# Transfection of cardiomyocytes using pCBA-ABOL-based polyplexes for cardiac regeneration

Cerissa van Walstijn[1], Matej Dzurov[1,2], Cristina Casadidio[1], Diego Aguilar Rodriguez[3], Mariona Estape Senti[3], Clara Labonia[4], Bas van Ravensteijn[1], Pieter Vader[3,4] and Tina Vermonden[1]

[1]Department of Pharmaceutics, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CG Utrecht, the Netherlands
[2] Central European Institute of Technology, Brno University of Technology, Purkyňova 656/123, 621 00 Brno, Czech Republic
[3]Central Diagnostic Laboratory, University Medical Center Utrecht, Utrecht University, 3584 CX Utrecht, the Netherlands
[4]Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, 3584 CX Utrecht, the Netherlands

**Abstract**

Conventional therapies for ischemic heart disease are focused on revascularization of the myocardium. This however is insufficient to halt disease progression and restoration of the damaged myocardium is necessary, because of the limited capability of cardiac tissue to regenerate itself upon injury. Cardiac repair can be achieved with the use of stem-cell based techniques, as well as by stimulation of cardiomyocyte proliferation and fibroblast reprogramming, which requires gene delivery systems for the uptake of nucleic acids that can induce such processes. The efficiency of cardiac regeneration therapies are currently still limited however, and therefore in this study we have investigated the transfection of cardiomyocytes using pCBA-ABOL-based polyplexes. These polyplexes were shown to have a high transfection efficiency in muscle tissue, especially when increasing the molecular weight of the polymers. In this study, we synthesized various pCBA-ABOLs employing multiple synthesis methods. pCBA-ABOL-based polyplexes consisting of low molecular weight polymers and mRNA showed heterogenous characteristics independent of the polymer size and a pH-dependent colloidal stability was observed. We studied the potential therapeutic effects of low molecular weight pCBA-ABOL-based polyplexes both in vitro and in vivo, but the transfection efficiency was limited. A discrepancy between in vitro and in vivo transfection results was caused by the colloidal instability of the polyplexes. Investigation of polyplexes generated from high molecular weight polymers is of great interest for a potential enhanced delivery of RNA to stimulate cardiac repair.

**Keywords:** polymer synthesis, polyplexes, transfection, cardiomyocytes, cardiac regeneration

## 1. Introduction

Cardiovascular diseases (CVDs) account for nearly one-third of all deaths globally, thereby making CVDs the major cause of death worldwide[1,2]. Ischemic heart disease (IHD) is a CVD which includes heart problems caused by narrowing or blockage of the coronary arteries, such as heart arrhythmias and myocardial infarction[3]. IHD is responsible for approximately half of all CVD-related deaths and therefore it is the leading cause of morbidity and mortality in the world[4]. Narrowing of the coronary arteries is most often caused by atherosclerosis, which restricts the blood flow to the myocardium[3]. Plaque rupture results in the formation of blood clots, which leads to complete obstruction of the coronary arteries[5]. As a consequence of narrowed or blocked coronary arteries, the heart muscle is deprived of blood and oxygen and this results in cardiomyocyte loss and fibrosis, leading to ventricular dysfunction and heart failure[3,4].

In order to prevent loss of the myocardium as a consequence of narrowed or blocked coronary arteries, numerous therapies have been developed, which include pharmacotherapies as well as surgical techniques[6,7]. The overlapping aim of these therapies is to restore the blood flow to the heart muscle, which has drastically improved patient outcomes[6,7]. Ischemic injury is irreversible however, since the adult

mammalian heart has a limited capability of self-repair, and because of this, disease progression is only slowed down by conventional treatments[8,9].

For this reason, therapies that stimulate regeneration of the myocardium are required to overcome the burden of IHDs. This can be achieved using stem cell-based techniques, in which stem cells are differentiated into cardiomyocytes and subsequently provided to the heart[10,11]. Other strategies include the stimulation of cardiomyocyte proliferation and the direct reprogramming of fibroblasts[4,10]. For this, gene delivery systems, such as viral vectors and nanoparticles, aid in the intracellular uptake of nucleic acids which induce the regeneration processes[4].

These novel strategies have been investigated in clinical studies already, in which partial restoration of the heart function and reduction of the fibrotic area was observed[4,10,12]. However, there are several major challenges that still need to be overcome before these therapies can be used as a safe therapy for IHD, including the oncogenic potential of the therapies as well as the rise of (lethal) immune reactions[4,10,12]. Additionally, the efficacy of these novel therapies is currently still limited, caused by improper delivery and retention, as well as an insufficient therapeutic efficacy[10,12,13].

