate of 1 mL/min at 50°C. The instrument was calibrated with Poly(methyl methacrylate) standards (Agilent EasyVials) between 955500–550 g mol^{-1} . Using conventional calibration, Cirrus GPC software, dispersity ($\mathcal{D}$) values, molecular weight (M_w) and experimental molar mass (M_n , SEC) were detected.

4.2.2.3 DLS and Zeta Potential

A Zetasizer nano-ZS90 (Malvern, Inc.) dynamic light scattering (DLS), instrument was used to characterise the particles in terms of their size, polydispersity index (PDI) and zeta potential. The instrument was used at 25°C

to determine the zeta potential, average hydrodynamic diameters, and PDI of polyelectrolyte complexes.

4.2.2.4 Transmission Electron Microscopy

Briefly, glow discharged Formvar/carbon coated TEM grids were used, on which were placed the formulation samples (13 μ L), which were left for 15 minutes, then the excess solution was removed and the samples allowed to dry at room temperature. Following this, 10 μ L of 2% aqueous uranyl acetate was applied to each grid and left for 10 seconds. After air drying, imaging was performed using a Tecnai G2 Spirit TEM with BioTwin lens configuration (Thermo Fisher Scientific, Eindhoven, The Netherlands) at an accelerating voltage of 100 kV.

4.2.3 Polymer Synthesis and Characterisation

4.2.3.1 Poly(BDD-5AP) (pBDD-6AP)

To BDD (1000 mg) in DMSO (0.95 mL), 5-AP (470 mg) in DMSO (2 mL) was added and the reaction was stirred at 90°C overnight. Then, the mixture was diluted with THF (2.94 mL), the product was isolated by precipitation from diethyl ether (40 mL), and further purified by re-dissolving in THF (3 mL) and precipitation in diethyl ether (40 mL) thrice. The product was dried under vacuum overnight yielding 1250 mg (85%) of a yellowish oil. Molecular weight determined by SEC: M_n = 6.2 kDa, M_w = 8.0 kDa, $\bar{D}$ = 1.3; for ^1H NMR see Spectrum S1 in Supplementary Information.

4.2.3.2 $\text{H}_2\text{N}-(\text{pBDD-5AP})-\text{NH}_2$

To produce amine end-capped PBAEs, an excess amount of 2,2'-(ethylenedioxy)bis(ethylamine) (300 mg) was added to the BDD-5AP (1000 mg)

in DMSO (10 mL) in a 20 mL vial and the mixture was stirred for 24 hours at room temperature. Polymers were isolated by diluting the reaction mixture into THF (10 mL) followed by dropwise precipitation in diethyl ether (40 mL) thrice. The polymer was collected via centrifugation and dried under reduced pressure at 37°C and a yellow viscous liquid was obtained. Finally, polymers were stored at -20°C as 100 mg/mL solution in DMSO. Molecular weight determined by SEC: $M_n = 6.6$ kDa, $M_w = 9.1$ kDa, $\bar{D} = 1.4$; for ^1H NMR see Spectrum S2 in Supplementary Information.

4.2.3.3 SH-(pBDD-5AP)-SH

To produce SH-end-capped PBAEs, an excess amount of mercaptopropanyl-N-hydroxysuccinimide ester (84.5 mg) was added to H_2N -(pBDD-5AP)- NH_2 (500 mg) in DMSO (5 mL) in a 20 mL vial, lastly TEA (16.8 mg) and THP (2.5 mg) were added the mixture. The mixture was stirred for 3 hours at room temperature under Argon and UV conditions to avoid polymer crosslinking. Polymers were isolated by diluting the reaction mixture into THF (5 mL) followed by dropwise precipitation in diethyl ether (40 mL) thrice. The polymer was collected via centrifugation and dried under reduced pressure at 37°C and a yellow viscous liquid was obtained. Finally, polymers were stored at -20°C as 100 mg/mL solution in DMSO. Molecular weight determined by SEC: $M_n = 6.9$ kDa, $M_w = 9.9$ kDa, $\bar{D} = 1.4$; for ^1H NMR see Spectrum S3 in Supplementary Information.

4.2.3.4 LOX-(pBDD-5AP)-LOX

Finally, to obtain LOX-end-capped PBAEs, an excess amount of Loxoribine (141 mg) was added to HS-(pBDD-5AP)-SH (500 mg) in DMSO (5 mL) in a 20 mL vial, lastly TEA (16.8 mg) and THP (2.5 mg) were added the mixture. The

mixture was stirred for 3 hours at room temperature under Argon and UV conditions to avoid polymer crosslinking. Then, the mixture was dialysed (molecular weight cut-off 3,5 kDa) against DMSO (1 L) for 24 hours and DMSO solution was changed once. Afterwards, the mixture was precipitated in diethyl ether (40 mL) and to remove remaining DMSO re-dissolved in THF (5 mL) and was precipitated in diethyl ether (40 mL) thrice. The product was dried under vacuum overnight and obtained a dark yellowish viscous liquid. Molecular weight determined by SEC: $M_n = 7.8$ kDa, $M_w = 11$ kDa, $\bar{D} = 1.4$; for ^1H NMR see Spectrum S4 in Supplementary Information.

4.2.4 Synthesis of Fluorescently Labelled Polymers

LOX-(pBDD-5AP)-LOX polymer was dissolved in 5 mL DMSO (0.5 mg/ml) in a 20 mL vial. FITC was added to vials at a 1:10 (moles of end amine: FITC) molar ratio and TEA was added to mixture as 0.1 molar ratio. The reaction mixture was allowed to stir for 24 hours in the dark at room temperature. The reaction mixture was then dialysed in the dark using molecular weight cut-off 3,5 kDa against 250 mL NaOAc buffer (pH:5.0). Purification was continued for 4 days and the dialysis medium refreshed 2 times in a day. After freeze drying, FITC-labelled polymers were collected as a yellow solid and were stored at -20°C as 100 mg/mL solution in DMSO.

4.2.5 Polymer Buffering Capacity Assay

The buffering capacity of the polymers was evaluated by acid-base titration over the pH range of 10.0–3.0. Briefly, 2 mg of polymer was dissolved in 30 mL of 0.1 M NaCl aqueous solution, and the solution was adjusted to pH 10.0 using 0.1 M NaOH. Then, precise volumes (between 20-40 μL) 0.1 M HCl were added

until a pH of 3 was achieved. The pH after each addition of HCl was recorded. 0.1 M NaCl was set as negative control.

4.2.6 Synthesis of saRNA

For firefly luciferase saRNA synthesis, luciferase encoded pDNA was linearised with Mlul-HF (NEB, UK) for 3 hours at room temperature. Uncapped RNA was synthesised in a MEGAscript reaction (Ambion, UK) using 1 µg of linearised DNA as a template. The RNA was then purified by LiCl precipitation (1:1 volume ratio) for 24 hours at -20°C. Then, transcripts were centrifuged at 14000 rpm at 4°C for 20 minutes, washed once with 70% ethanol, centrifuged for 5 minutes, the ethanol wash was removed, and the pellet was dried at room temperature for 5 minutes. The purified RNA was then capped using ScriptCap Cap 1 capping system kit (CellScript, Madison, WI, USA) and used according to the manufacturer's protocol. Capped transcripts were again purified by LiCl precipitation. The purified capped saRNA was resuspended in RNAase/DNAase free water and stored at -80°C at 800 µg/mL.

4.2.7 Preparation of Polyplexes

Polyplexes were prepared by electrostatic interactions between positively charged polymers and negatively charged saRNA by mixing various w/w ratios (32, 64 and 128). Firstly, the working dilutions of polymers and saRNA were prepared in 25 mM NaOAc buffer (pH:5.0). Depending on the desired w/w ratio, differing amounts of polymer stock solutions (100 mg/mL in DMSO) were mixed with nucleic acids in the buffer, gently mixed using a pipette and incubated at room temperature for 30 minutes. Whilst preparing the PBAE/saRNA complexes, taking a w/w 64 ratio as an example, 10 µg/mL of the RNA stock

solution was mixed with 2.560 mg/mL PBAE by pipette using a 25 mM NaOAc buffer.

4.2.8 Encapsulation Efficiency Using Ribogreen Assay

A fluorescence-based Quant-iT RiboGreen assay (ThermoFisher, UK) was utilised to be able to detect free RNA in solution after polymer encapsulation. Samples were diluted in 1× TE buffer (v/v 1:1) and the assay was performed according to the manufacturer's protocol. Samples were then loaded on a black, 96-well plate, and analysed for fluorescence on a microplate reader at an excitation of 485 nm and emission at 528 nm.

4.2.9 Determination of Drug Loading

The evaluation of loxoribine amount in LOX-(pBDD-5AP)-LOX nanoparticles was conducted through high-performance liquid chromatography (HPLC) quantification. Initially, 1 mg of the LOX-end-capped formulation was suspended in 1 mL of 1M HCl and subjected to a 4-hour incubation at 37°C to facilitate nanoparticle degradation and release of the therapeutic agent. Subsequently, the drug content was analysed using a Shimadzu UFLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a Hichrom 5 C18 column (250 × 4.6 mm) at room temperature. The mobile phase, composed of water and acetonitrile in a 20:80 ratio, flowed at 1 mL/min. A 20 µL injection volume was used, and the drug was monitored at 254 nm. Loxoribine exhibited a retention time of 2 minutes. The method demonstrated linear responses within the concentration range of 0.6-200 µg/ml. The results are presented as mean values ± standard deviation (SD). The drug loading was calculated using the following equation:

$$\text{Drug load (\%)} = \frac{\text{weight of drug (mg)}}{\text{weight of formulation (mg)}} \times 100$$

4.2.10 Determination of Drug Loading Using Ultraviolet-visible

Polymers were dissolved in 25mM NaOAc buffer (pH:5.0) at 1 mg/ml. Serial dilutions of loxoribine (3 – 100 µg/mL) were also prepared using the same buffer. Solutions were placed in quartz vial, and irradiated with UV light (λ_{max} = 251, 300 nm) using Tecan-Spark Ultraviolet-visible (UV-vis).