To overcome this limitation, we have investigated the use of p(N,N'-cystamine bysacrylamide-4-amino-1-butanol) (pCBA- ABOL)-based polyplexes for the transfection of cardiomyocytes. In previous research, pABOL-based polyplexes have shown high transfection efficiencies in various cell types and animal studies for multiple applications[14-16]. In particular, high molecular weight pCBA-ABOL-based polyplexes showed excellent in vivo transfection efficiencies after an intramuscular injection in mice[17]. Polymers up to 167 kDa were synthesized using optimized reaction conditions, which encompasses the use of triethylamine (TEA) as a catalyst and a high monomer concentration. It was found that a higher molecular weight of the polymer resulted in a higher transfection efficiency of the polyplexes. Based on these findings, we found inspiration to investigate whether these polyplexes can

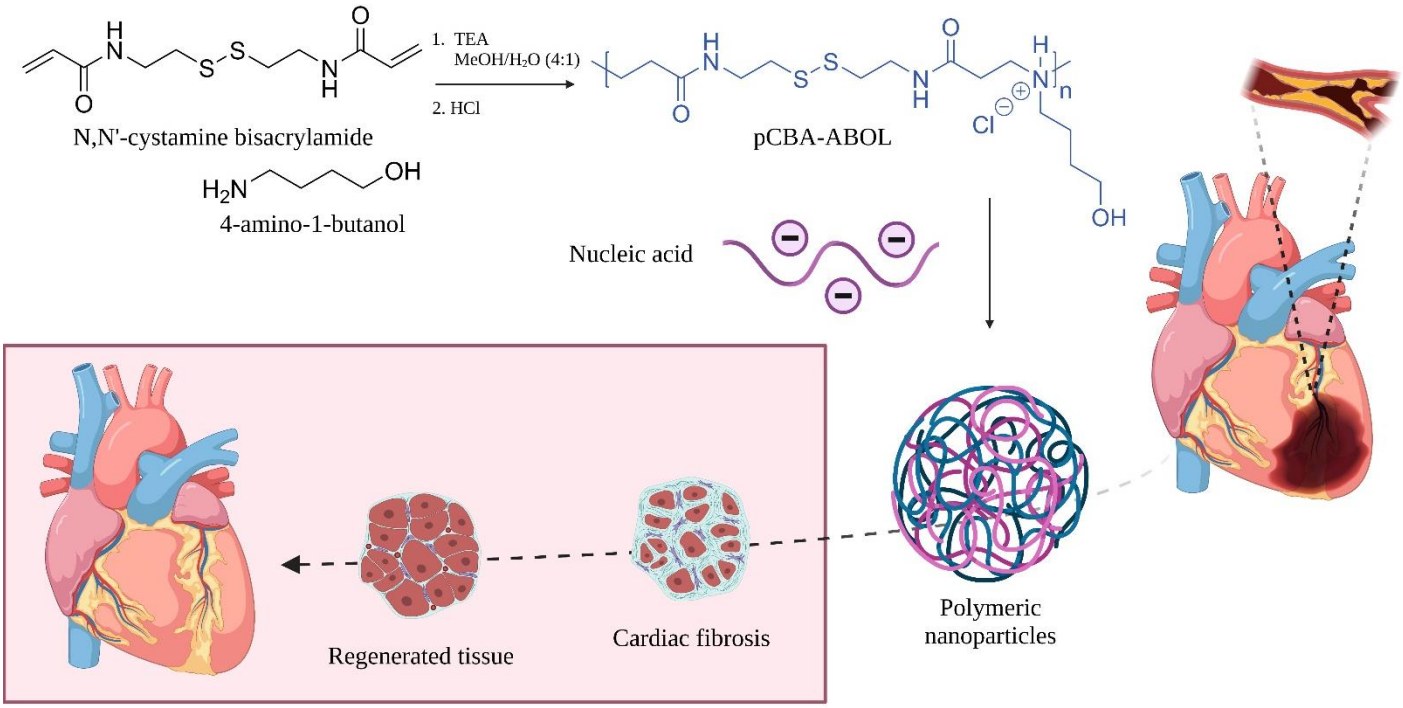


**Figure 1**. Regeneration of injured myocardial tissue in IHD using pCBA-ABOL-based polymeric nanoparticles that promote cardiomyocyte proliferation. Figure created with BioRender.com.

exert similar benefits on cardiomyocytes and can therefore be used as a potential treatment for cardiac regeneration.

The polyplexes consist of positively charged polymers as well as negatively charged nucleic acids (Figure 1). Polymers are synthesized via aza-Michael polyaddition of N,N'-cystamine bysacrylamide (CBA) and 4-amino-1-butanol (ABOL), with a subsequent acidification step for the polymer to become positively charged. The nucleic acids are the therapeutic agent as they contain information to induce cardiomyocyte proliferation, resulting in the restoration of the injured myocardium (Figure 1). Delivery of nucleic acids into cells requires a carrier such as a polymer however, because of the low in vivo stability of nucleic acids as well as the rapid host clearance and the poor cell-membrane permeability[18]. A carrier provides protection from nucleases in the bloodstream, assists with cellular uptake and promotes endosomal escape once entered into the cell[19]. pCBA-ABOL is degraded intracellularly by glutathione due to the presence of the disulfide bond in CBA, thereby releasing the mRNA[17].

Hence, based on the finding that pCBA-ABOL-based polyplexes have a high transfection efficiency in muscle cells, in this current study, we have investigated the use of these polyplexes for the transfection of cardiomyocytes. In this way, the limited efficacy of previously investigated therapies for cardiac regeneration can be overcome.

## 2. Experimental section

### 2.1. Materials

All solvents and reagents were obtained from commercial sources (Sigma-Aldrich and Thermo Fisher Scientific) and used as received unless stated otherwise. Dialysis tubing (3.5 kDa molecular weight cut-off; MWCO) was bought from Repligen (MA, USA). Poly(methyl methacrylate) (PMMA) standards were obtained from Polymer Laboratories (UK) and 0.2 µm Mini-UniPrep filters were from Whatman (UK). Firefly Luciferase (fLuc) mRNA was gifted by eTheRNA (Belgium) and induced pluripotent stem cell derived cardiomyocytes (iPS-cardiomyocytes) were provided by the Hubrecht Institute (the Netherlands).

### 2.2. Methods

#### 2.2.1. pCBA-ABOL synthesis

pCBA-ABOL was first synthesized according to the protocol of Blakney et al. (2020). In a general procedure, CBA and ABOL were reacted in a 1:0.99 molar ratio together with 0.1 equivalents of TEA. A methanol water mixture with a v/v ratio of 4:1 ($MeOH/H_2O$ 4:1) was added to a final concentration of the reagents of 3.2M. Polymerization was carried out in the dark at 45 °C under a static nitrogen atmosphere. Reactions were performed for 6, 8, 12 and 14 days. The reaction was quenched by the addition of 0.1 equivalents of ABOL. After 24 hours, the reaction mixture was diluted with $MeOH/H_2O$ (4:1) and acidified to pH 4 using 1M hydrogen chloride (HCl). Purification by dialysis was performed (MWCO: 3.5 kDa) against acidic water (pH 4). The water was refreshed twice daily for the duration of 3 days. After freeze-drying, the purified polymers were obtained in their protonated form (polymer IDs: P1-6d, P1-8d, P1-12d and P1-14d).

Additional pCBA-ABOL polymerization reactions were carried out under adjusted reaction conditions. Firstly, polymerization was carried out under similar conditions to the original protocol, but a 1:1 molar ratio of CBA and ABOL was used instead. The reaction was continued for 8 days (polymer ID: P2). Another reaction was performed in which CBA was first suspended in $MeOH/H_2O$ (4/1). TEA (0.1 equiv.) was then added along with ABOL in an equal molar ratio to CBA. The final concentration of the reagents was 0.6M. The reaction was stirred for 8 days in the dark at 45 °C under a static nitrogen atmosphere (polymer ID: P3). In

| | Polymer ID | [CBA]/ [ABOL] | [M] | Order of addition | Atmosphere | Temperature | Reaction time |
|---|---|---|---|---|---|---|---|
| **Original synthesis** | P1-6d | 1:0.99 | 3.2M | CBA, ABOL, TEA, solvent | $N_2$ | 45 °C | 6 days |
| | P1-8d | 1:0.99 | 3.2M | CBA, ABOL, TEA, solvent | $N_2$ | 45 °C | 8 days |
| | P1-12d | 1:0.99 | 3.2M | CBA, ABOL, TEA, solvent | $N_2$ | 45 °C | 12 days |
| | P1-14d | 1:0.99 | 3.2M | CBA, ABOL, TEA, solvent | $N_2$ | 45 °C | 14 days |
| **Adjusted synthesis** | P2 | *1:1* | 3.2M | CBA, ABOL, TEA, solvent | $N_2$ | 45 °C | 8 days |
| | P3 | *1:1* | *0.6M* | *CBA, solvent, TEA, ABOL* | $N_2$ | 45 °C | 8 days |
| | P4 | *1:1* | *0.6M* | *Solvent, ABOL TEA, CBA* | $N_2$ | *50 °C* | 14 days |
| | P5 | *1:1* | *0.6M* | CBA, ABOL, TEA, solvent | *Air* | *60 °C* | 2 days |
| **Microwave synthesis** | P6 | *1:1* | *0.6M* | CBA, ABOL, TEA, solvent | *Air* | *100 °C ** | ** |
| | P7 | *1:1* | *0.6M* | CBA, ABOL, TEA, solvent | *Air* | *60 °C ** | *** |