4.2.11 In vitro experiments

4.2.11.1 Cell Culture

Human embryonic kidney 293T (HEK293T) cells were a gift from Imperial College London and cultured in 10 % (v/v) FBS containing high glucose DMEM cell culture medium. DC2.4 cells were obtained from ATCC and cultured in RPMI 1640 cell culture medium. Media containing L-glutamine was supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), non-essential amino acids (0.1 mM) and 10 % FBS. Human TLR7 transfected HEK cells (HEK-Blue™) cells were purchased from InvivoGen and cultured in 10 % (v/v) FBS containing high glucose DMEM cell culture medium. Absence of mycoplasma was confirmed using polymerase chain reaction. Cells were grown to 90 % confluence in a humidified incubator at 37°C (5% CO₂) and detached with 1x trypsin/EDTA. Viability was assessed using Trypan Blue staining.

4.2.11.2 Cell Viability Assays

To investigate the cytotoxicity of the formulations, a cell viability assay was performed in the HEK293T, DC2.4 and HEK-BLUE cell lines. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells/well. For the treatment, the medium was aspirated, and cells were treated with 200 μ L of Opti-MEM containing various w/w ratios of formulations complexed with 10 μ g/ml fluc saRNA concentration (20 μ L of polyplex solution). After 4 hours, nanoparticles were aspirated and 100 μ L of complete medium was added to wells. At 24 hours posttreatment, each well was treated with 100 μ L of 10 % PrestoBlue reagent and allowed to incubate for 1 hour. Then, the total volume was transferred to a black 96-well plate and the FLUOstar Omega plate reader (BMG LABTECH, UK) was used to determine the fluorescent intensity (540 nm – 590 nm) of each well and normalized to the medium control.

4.2.11.3 In Vitro Transfection Experiments

In vitro transfection experiments were performed in HEK293T and DC2.4 cell lines, and the commercially available transfection reagent Lipofectamine Messenger MAXTM was used as positive control. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells per well. For the treatment, the medium was aspirated, then cells were transfected with 200 μ L of Opti-MEM containing various w/w ratios of formulations complexed with 10 μ g/mL fluc saRNA concentrations (20 μ L of polyplex solution). After 4 hours, nanoparticles were aspirated and 100 μ L of medium was added to wells. At 24 hours post-treatment, 50 μ L of media was removed then 50 μ L of the ONE-Glo D-luciferin substrate was placed into each well and mixed well by pipette. Finally, the total 100 μ L was placed in a 96-well plate and FLUOstar

Omega plate reader (BMG LABTECH, UK) was used to determine the luminescence.

4.2.11.4 TLR7 Activation Assay

TLR7 activation was tested on human TLR7 transfected HEK cells (HEK-BLUE). The commercially available transfection reagent Lipofectamine Messenger MAXTM was used as positive control. 24 hours prior to treatment, cells were seeded in a clear 96-well plate at a density of 20×10^3 cells per well. For the treatment, the medium was aspirated, then cells were transfected with 200 μ L of HEK-Blue Detection medium containing polymers only (without saRNA, 20 μ L) with different w ratios and various w/w ratios of formulations complexed with 10 μ g/mL fluc saRNA concentrations (20 μ L of polyplex solution). At 24 hours post-treatment, absorbance was measured at 620 nm using a FLUOstar Omega plate reader (BMG LABTECH, UK).

4.2.11.5 Cellular Uptake of saRNA using Confocal Microscopy

HEK293T and DC2.4 cells were seeded in CellViewTM 35 mm diameter glass bottom cell culture dishes at a density of 30×10^4 cells per well. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing FITC labelled LOX-end-capped formulation (LOX-(pBDD-5AP)-LOX) w/w 128 at 0.01 mg/mL. Cells were incubated for 2 hours at 37°C with 5% CO₂. Then, nanoparticles were aspirated, and cells washed 3 times with PBS and stained with 10 μ g/mL Hoechst 33342 and 75nM LysoTracker red applied in PBS solution. After 30 minutes, solution was aspirated, and cells were washed 3 times with PBS then FluoroBrite DMEM was added to cells. Imaging was performed using Leica confocal microscopy with LAS X software

on DAPI, FITC and LysoTracker filters. ImageJ software was used to process the pictures.

4.2.11.6 Imaging Flow Cytometry

DC2.4 cells were seeded in a clear 6-well plate at a density of 40×10^5 cells per well. For the treatment, the medium was aspirated, and cells were transfected with Opti-MEM containing LOX-(pBDD-5AP)-LOX w/w 128 (0.01 mg/mL) formulation. After 4 hours, nanoparticles were aspirated and 150 μ L of 0.05% trypsin was used for detachment of cells from wells. After incubation for 15 minutes, 300 μ L FACS buffer was used to neutralise the trypsin and cells were centrifuged at 1500 rpm for 10 minutes. Following this, cells were stained with Zombie Violet (1:500) dye for 30 minutes at room temperature in order to help with gating on live cells. Then, cells were fixed with 100 μ L of 4% paraformaldehyde in PBS for 20 minutes. Fixed cells were centrifuged, and pellets resuspended in 50 μ L of PBS. Data were acquired using an Image Stream 100 (Amnis, Seattle, US) and on single cell in focus events was used for analysis. Ideas Software (Amnis, Seattle, WA, USA) was used for data analysis. Total cell fluorescence was calculated by default total cell masks for the measurement of the nanoparticle internalisation and cell interiors were identified using the area erode tool (brightfield channel). Internalisation index was shown as percentage of maximum internalisation (percentage of interior cell fluorescence to total cell fluorescence).

4.2.12 Statistical analysis

Graphs and statistics were prepared in GraphPad Prism 9.5.1 version or higher. Statistical differences were analysed using either one-way or two-way ANOVA

adjusted for multiple comparison (either Tukey's or Dunnet's), p value lower than 0.05 was considered as statically significant and the levels of statistical significance were set $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

4.3 Results and discussion

4.3.1 Synthesis and Characterisation of PBAEs

To synthesise the targeted poly(beta amino ester) (PBAE), modified covalently with TLR7 agonist loxoribine (LOX), a synthetic pathway consisting of four steps was employed (Figure 4.2A). For the first step, 1,4-butanediol diacrylate (BDD) was reacted with 5-aminopentanol (5AP) using a molar excess of BDD to yield a PBAE terminated with acrylic groups, pBDD-5AP. This PBAE was chosen due to previous preclinical studies showing high mRNA transfection capability both *in vitro* and *in vivo* [27, 28]. The successful synthesis of the acrylic terminated PBAE, pBDD-5AP was confirmed using ^1H NMR spectroscopy, as signals around 6.3 - 5.9 ppm on the ^1H NMR spectrum indicated the presence of double bonds (Spectrum S4.1). p(BDD-5AP) was then end-capped with 2,2'-(ethylenedioxy)bis(ethylamine) via a further aza-Michael addition to yield the amine terminated PBAE $\text{H}_2\text{N}-(\text{pBDD-5AP})-\text{NH}_2$ which was confirmed as the ^1H NMR spectrum indicated complete disappearance of acrylic end-groups (Spectrum S4.2). $\text{H}_2\text{N}-(\text{pBDD-5AP})-\text{NH}_2$ was further transformed with *N*-hydroxysuccinimidyl- 3-mercaptopropanoate to yield the thiol end-capped PBAE $\text{HS}-(\text{pBDD-5AP})-\text{SH}$ polymer. The success of this modification was proved by the appearance of two signals characteristic to 3-mercaptopropionate group - at 2.90 ppm coming from alpha-methylene group

to the thiol moiety and 2.20 ppm coming from the thiol group (Spectrum S4.3). The thiol groups were then used as a reactive handle to conjugate loxoribine to the PBAEs, which contain a double bond making it suitable for thiol-ene chemistry under UV irradiation leading to the final product LOX-p(BDD-5AP)-LOX. Due to difficulties with analysis of an NMR spectrum of the final product, as a small molecule was conjugated with the polymer (Figure 4.2), the successful conjugation with loxoribine was demonstrated using HPLC and UV-Vis spectroscopy experiments (see Supplementary Information Figure S4.2). These methodologies allowed to determine the total content of loxoribine in the final polymer which was found as approximately 27 μg per mg PBAE (w/w 2.7%). All intermediate products and the final product were characterised by means of GPC (Figure 4.2B and 2C) where the molar mass was continuously increased after each end modification step (6200, 6600, 6900 and 7800 g mol^{-1}) and monomodal chromatograms with around $\bar{M}_w/\bar{M}_n \approx 1.4$ were revealed. The buffering capacities were assessed for $\text{H}_2\text{N}-(\text{pBDD-5AP})-\text{NH}_2$ and $\text{LOX}-(\text{pBDD-5AP})-\text{LOX}$ as the pK_a of polycations has been recognised as crucial for enhancing endosomal buffering and endo/lysosomal escape of polymer-based nucleic acid delivery systems (Figure 4.2D). Acid-base titration in the pH range of 10.0-3.0 for amine-end-capped PBAE titration curve was observed between the negative control (NaCl) and positive control (PEI). However, with LOX-end-capped PBAE the titration curve was similar to that of PEI, indicating that LOX-end-capping increased their buffering capacity (Figure 4.2D), likely due to the purine rings of LOX even though only 2 drug molecules were added to each polymer chain.