**Table 1.** The conditions of all reactions that were performed in this study. Alterations of reactions conditions to the original protocol are highlighted in *Italic*. * microwave heating. ** Time points: 10 min, 20 min, 30 min, 1 hr, 4 hrs, 7 hrs, 16 hrs, 24 hrs. *** Time points: 30 min, 1 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 12 hrs, 16 hrs.

another reaction, MeOH/$H_2O$ (4:1), ABOL and TEA (0.1 equiv.) were first added together in the reaction vessel. CBA was then added in an equal molar ratio to ABOL. The final concentration of the reagents was 0.6M. The mixture was reacted for 14 days in the dark at 50 °C under a static nitrogen atmosphere (polymer ID: P4). In a fourth reaction, CBA and ABOL were added together in a 1:1 molar ratio. TEA (0.1 equiv.) was added as well as MeOH/$H_2O$ (4/1) to a final concentration of 0.6M. The reaction was continued for 2 days in the dark at 60 °C (polymer ID: P5). All aforementioned reactions were quenched, acidified and purified according to the same procedures of the original protocol described previously.

pCBA-ABOL was also synthesized using microwave heating. For this, CBA and ABOL were added together in a 1:1 molar ratio. TEA (0.1 equiv.) was then added into the reaction vessel together with MeOH/$H_2O$ (4:1) to a final concentration of the reagents of 0.6M. Polymerization was carried out at 60 °C and 100 °C using an Initiator Microwave Synthesizer (Biotage, Sweden). Samples were taken at various time points (polymer IDs: P6 and P7). The conditions of all reactions that were performed in this study are summarized in Table 1.

2.2.2. Microwave degradation study

Polymers P1-12d and P1-14d were dissolved in MeOH/$H_2O$ (4:1) to a final concentration of 10 mg/ml. Subsequently, the samples were submitted to microwave heating at 100 °C for 4 to 8 hours and 12 to 16 hours for P1-14d and P1-12d respectively.

2.2.3. pCBA-ABOL characterization

The polymers were characterized via $^1$H Nuclear Magnetic Resonance (NMR) spectroscopy using a 400MHz NMR spectrometer (Agilent, CA, USA). Deuterated $H_2O$ was used to dissolve purified samples. MestReNova software (version 14.2) was used to analyze the spectroscopy data.

Gel permeation chromatography (GPC) was performed to obtain the molecular weight distributions of the polymers using narrow PMMA standards and dimethylformamide (DMF) + 10mM LiCl as eluent. For this, crude products were dissolved in DMF + 10mM LiCl and filtered through 0.2 µm filters. Samples were analyzed using an Alliance e2695 Seperations Module (Waters, MA, USA) with a PLgel 5 µm mixed-D column (Agilent, CA, USA) and a 2414 RI Detector (Waters, MA, USA). The temperature of the system was 65 °C. Empower 3 software (version 7.5) was used to analyze the chromatography data.

2.2.4. Polyplex formation and characterization

In a general procedure, polymers were directly mixed with fLuc mRNA in a 4:1 v/v ratio, using an N/P ratio of 8:1 and 37:1. Polyplexes were prepared in 20 mM HEPES buffer at pH 7.4 and the final concentration of mRNA was 10 mg/ml. Another batch of polyplexes were also prepared using 20 mM HEPES buffer but with

a pH of 6. Of note, samples that were used in a stability study were incubated at 4 °C in-between measurements.

The particle size (hydrodynamic diameter, $D_h$) and polydispersity (Đ) were obtained using dynamic light scattering (DLS). Measurements were performed on a Zetasizer Nano-S (Malvern Instruments, UK) and the scattering angle was fixed at 173°. Zeta potential measurements were conducted using the Zetasizer Nano-Z (Malvern Instruments, UK) in HEPES buffer at 25 °C.

2.2.5. In vitro transfection

Transfections were performed on HEK293T cells as well as iPS-cardiomyocytes. HEK293T/17 (ATCC CRL-11268) cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM)-high glucose containing 10% (v/v) fetal bovine serum (FBS). The cells were seeded in a 96-well plate 24 hours prior to transfection at a density of 10 000 cells per well in DMEM-high glucose containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. iPS-cardiomyocytes were obtained in a 96-well plate at a density of 100 000 cells per well in Roswell Park Memorial Institute (RPMI)-1640 medium containing 2% (v/v) B-27 Supplement and 1% (v/v) penicillin/streptomycin.

For the transfection of both cell types, medium was completely removed from the cells and 75 µL of fresh medium was added. Subsequently, a 75 µL amount of the polyplex solution containing 250 ng of mRNA was added to each well. The cells were incubated for 24 hours at 37 °C (5% $CO_2$).

Using the Luciferase Assay System (Promega, WI, USA), the transfection efficiency of various polyplexes was analyzed on both HEK293T and iPS-cardiomyocytes. In brief, cell culture medium was completely removed from each well 24 hours after transfection. Then, HEK293T cells were lysed with 20 µL 1× Reporter Lysis Buffer followed by a freeze-thaw cycle. Luminescence emission from cell lysates was analyzed using Fluostar Optima (BMG Labtech, Germany) after 100 µL of Luciferase Assay Reagent was added. For iPS-cardiomyocytes, cells were lysed using 20 µL 1× Cell Culture Lysis Reagent and the luminescence emission from cell lysates were analyzed on a SpectraMax iD5 (Molecular Devices, CA, USA) after adding 100 µL of Luciferase Assay Reagent.

2.2.6. In vivo transfection

Male BALB/cByJ mice (Charles River Laboratories, 25-30 g, 12-13 weeks) were intramyocardially injected with 10 µl containing 2 µg of fLuc mRNA encapsulated in polyplexes. The mice were anesthetized with an intraperitoneal injection of fentanyl (0.07 mg/kg body weight), midazolam (6,67 mg/kg body weight) and dexdomitor (0.67 mg/kg body weight), followed by intubation and connection to a respirator with a 170:1 oxygen-air ratio (times/min). In order to access the heart, a left lateral thoracotomy was performed. The treatments were injected into the left ventricular wall using an infuse/withdraw syringe pump (Pump 11 Elite Nanomite). Once finished with the surgeries, the mice were subcutaneously injected with atipamezole hydrochloride (3.3 mg/kg weight) and flumazenil (0.5 mg/kg body weight) as antagonists and buprenorphine (0.2 mg/kg body weight) for pain relief.

24 hours after surgeries, the mice were anesthetized with isoflurane and intraperitoneally injected with 100 µl of D-luciferin (Promega, WI, USA) at a concentration of 25 mg/ml in Dulbecco's Phosphate Buffered Saline (DPBS). Luminescence emission was analyzed using a PhotonImager (Biospace Lab, France). After imaging, the heart, liver, lungs, spleen and kidneys were extracted and snap-frozen in liquid nitrogen and stored at -80ºC for further analysis of the luciferase activity in the mice organs tissue lysates.

## 3. Results and Discussion

3.1. pCBA-ABOL synthesis and characterization

pCBA-ABOL was synthesized from CBA and ABOL via aza-Michael polyaddition, as illustrated in Figure 1.

| Polymer ID | Mw (kDa) | Đ | Polymer ID | Mw (kDa) | Đ | Polymer ID | Mw (kDa) | Đ |
|---|---|---|---|---|---|---|---|---|
| P1-6d | 6.0 | 2.07 | P6-10min | 4.1 | 1.60 | P7-30min | 2.1 | 1.34 |
| P1-8d | 7.1 | 2.29 | P6-20min | 4.2 | 1.63 | P7-1hr | 2.3 | 1.37 |
| P1-12d | 10.8 | 2.00 | P6-30min | 3.7 | 1.62 | P7-2hrs | 3.1 | 1.48 |
| P1-14d | 7.5 | 1.89 | P6-1hr | 4.1 | 1.62 | P7-4hrs | 4.1 | 1.54 |
| P2 | 7.9 | 1.90 | P6-4hrs | 3.3 | 1.59 | P7-6hrs | 4.0 | 1.52 |
| P3 | 6.3 | 1.79 | P6-7hrs | 3.4 | 1.88 | P7-8hrs | 4.3 | 1.58 |
| P4 | 5.6 | 1.73 | P6-16hrs | 2.4 | 1.54 | P7-12hrs | 4.6 | 1.64 |
| P5 | 7.2 | 1.81 | P6-24hrs | 1.5 | 1.26 | P7-16hrs | 5.0 | 1.69 |

**Table 2.** The molecular weight and polydispersity (Đ) of the polymers that were synthesized, obtained from analysis of crude products by GPC in DMF + 10mM LiCl and calibrated using monodisperse PMMA standards.