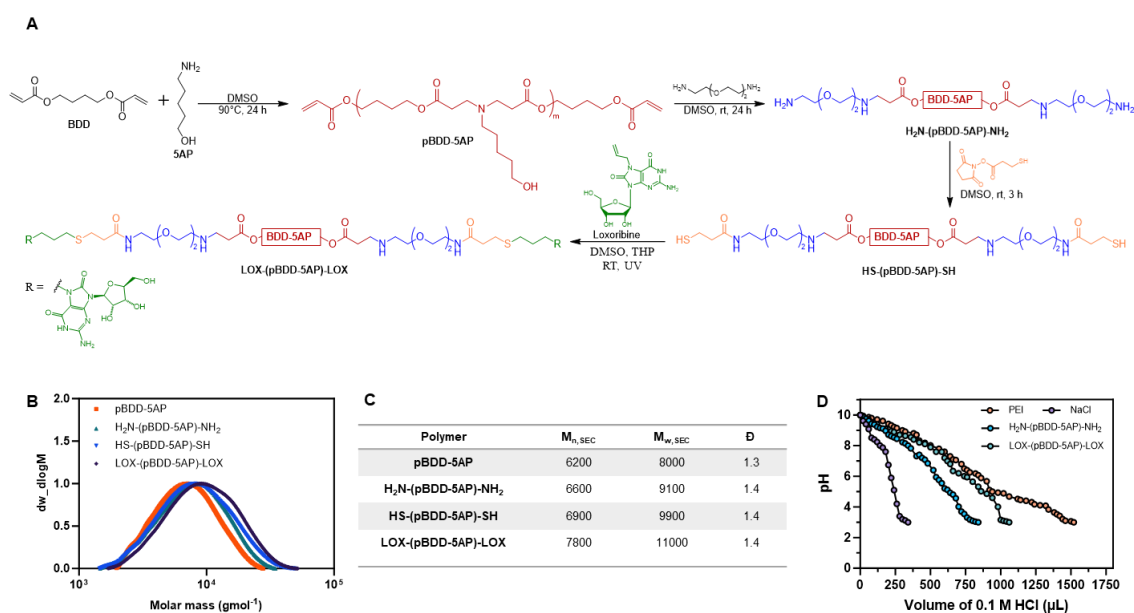


Figure 4.2. Synthesis and characterisation of PBAEs. (A) Synthetic scheme for step-growth Michael-addition polymerisation of BDD, 5-AP (Step 1) then end-capped utilizing 2,2'-(Ethylenedioxy)bis(ethylamine) (Step 2) and 3-Mercaptopropionyl-N-hydroxysuccinimide ester (Step 3) to produce LOX-end-capped PBAE (Step 4). (B) Molecular weights of PBAEs determined using GPC and (C) the molecular weight distribution of pBDD-5AP, H₂N-(pBDD-5AP)-NH₂, SH-(pBDD-5AP)-SH and LOX-(pBDD-5AP)-LOX. (D) Buffering capacity of H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX compared to polyethylenimine (PEI) and negative control of a 0.1 M NaCl(aq) solution.

4.3.2 Polyplex Physicochemical Properties of different end-capped polyplexes

Polyplex preparation was performed at two different polymer / fluc saRNA ratios weight-to-weight ratios (w/w ratios of 32, 64 and 128) for H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX PBAEs. Solutions of saRNA and the polymers were prepared using 25 mM NaOAc buffer then the nucleic acid solution was dropwise added to polymer solution as 1:1 volume ratio using pipette. The prepared nanoparticles were incubated at room temperature for 30 minutes prior to use. Experiments were then performed to define physicochemical and biological characteristics of the polyplexes, encompassing particle size, surface

charge, encapsulation efficiency, biocompatibility, and RNA expression (Figure 4.3A).

Hydrodynamic diameters of all nanoparticles were observed to be between 100-200 nm with low polydispersity index (PDI) values (< 0.2) determined by dynamic light scattering (DLS) (Figure 4.3B), with no major changes in particle size observable across different w/w ratios. Notably, LOX-(pBDD-5AP)-LOX polyplexes were larger than the H₂N-(pBDD-5AP)-NH₂ polyplexes, suggesting that end modification of the polymer with LOX affected the structures of the polyplexes. We reasoned that this could be due to the removal of the free NH₂ end groups to the more bulky but possibly less protonated (at ambient pH) purine groups of LOX which changed the sterics and electrostatics of the complexation interactions with RNA. However, the hydrodynamic diameters of LOX-(pBDD-5AP)-LOX polyplexes were significantly decreased with increasing w/w ratios, possibly due to the increased cation content that can induce more compact structures, consistent with previous reports on polyplexes [29].

Next, the zeta potentials of the particles were measured using DLS. Notably, higher zeta potential values were revealed for LOX-(pBDD-5AP)-LOX polyplexes (20 ± 2 mV) compared to H₂N-(pBDD-5AP)-NH₂ (24 ± 8 mV), which could be due to the LOX addition, as mentioned above due to the purine rings of LOX and increased surface-exposed charges on the polyplexes (Figure 4.3C). The encapsulation efficiency of nanoparticles was analysed utilizing a Ribogreen assay and showed full RNA encapsulation for all nanoparticles produced at all ratios (Figure 4.3D). Finally, TEM micrographs of all polyplexes prepared at a w/w ratio of 128 indicated mostly spherical complexes between 25 to 280 nm in diameter consistent with DLS analysis, and allowing for particle

shrinking during dehydration for TEM (Figure 4.3E). Furthermore, as DLS analysis can over represent larger particles present in heterogeneous particle distributions, it is likely that any smaller species visible by TEM may not have been included in the particle size averages [30]. Overall, these results indicate that all six polyplexes were capable of encapsulating saRNA and producing particle sizes below the typical 200 nm diameter threshold for efficient gene delivery.

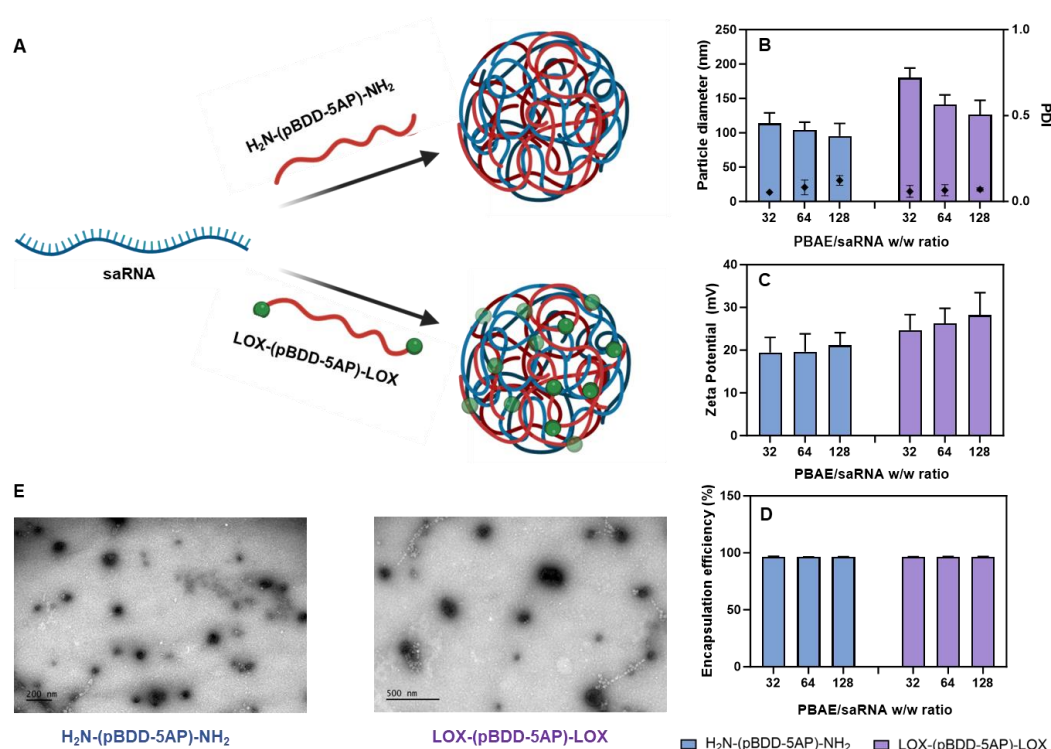


Figure 4.3. Formulation of PBAEs with saRNA to produce polyplex nanoparticles. (A) Schematic representation of saRNA formulation with H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX prepared in pH 5.0 25 mM sodium acetate at a final saRNA concentration of 10 µg/mL. (B) Particle size and (C) zeta potential for the H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX polyplexes at a PBAE/saRNA w/w ratio of 32, 64 and 128 determined by dynamic light scattering at 25°C. Bars represent the mean ± SD (n=3). (D) Encapsulation efficiency of saRNA within polyplexes evaluated using Ribogreen assay. (E) Representative transmission electron micrograph of the polyplexes H₂N-(pBDD-5AP)-NH₂ and LOX-(pBDD-5AP)-LOX.

4.3.3 In Vitro Evaluation of saRNA Delivery

4.3.3.1 Transfection and metabolic activity of different end-capped polyplexes

The acute toxicity and saRNA delivery efficiency of individual formulations OH-(pBDD-5AP)-OH and PEG-(pBDD-5AP)-PEG were evaluated in HEK293T cells (as a model cell line) and DC2.4 cells (a relevant dendritic cell line for vaccines). Similar to the formulation characterisation study described earlier, polyplexes were prepared at polymer/saRNA w/w ratios of 32, 64, and 128 using firefly luciferase encoding saRNA. These were compared against lipofectamine (as the positive expression control) and Triton X-100 (as the positive toxicity control).

All formulations demonstrated higher viability values (over 80% as inferred from metabolic activity) compared to positive control Lipofectamine (50%) (**Figure 4.4A** and **B**). It can be implied that the conjugation of loxoribine did not affect the metabolic activity in these cell lines. Similar to previous studies, increasing w/w ratios of H₂N-(pBDD-5AP)-NH₂ resulted in increased toxicity due to increased polycation concentration which is known to disrupt the negative phospholipid bilayer [31, 32] (**Figure 4.4A**). Interestingly, there was no substantial difference detected for LOX-(pBDD-5AP)-LOX formulations despite the increasing w/w ratios. In terms of transfection efficiency, in general, saRNA expression was slightly lower for the LOX-(pBDD-5AP)-LOX formulations compared to amine end-capped analogues (**Figure 4.4C** and **D**). This could be due to the above-mentioned slightly bigger nanoparticle diameters of the LOX-end-capped formulations where the cellular uptake could be lower. Notably, increasing w/w ratios of the formulations in both cell lines resulted in higher

transfection [33]. In HEK293T cells, the transfection efficiencies of w/w ratio 128 were lower than Lipofectamine; however, both formulations (w/w 128) showed as high transfection efficiency as positive control.

Given the lower transfection efficiencies of the loxoribine conjugated PBAE in both HEK293Ts and DC2.4s, we wanted to evaluate if conjugation of loxoribine caused the same effect. To investigate this, we treated cells with formulations containing non-loxoribine conjugated amine-end-capped PBAE H₂N-(pBDD-5AP)-NH₂/saRNA formulation at w/w 128 in the presence of different concentrations of free drug loxoribine (0 – 100 µg/mL) and compared this to negative control naked saRNA (**Figure 4.4E and F**). Loxoribine addition at all concentrations did not meaningfully influence transfection efficiency in both HEK293Ts and DC2.4s. This contrasts with other studies which when an mRNA delivery system is co-formulated with a TLR agonist, the expression of the delivered mRNA reduces by several orders of magnitude due to the downstream expression of pro-inflammatory cytokines inhibiting the cells ribosomal protein synthesis machinery [34]. This effect seems to be TLR dependent with TLR3 activation exhibiting the most potent reduction in RNA expression, while TLR7 and TLR9 have a moderate effect and minimal effect respectively. Given that once stimulated, most TLR pathways lead to the same pro-inflammatory pathways, this TLR dependent mechanism may instead be due to the non-selective activation of other PRRs by specific adjuvants, such as polyI:C which not only antagonises TLR3 but also cytosolic RNA sensors. In our case, the minimal effect on transfection efficiency when spiked with free loxoribine, especially in DC2.4's which are known to express TLR7, was an

encouraging indication that adjuvantation with loxoribine should not compromise protein expression [17].

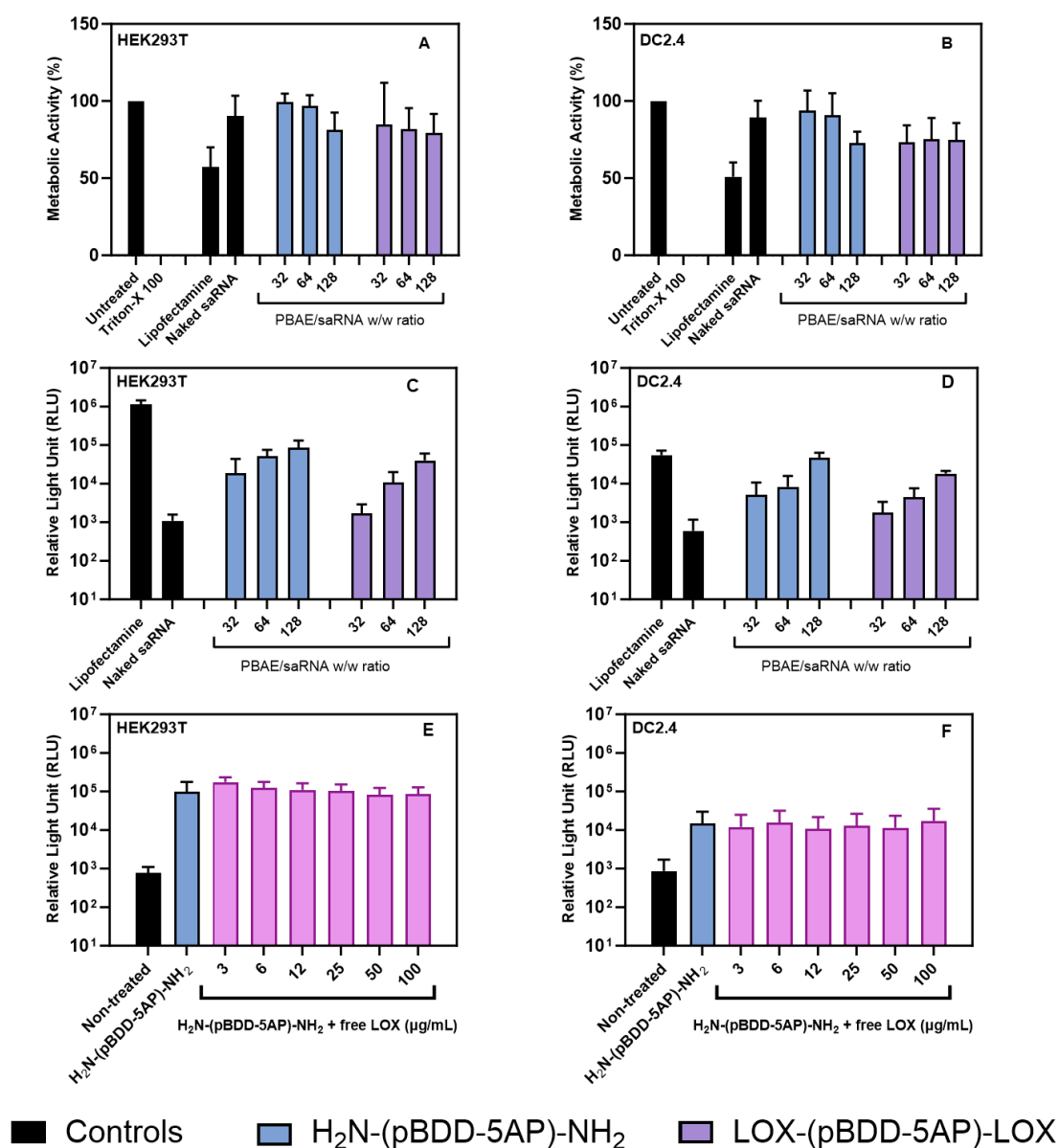


Figure 4.4. Metabolic activity and transfection efficiency of PBAE derived saRNA polyplexes. Effect of PBAE polyplexes formulated with firefly luciferase encoding saRNA at PBAE/saRNA w/w ratios of 32, 64 and 128 on metabolic activity (A and B) and transfection efficiency (C and D) on HEK293T cells (A and C) and DC2.4 cells (B and D). Cells were treated with polyplexes containing saRNA at a concentration of 500 ng/mL (100 ng/well) in serum-free Opti-MEM. Metabolic activity was compared against Triton X (positive control) and untreated cells (negative control) and calculated by normalizing metabolic activity to untreated cells. In vitro transfection efficiency was analysed after 24 h post-transfection using the Promega ONE-GLO luciferase assay and compared against Lipofectamine Messenger MAXTM (positive control) and naked saRNA (negative control) expressed as relative light units (RLU) for luciferase expression. (E-F) RLU for H₂N-(pBDD-5AP)-NH₂ and H₂N-(pBDD-5AP)-NH₂ + free LOX dilutions (3-100 µg/mL) in HEK293T and DC2.4 cells. Bars represent the mean ± SD (n=3).

4.3.3.2 TLR7 Activation Assay

Having observed high transfection efficiencies from the loxoribine conjugated PBAEs in both HEK293T and DC2.4 cell lines, we then evaluated the loxoribine conjugated PBAEs, LOX-(pBDD-5AP)-LOX and its counterpart polyplexes to stimulate intracellular TLR7 compared against free loxoribine. This was achieved by using an engineered HEK cell line, HEK-Blue™ TLR7, which has been transduced to co-express TLR7 with an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene which can be detected by the conversion of the SEAP-induced hydrolysis of its corresponding substrate into a UV (650 nm) absorbing product (Figure 4.5A). Similar to the formulation characterisation studies described earlier, polyplexes were prepared at PBAE/saRNA w/w ratios of 32, 64, and 128 using firefly luciferase encoding saRNA. Using the data from our quantification assays to determine loxoribine conjugation, we compared the polyplexes produced to the equivalent concentrations of non-formulated LOX-(pBDD-5AP)-LOX to examine the potential synergy when complexed with saRNA, which itself can stimulate TLR7, and against free loxoribine to determine the effect of conjugation (Figure S4.3D). These were assessed against lipofectamine (as the positive expression control) and Triton X-100 (as the positive toxicity control). High metabolic activity values (> 90%) were demonstrated for all treatments with no major decrease in viability for all free loxoribine, LOX-(pBDD-5AP)-LOX and saRNA polyplex concentrations in accordance with the non-transgenic HEK293T cells tested above and therefore would not impact the expression of SEAP to assess TLR7 activation (Figure S4.3A and S4.3C and S4.5C).

Quantification with the SEAP reporter revealed a dose dependent increase in OD_{650 nm} with increasing free loxoribine concentrations (Figure S4.3B). Both free H₂N-(pBDD-5AP)-NH₂ and the resulting polyplex exhibited lower or a statistically insignificant different in OD₆₅₀ compared to the equivalent concentrations of free loxoribine due to the absence of loxoribine or weak activation of TLR7 alone by saRNA when encapsulated. Free LOX-(pBDD-5AP)-LOX exhibited a dose-dependent increase in TLR7 activation, with free polymer concentrations at the equivalent of polyplex at w/w 128 exhibiting 50% higher OD_{650 nm} than the corresponding concentration of free loxoribine (25 µg/mL). This indicated that conjugation of LOX to the polymer enhances its TLR7 antagonisation effect (Figure 4.5B). This may be due to enhanced uptake of loxoribine, or enhanced trafficking to the endosome where the receptor resides due to an increase in apparent size when conjugated. In addition, the LOX-(pBDD-5AP)-LOX polyplexes at w/w 128 exhibited the highest OD_{650 nm} of 0.55; this was over 2-fold higher than the equivalent concentration of free loxoribine and was likely caused by the combination of saRNA and loxoribine. From these results it is reasonable to suggest that TLR7 activation of conventional mRNA polyplexes can be boosted by the addition of a TLR7 agonist, which also exhibits an increase in activity from being transported to the endosome in either nanoparticle or polymer form. Interestingly, lipofectamine delivered saRNA did not exhibit any statistically significant TLR7 activation over the untreated control, which may suggest a difference between lipid and polymer delivery systems (Figure 4.5B).