$^1$H NMR spectroscopy confirmed the successful synthesis of the pCBA-ABOLs (Figure S1) and the molecular weight distributions of the polymers were obtained from analysis by GPC, as summarized in Table 2.

Previously, pCBA-ABOLs up to 167 kDa were synthesized with the use of TEA as a catalyst and a high monomer concentration[17]. In fact, higher molecular weight pCBA-ABOLs were shown to exert a higher transfection efficiency both in vitro and in vivo compared to pCBA-ABOLs with a lower molecular weight[17]. Inspired by these findings, the aim of this work was to synthesize pCBA-ABOLs with a high molecular weight. Polymers P1-6d up to P1-14d were synthesized according to the procedures as reported by Blakney et al.[17]. Employing the same reaction strategies and characterization methods, we obtained polymers with a molecular weight ranging from 6 to 10.8 kDa after 6 to 14 days of synthesis (Table 2; P1). These molecular weights deviate tremendously from the molecular weights reported by Blakney et al., as 14 days of synthesis led them to a 167 kDa polymer[17].

Theoretically, CBA and ABOL react in a 1:1 molar ratio with each other and therefore an unequal ratio between the reagents terminates the elongation process. For this reason, we adjusted the ratio between CBA and ABOL from 1:0.99 to an equal molar ratio. Employing this strategy, after 8 days of reacting, a polymer with a molecular weight of 7.9 kDa was obtained (Table 2; P2). This is similar to the molecular weight of P1-8d, which was synthesized using the same reaction conditions as P2, but with a molar ratio of 1:0.99. Our results were therefore still not in line with the results reported by Blakney et al., where 8 days of reacting resulted in a 25 kDa polymer[17]. Hence, using an equal molar ratio did not improve the synthesis conditions to reach the high molecular weight polymers.

For this reason, we optimized the reaction conditions further based on other reported pABOL synthesis methods[14-16]. Variables that were adjusted included temperature as well as atmosphere. An increase in temperature accelerates the reaction, thereby stimulating the growth of the polymer chains. An air atmosphere was shown to prevent the formation of branched polymers, due to the radical scavenging mechanism of oxygen, in contrary to an inert atmosphere, which activates radical polymerization, leading to branching[14]. Additionally, we lowered the final monomer concentration and we explored the order by which the reagents were added. CBA is poorly soluble in the solvent and we rationalized that because of this, the monomer availability would be limited, thereby halting the elongation process. Despite these changes to the protocol, the polymers did not exceed a molecular weight of 7.2 kDa (Table 2; P3-P5).

We also evaluated the use of microwave heating for the synthesis of pCBA-ABOLs with a high molecular weight. In a previous study, high molecular weight pCBA-ABOLs were obtained by making use of microwave synthesis[14]. Microwave heating drastically