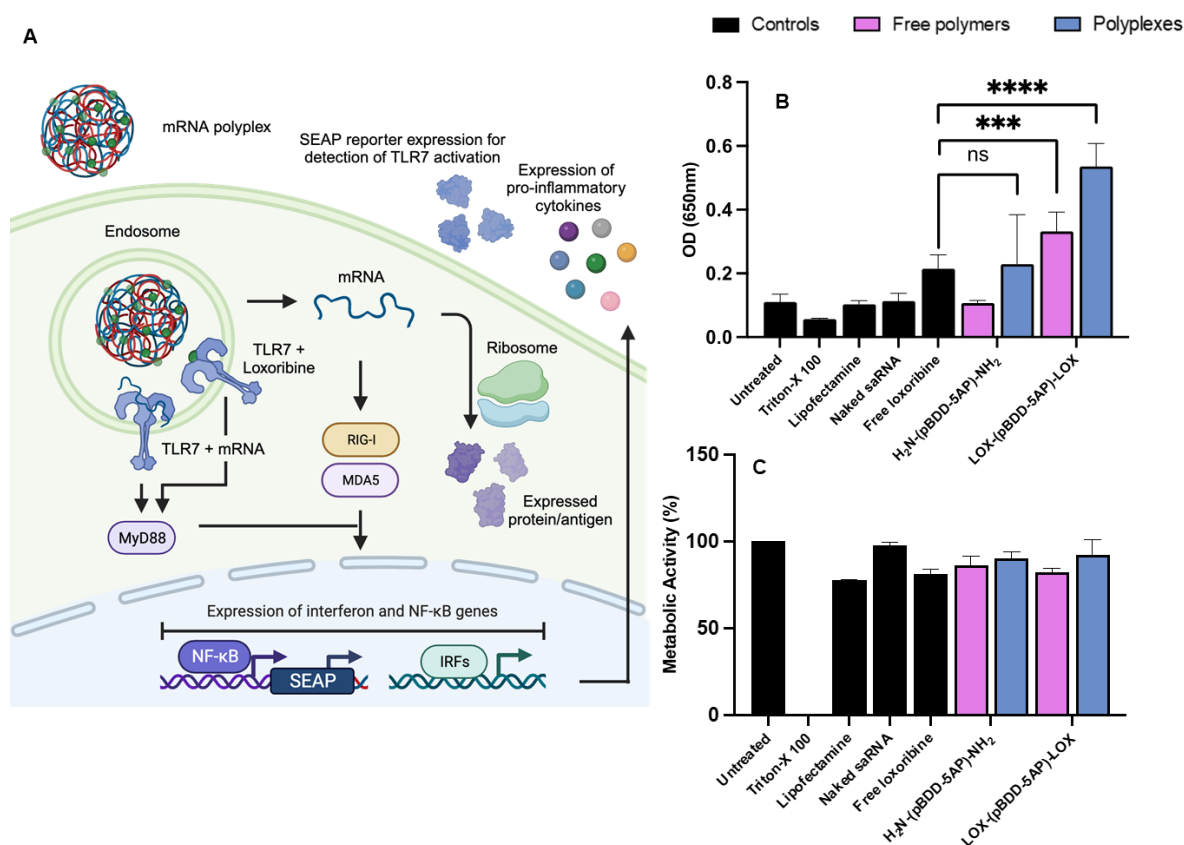


Figure 4.5. TLR7 Activation Assay using HEK-BLUE cells. (A) Schematic representation of nanoparticle uptake and TLR7 activation. (B) The determination of the TLR7 activation capacity of amine and loxoribine-end-capped polymers and nanoparticles (w/w 128) compared to controls in HEK-BLUE cells. The concentration of free lox was 25 μ g/mL (C) Metabolic activity of the amine and loxoribine-end-capped polymers and nanoparticles (w/w 128) compared to controls in HEK-BLUE cells. Bars represent the mean $\pm$ SD (n=3).

4.3.3.3 Cellular uptake of polyplexes

Due to the encouraging transfection, toxicity and TLR7 receptor activation profiles of LOX-(pBDD-5AP)-LOX polyplex at w/w 128, we investigated further the extent of internalisation of these polyplexes. Therefore, we first generated a FITC labelled LOX-(pBDD-5AP)-LOX through alcohol isothiocyanate coupling on the pendant hydroxyl groups of the 5-AP monomers within the core PBAE. Imaging flow cytometry was utilised to evaluate the cellular uptake of selected polyplex as 2 h and 4 h after treatment (Figure 4.6A to C). The successful cell association of the nanoparticles using DC2.4 cells was evaluated through FITC

intensity, and the histogram of the 2 h and 4 h indicates the increased intensity of FITC fluorescence observed (Figure 4.6B). The median fluorescence intensity value at 2 h incubation was around 60000, and expectedly, the intensity further increased to around 80000 at 4 h incubation time. High internalisation can explain the high transfection efficiencies of the LOX-(pBDD-5AP)-LOX w/w 128 polyplexes. In addition to cellular uptake scores, the internalisation vs membrane association of nanoparticles for each cell can be analysed using imaging flow cytometry. In this study, the median of internalisation score of the polyplexes was approximately 1.7 indicating a high proportion of nanoparticles were internalised inside the cells since having a score less than 0.3 can be considered to be surface bound (Figure S4.3) [35].

Finally, to assess further the intracellular internalisation mechanism of the selected nanoparticle LOX-(pBDD-5AP)-LOX w/w 128 in HEK293T and DC2.4 cells, we employed confocal microscopy to evaluate colocalization with a lysosomal (Lysotracker™ red) and nuclear (Hoecht 33342) stain (Figure 4.6D). After 2 h post administration, confocal micrographs showed that polyplexes successfully internalised within the cells and merged images demonstrated with minimal co-localisation with the acidic lysosomes. This was analysed with imageJ indicating Pearson's Correlation Coefficient between FITC labelled polyplexes and Lysotracker™ stained lysosomes were below 0.5 (0.3 for HEK293T and 0.4 for DC2.4 cells). Slightly higher co-localisation values were detected for DC2.4 (0.4) cells compared to HEK293T cells (0.3), which may be one of the reasons of low transferability of dendritic cells. Since, given the ~200 nm particle diameters it is likely that these formulations were internalised by an endocytosis mechanism, however the apparent low colocalization with the

acidic lysosomes may indicate that the particles escaped before endosomal maturation to lysosomal compartments. This successful endosomal escape could also be due to TLR7 activation as this is where the TLR7 receptor is and would be activated with LOX-conjugated carriers [36]. Although further endosomal escape studies would be needed to investigate this phenomenon in more detail, it is possible that enhanced endosomal escape was a factor in the high expression efficiencies observed for these complexes.