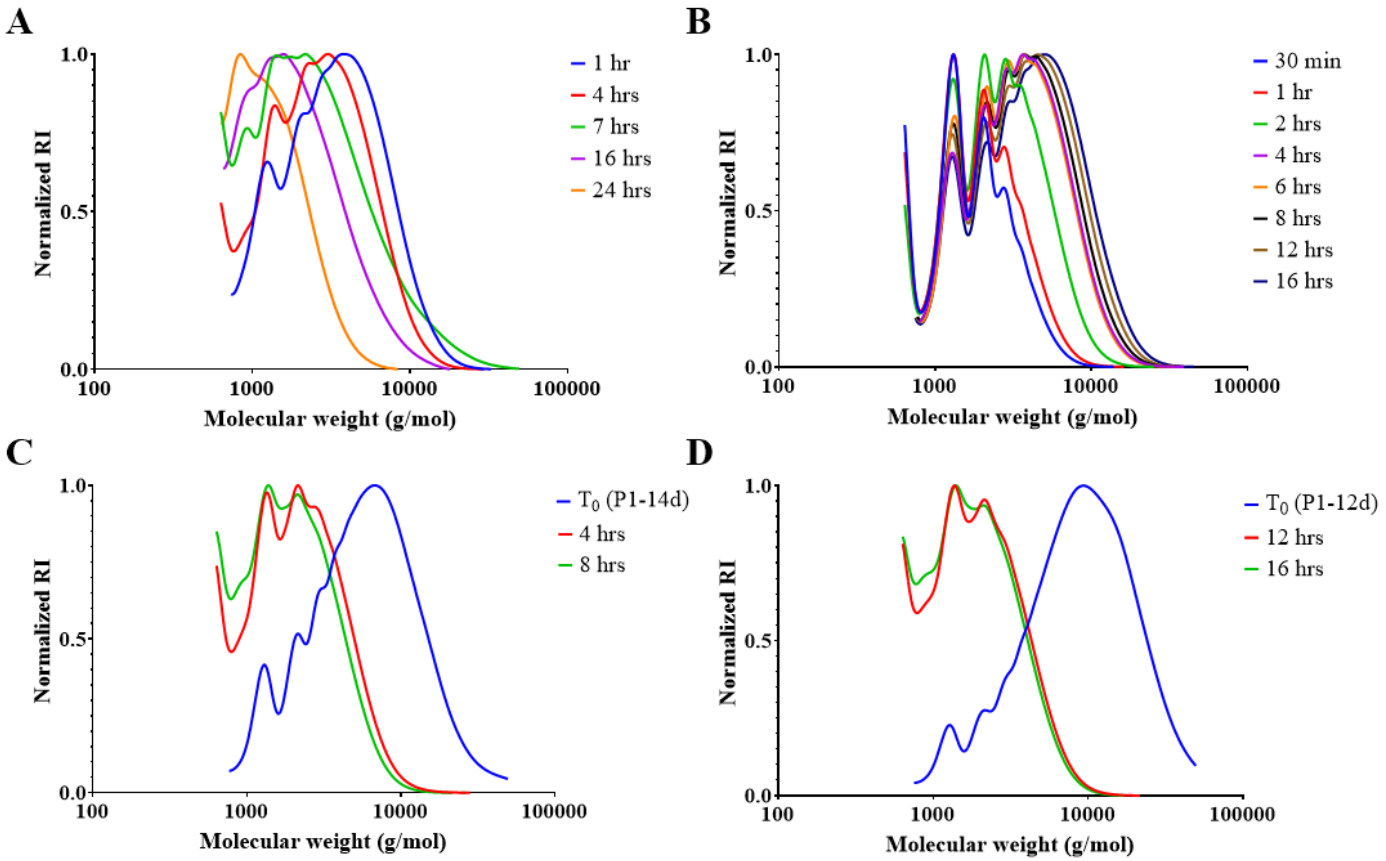


**Figure 2.** Molecular weight distributions of (a) microwave synthesis at 100 °C and (b) 60 °C over time, and in a degradation study where (c) P1-14d and (d) P1-12d were submitted to microwave heating at 100 °C for various durations. RI = refractive index.

speeds-up the reaction, thereby reaching the high molecular weight polymers within shorter reaction times. Employing this strategy, we also could not obtain the high molecular weight polymers however (Table 2; P6). After 10 minutes, a 4.1 kDa polymer had already formed and there was no significant additional growth up until 1 hour of reacting. Surprisingly, after 4 hours, there was a decline in the molecular weight, suggesting degradation of the polymer chains (Figure 2a). After 24 hours, only a 1.5 kDa polymer was left, whereas in the previous study on which we based our methods, a polymer size of 23 kDa was reported for a reaction time of 24 hours. 'Shoulders' in the graphs belong to oligomers that are formed.

In order to investigate whether there was degradation of the polymer chains during microwave synthesis, a degradation study was performed in which P1-12d and P1-14d were subjected to microwave heating. After 4 and 8 hours of microwave heating at 100 °C, the molecular weight of P1-14d had decreased from 7.5 kDa to 2.6 and 2.3 kDa respectively (Đ = 1.49 and 1.46). P1-12d decreased from 10.8 kDa to 2.3 and 2.2 kDa (Đ = 1.45 and 1.44) after 12 and 16 hours of microwave heating respectively (Figure 2c and 2d). Hence, this confirmed the degradation of the polymer upon microwave heating at 100 °C.

For this reason, we optimized the microwave synthesis reaction conditions, for which we lowered the temperature to 60 °C, which was rationalized to prevent an excessive build-up of pressure inside the reaction vessel. Over time the molecular weights remained in an increasing trend. Besides, the amount of oligomers present was decreasing with longer reaction times, which is because of the step-growth nature of the polymerization reaction[14]. After 16 hours of reacting we obtained a polymer size of 5 kDa (Table 2; P7 and Figure 2b). Although this synthesis method was not investigated further, we strongly encourage future

research on this, as these optimized microwave synthesis reaction conditions can provide a more robust and reproducible method towards the high molecular weight polymers.