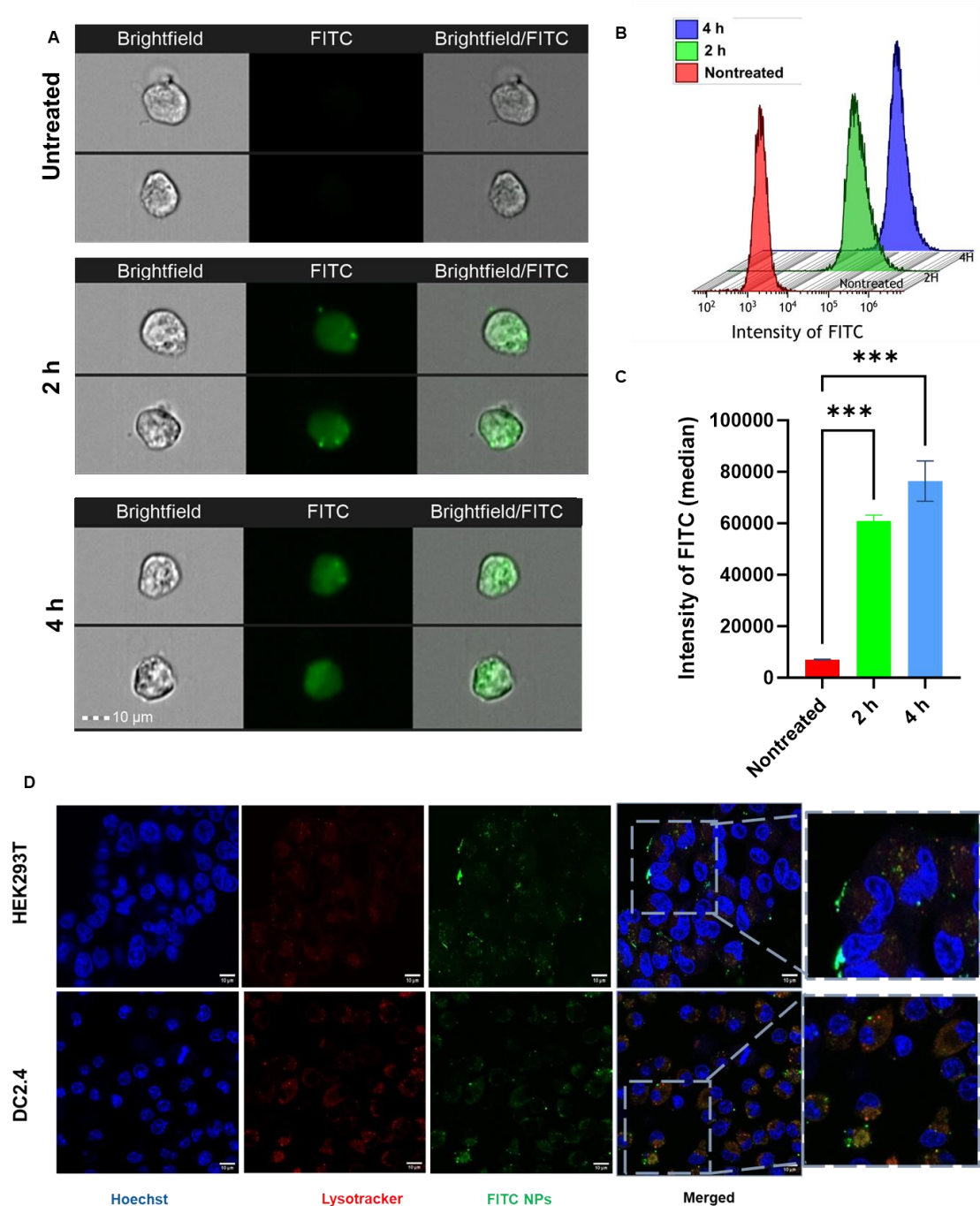


Figure 4.6. Cellular uptake of PBAE derived saRNA polyplexes. (A) Imaging flow cytometry images for DC2.4 cells after 2 h and 4 h following treatment with FITC-labelled LOX-(pBDD-5AP)-LOX polyplexes at a PBAE/saRNA w/w ratio 128 at 500 ng/well (500 ng/mL) (Scale bar 10 μ m) (B) histograms of fluorescence intensity and (C) median fluorescence values Data were analysed using IDEAS software and Kaluza. (D) Representative live cell confocal microscopy images of HEK293T and DC2.4 cells 2 h post-transfection with a firefly luciferase encoding saRNA formulated with LOX-(pBDD-5AP)-LOX at a PBAE/saRNA w/w ratio of 128 at 100 ng/well (500 ng/mL) saRNA. Scale bar 10 μ m (63x).

4.4 Conclusion

In conclusion, the exploration of messenger RNA technology has lined the way for innovative vaccines and treatments. The search for safe and effective delivery systems for RNA medicines has led to a focus on nanoparticle-based carriers, particularly the promising avenue of polycations like PBAEs. Despite challenges related to immune activation and translation efficiency, recent advances have been made towards improving these delivery systems. This study introduces a new and potentially important advance - a TLR7a-conjugated polycation using loxoribine for self-amplifying mRNA formulations. Through the synthetic pathway and formulation process, these polymers successfully encapsulated the cargo, resulting in small polyplex nanoparticles with notable transfection efficiency and minimal cytotoxicity. Importantly, the TLR7 activation assays demonstrated the significant potential of LOX-end-capped formulations to induce TLR7 signalling, surpassing amine-end-capped PBAE-based formulations and Lipofectamine. These findings highlight the promise of LOX-end-capped formulations at w/w ratio 128 for not only enhancing mRNA delivery but also activating crucial immune pathways. This development marks a considerable step forward in the pursuit of mRNA-based therapeutics and vaccine development, exhibiting the potential of tailored polycation systems in shaping the future of medicine.

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4.6 Author Contributions

Acknowledgement of research work done by co-authors:

- H. Bayraktutan: Performed all the experiments unless stated otherwise. Data curation, formal analysis, investigation, methodology, validation, visualization, writing of the original draft of the manuscript.
- R.J. Kopiasz and P. Gurnani helped with polymer synthesis and critically reviewed and edited the manuscript.
- A. Elsherbeny and N. Gumus helped with in vitro studies. A. Elsherbeny performed confocal imaging and N. Gumus performed cellular uptake experiments. Critically reviewed and edited the manuscript.
- K. Polra, P. F. McKay and R. J. Shattock: synthesised and purified saRNA and critically reviewed the manuscript.
- A. Mata, C. Alexander, and P. Gurnani: Supervisors of H. Bayraktutan, supervised the student while conducting the work, critically reviewed and edited the manuscript.

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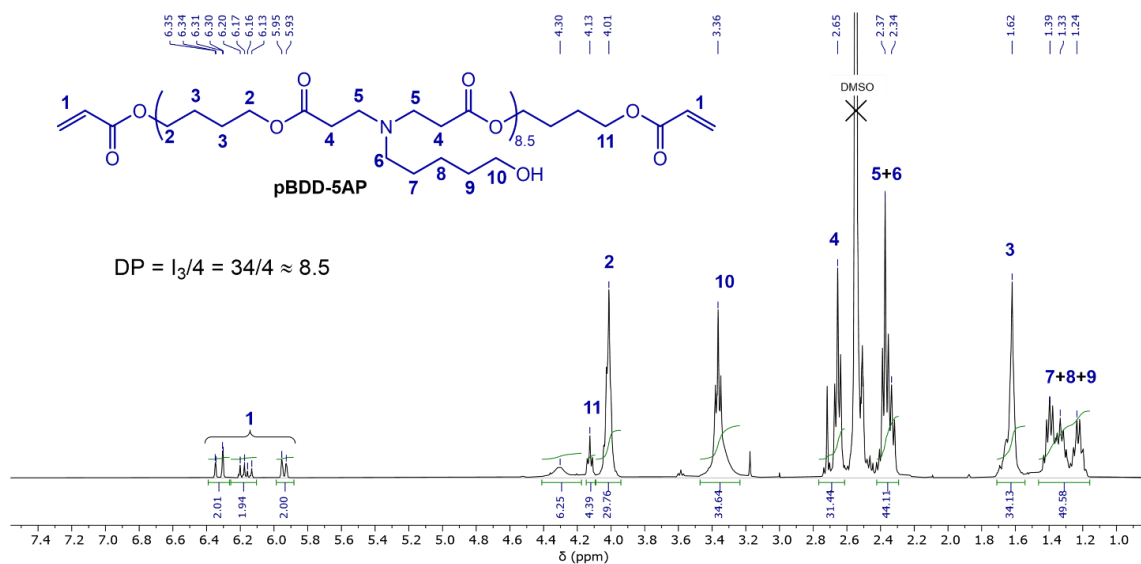
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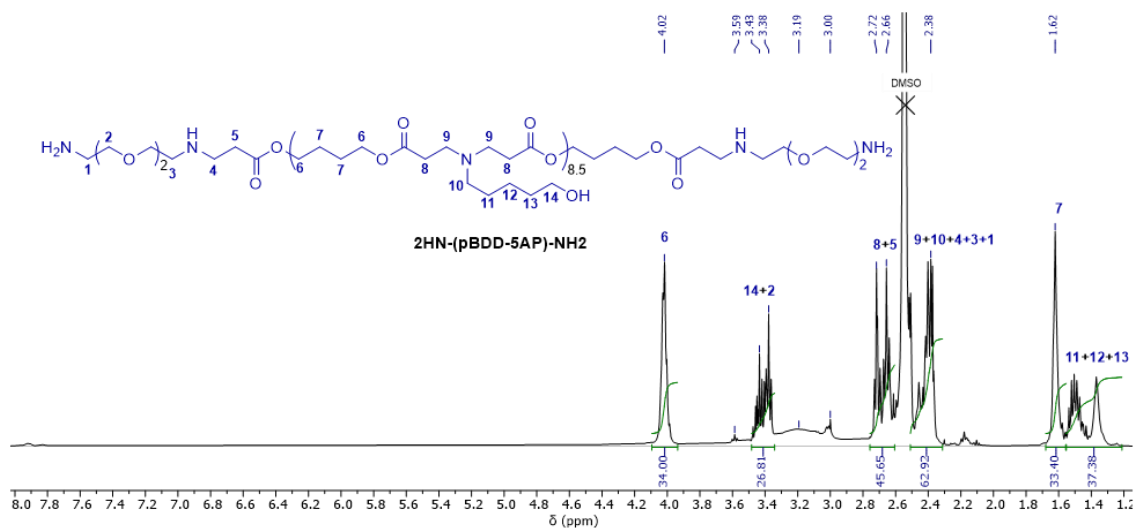
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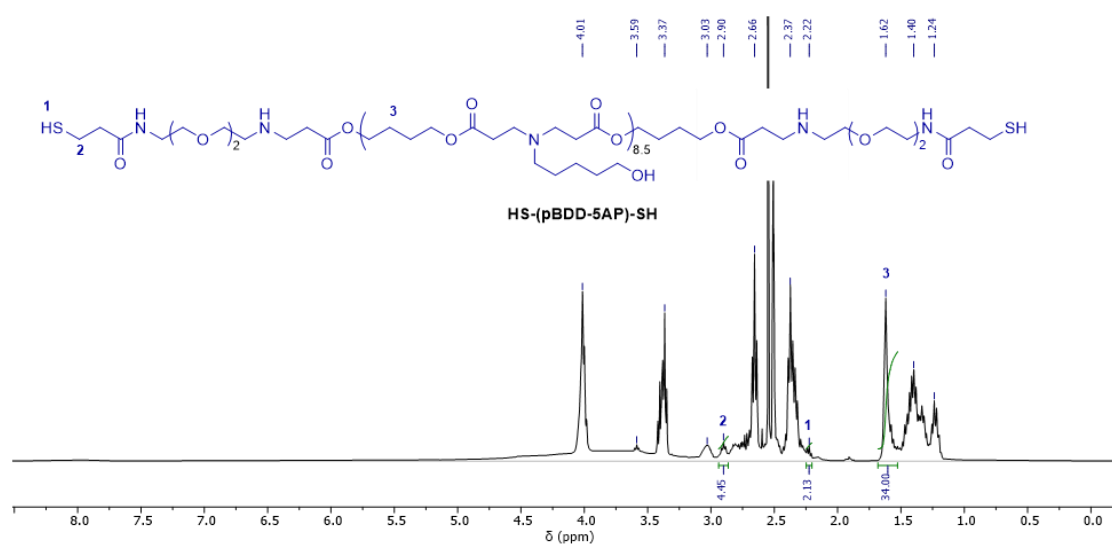
4.8 Supporting Information



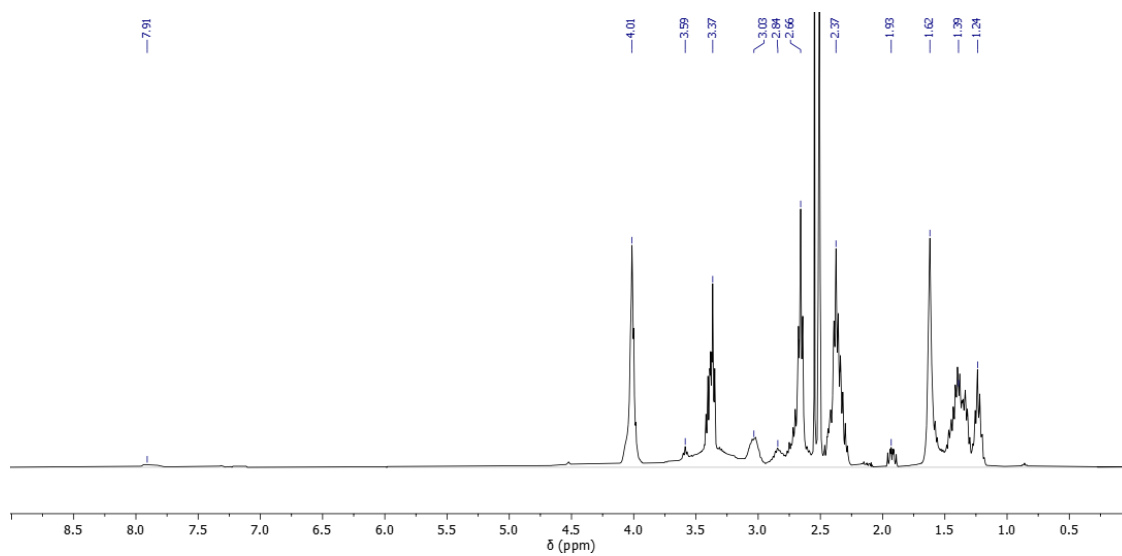
Spectrum S4.1. ¹H NMR (400 MHz, DMSO-d₆) of pBDD-5AP.



Spectrum S4.2. ¹H NMR (400 MHz, DMSO-d₆) of H₂N-(pBDD-5AP)-NH₂.



Spectrum S4.3. ^1H NMR (400 MHz, DMSO- d_6) of HS-(pBDD-5AP)-SH.



Spectrum S4.4. ^1H NMR (400 MHz, DMSO- d_6) of LOX-(pBDD-5AP)-LOX.

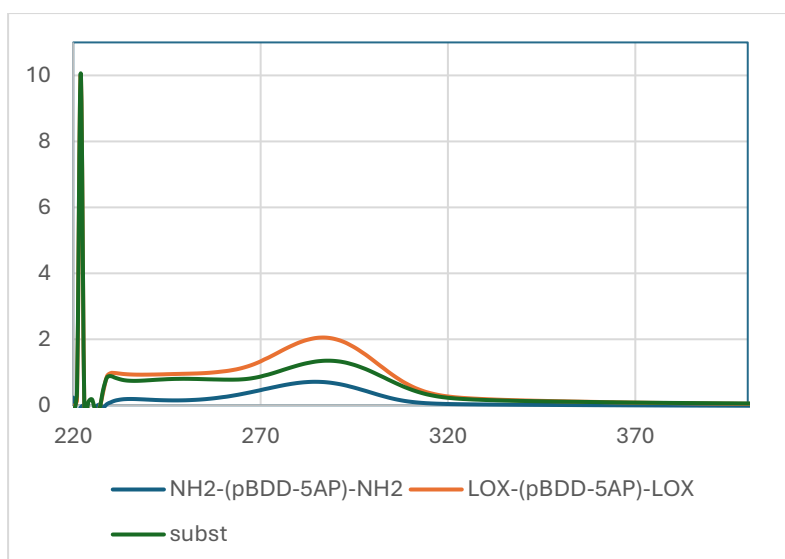


Figure S4.1: Determination of Drug Loading Using UV-vis spectroscopy.

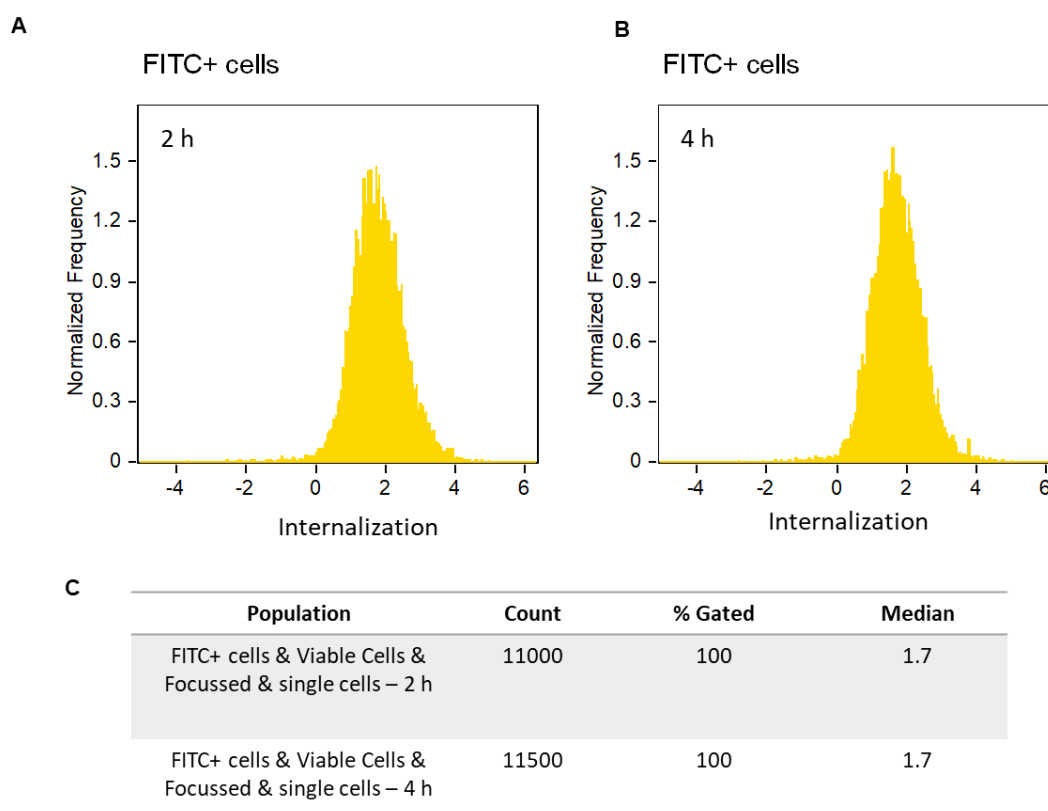


Figure S4.2. Cellular uptake of PBAE derived saRNA polyplexes. (A-B) Imaging flow cytometry internalisation histograms and (C) statistical values for DC2.4 cells after 2h and 4 h following

treatment with FITC-labelled LOX-(pBDD-5AP)-LOX polyplexes at a PBAE/saRNA w/w ratio of 128 at a concentration of 500 ng/mL saRNA. Data were analysed using IDEAS software.

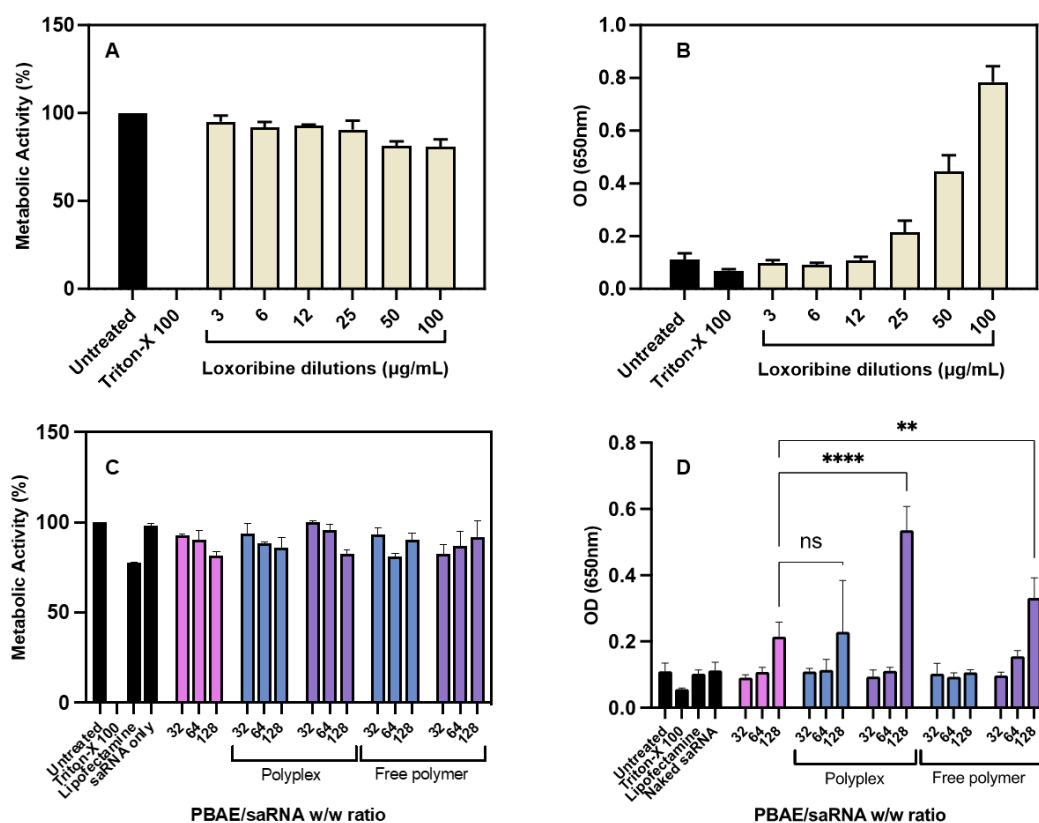


Figure S4.3. TLR7 Activation Assay using HEK-BLUE cells. (A) The determination of the TLR7 activation capacity of loxoribine dilutions (3 – 100 μg/mL) compared to controls in HEK-BLUE cells. (B) Metabolic activity of loxoribine dilutions (3 – 100 μg/mL) compared to controls in HEK-BLUE cells. (C) The determination of the TLR7 activation capacity of amine and loxoribine-end-capped polyplexes and free polymers (w/w 32, 64 and 128) compared to controls in HEK-BLUE cells. (D) Metabolic activity of the amine and loxoribine-end-capped polyplexes and free polymers (w/w 32, 64 and 128) compared to controls in HEK-BLUE cells.

Chapter 5

5.1 Conclusions and future prospects

The exploration of nucleic acid technology has facilitated for groundbreaking vaccines and treatments. The search for safe and efficient delivery systems for RNA medicines has led to a significant focus on nanoparticle-based carriers. Lipid nanoparticles (LNPs) are the most popular delivery systems, consisting of self-assembled particles made from various lipids. These particles encapsulate nucleic acids in core-shell structures, protecting them and facilitating their transfer across cell membranes. LNPs are the most clinically advanced delivery method, exemplified by their use in siRNA therapy, Onpattro and the mRNA COVID-19 vaccines, SpikeVax and Comirnaty.

However, alternative delivery methods are needed due to the stringent intellectual property constraints and limited chemical diversity of lipid nanoparticles (LNPs). Lipids inherently have lower chemical versatility because they must include a lipid tail. Additionally, lipid nanoparticles (LNPs) typically consist of multiple components that are widely used across various platforms. While some components, such as helper lipids and cholesterol, are off-patent, others like ionizable lipids and PEG lipids are still protected, creating a challenging intellectual property landscape. In contrast, polymer delivery systems have fewer strict requirements aside from being cationic, offering greater flexibility to modify structures and develop novel intellectual properties.

Therefore, cationic polymers are gaining attention as they can be easily modified in terms of chemical composition, molecular weight, and architecture to offer multiple functions. Particularly the promising avenue of polycations such as Poly(beta-amino esters) PBAEs. PBAEs stand out in this regard due to their straightforward synthesis process, adaptability in structure, exceptional compatibility with biological systems, and biodegradability. Therefore, the aim of this PhD was to explore and develop PBAE-based nucleic acid medicines delivery platforms to overcome current limitations, which was investigated through various end-modifications approaches.

The initial chapter detailed the properties of PBAEs and their synthesis methods using variations in monomer content, reaction temperature, reaction solvent and time. In addition, the structural features of these polymers when expanded via changes in backbones, side chains, end groups (small molecules or hydrophilic polymers), topology (degree of branching), molar mass, and the hydrophilic/hydrophobic balance of the PBAEs were considered. Then, the current state of these carriers for nucleic acid delivery platform was discussed in the context of previous studies, and the stated limitations of these carriers, was used to define the focus on property optimisation of PBAEs in the next research chapters.

In Chapter 2, we developed various polymer-based nanocarriers using the PBAE polycation platform to investigate the impact of surface PEG density on pDNA encapsulation, formulation properties, and gene transfectability both in vitro and in vivo. We synthesized non-PEGylated and PEGylated PBAEs via end-modification and formulated them into polyplexes, including mixed formulations to adjust surface PEG density. All formulations produced small

polyplex nanoparticles with high cargo encapsulation efficiency. In addition, zeta potential data showed no significant difference between non PEGylated and PEGylated nanoparticles, which could be due the low molecular weight of used PEG (800 Da). While high gene transfection was observed in HEK293T cells, only fully PEGylated and mixed formulations showed significantly higher gene expression in dendritic cells compared to the negative control. In vivo studies with a bivalent SARS-CoV-2 pDNA vaccine demonstrated strong antigen-specific T-cell responses only with the mixed formulation, suggesting the need for further investigation to improve immunization using polymer delivery systems.

In Chapter 3, due to the challenges associated with PEG-induced reactogenicity, pSar was utilized as an end-capped regent to increase colloidal stability and improve toxicity. Previously, pSar has shown promise as a PEG alternative, demonstrating reduced reactogenicity when incorporated into lipid nanoparticles. We therefore developed a synthetic route to conjugate pSar on to PBAE end-groups, resulting in a pSar-PBAE-pSar triblock copolymer. Polyplexes containing various ratios (0, 20, 50, 80, 100% pSar) of the pSarylated and a non-pSarylated PBAE were prepared with self-amplifying messenger RNA, which has been shown more reactive compared to pDNA, to examine the effect of surface pSar density. RNA encapsulation efficiencies, formulation properties and gene transfectability in vitro were then assessed indicating complete encapsulation of the cargo and sub-200 nm particle diameters in all cases and zeta potential data showed no significant difference between non pSarylated and pSarylated nanoparticles, which could be due the low molecular weight of used pSar (800 Da). While HEK293T cells exhibited

high transfection rates, only 50%-pSar showed significant gene expression in Caco-2 cells. Further testing in 3D colorectal cancer organoids demonstrated strong nanoparticle penetration into the core, suggesting pSar-conjugated PBAEs as promising for nucleic acid therapeutics delivery enhancement.

Finally, in Chapter 4, we introduced a potent TLR7 agonist, loxoribine, as an end-capping agent for PBAE to enhance immune responses of the carrier. The loxoribine-conjugated PBAE efficiently condensed self-amplifying RNA into small polyplex nanoparticles (100 – 200 nm) with complete encapsulation efficiency. Formulations, particularly at a w/w ratio of 128, demonstrated significant transfection rates in HEK293T and DC2.4 cell lines with minimal cytotoxicity. Furthermore, TLR activation assays using TLR7 reporter HEK-Blue cells revealed efficient TLR7 agonism by loxoribine-conjugated formulations, indicating their potential for augmenting mRNA vaccine efficacy.

The presented thesis demonstrates that the PBAE-based polymers are highly effective for nucleic acid delivery platform and for their biomedical applications. However, we have shown that the properties of these polymer-based carriers can significantly depending on the end-capping agent. The stability of the polymers can be enhanced using hydrophilic end-capping polymers where we utilized PEG and pSar. Our investigation into PEGylation density's role in pDNA formulation and delivery highlights the importance of surface properties in polymer delivery systems. Polyplexes derived from PEG-(pBDD-5AP)-PEG or a mixed formulation demonstrated favorable characteristics in vitro, indicating their potential for vaccination. However, further research is needed to enhance immune response quality and tailor polymer properties for specific immune effectors. In addition, the development of pSarylated-PBAE as an alternative to

PEGylation presents a promising avenue for gene delivery. These pSar-PBAE polyplexes exhibited high transfection efficiencies in vitro and demonstrated potential for tumor delivery, suggesting their suitability for intratumoral delivery of genetic material in immuno-oncology applications. However, future investigations should focus on elucidating immunogenic differences between PEG-PBAE and pSar-PBAE conjugates, especially relevant for vaccine and immunotherapy formulations.

Lastly, we showed that the effectiveness of these PBAE-based carriers can be altered via conjugation of desired agonists which can assist for targeted delivery and increase adjuvant properties of the carriers. The introduction of a TLR7a-conjugated polycation using loxoribine for self-amplifying mRNA formulations represents a significant advancement in mRNA-based therapeutics. These LOX-end-capped formulations exhibited superior TLR7 activation and transfection efficiencies compared to conventional formulations, offering promise for enhancing mRNA delivery and activating crucial immune pathways. However, future studies should be performed using 3D in vitro models or in vivo to evaluate the potential applications of newly synthesised nanocarriers and their effectiveness in immune cells.

Together, these findings highlight the diverse potential of tailored polymer delivery systems in shaping the future of medicine, from vaccination strategies to targeted cancer therapies. Continued research in this field holds immense promise for advancing nucleic acid-based therapeutics and vaccines. The now large datasets emerging from countless high throughput screens, will certainly be exploited for machine learning based predictive tools and in-depth correlative analysis to begin to predict the performance of novel unseen PBAEs,

or structures which can intrinsically target immune cells, with early work in these areas now emerging.

As limitation of the studies, we have utilised only one type of PBAE-based polymer core; however, future studies can be conducted using different monomers (maybe more hydrophobic polymer cores) and then end-modifications can be evaluated. The use of use more hydrophobic polymer cores can interact more favorably with these membranes, facilitating easier penetration and cellular uptake. This is due to the cell membranes are primarily composed of lipid bilayers with hydrophobic properties. Besides, the chemistry itself of PBAEs has not been a limitation in terms of design space, however the step-growth polymerisation itself, although simple, yields a broad molar mass distribution which may be challenging for predicting the ADME (absorption, distribution, metabolism and excretion) properties of these systems. Therefore, future research could also entail new synthetic methodologies for PBAEs, some of which already exist, including radical based ring-opening polymerisations. Furthermore, the molecular weights (repeating units) of the utilized hydrophilic polymers (PEG and pSar) could be changed for further investigation of nanocarriers. The merge of nucleic acid medicines and PBAEs presents exciting opportunities for the development of the next-generation therapies. Continued interdisciplinary research efforts both in technology development, but also in regulation and manufacturing will be essential in unlocking the full potential of PBAEs in immunology applications, ultimately translating into improved clinical outcomes for patients. Overall, this work reveals diverse advancements in the field of PBAE-based nucleic acid delivery systems, each offering unique insights and potential applications.