3.2. Polyplex formation and characterization

In previous research, polyplexes were prepared by direct mixing of the polymers with RNA or via titration[17]. For polymers with a high molecular weight, the polyplexes prepared using the titration method showed higher transfection efficiencies than the ones prepared by the direct mixing method. We prepared polyplexes using the direct mixing method however, since we only obtained low molecular weight polymers and therefore the titration method did not have advantages over the direct mixing method[17].

Additionally, the polyplexes were prepared using an N/P ratio of 8:1 and 37:1. A high N/P ratio is required, due to the highly sterically hindered positive charge on pCBA-ABOL, and therefore the binding efficiency to the RNA is low[17]. However, we also included a lower N/P ratio in order to analyze the differences. Polymers obtained from microwave synthesis were inadequate for further studies due to probable degradation (P6) or the reaction conditions were not fully optimized (P7). For this reason, polyplexes were only prepared using polymers P1-P5. The size, polydispersity index and zeta potential of the polyplexes were measured, of which the results are shown in Figure 3.

In general, polyplexes with an N/P ratio of 37:1 showed favorable characteristics for further cell studies, i.e. small size, low polydispersity and high zeta potential, contrary to P1-14d and P5 which exhibited relatively large sizes and low zeta potential. In addition, polyplexes with an N/P ratio of 8:1 displayed much less favorable characteristics for a high transfection efficiency, as these polyplexes are generally large and in most cases have a negative zeta potential (Figure 3). P1-6d, P1-8d and P2 on the other hand do have a small size and a positive zeta potential. For both N/P ratios, there is a correlation between the size and the zeta potential of the polyplexes, as the zeta potential in general is higher when the size of the polyplexes is smaller.

We hypothesize that the differences between the characteristics of the polyplexes are not related to the molecular weights of the polymers, because of the minor differences between the molecular weights of the various polymers we synthesized. Also, there is no clear trend visible between the molecular weight of the polymers and the size, polydispersity and zeta potential of the polyplexes. For instance, P1-8d and P5 have a similar molecular weight (7.1 and 7.2 kDa respectively; Table 2), but the polyplexes consisting of these two polymers have completely different characteristics, suggesting that the differences between the polyplexes could likely be caused by another factor. We can speculate that that the polyplex formation could be very

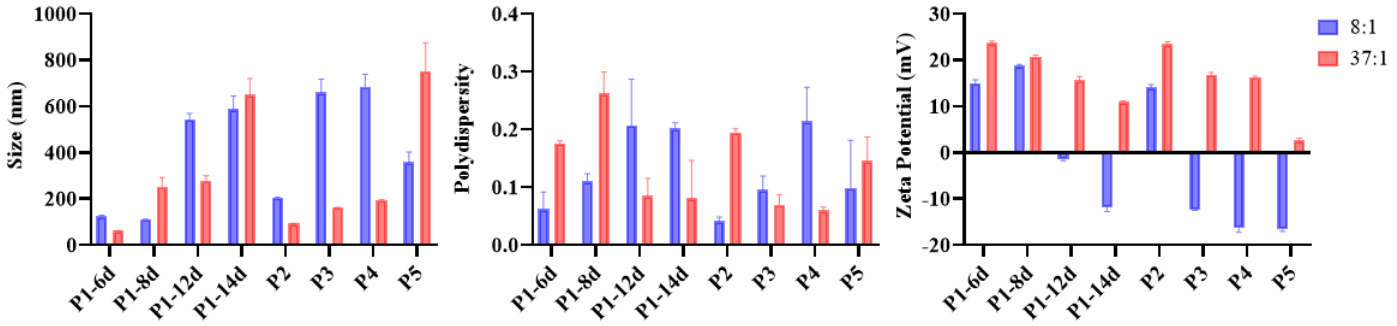


**Figure 3.** The size, polydispersity index and zeta potential of polyplexes prepared from polymers P1-P5 and fLuc mRNA in a 8:1 or 37:1 N/P ratio using the direct mixing method. Technical replicates (3) were analyzed and the mean ± SD is shown.

sensitive to small changes, or that the polymers differ from each other in another way than their size.

Moreover, the colloidal stability of the polyplexes was evaluated at various time points (Figure 4). For this, polyplexes were prepared from polymer P1-8d using an N/P ratio of 37:1. These polyplexes were selected based on their characteristics, since they exhibited an average size and zeta potential profile. The size of the polyplexes increased with longer incubation times, implicating aggregation of the polyplexes over time. This can be caused by precipitation of the polymer due to its pH-sensitivity. pCBA-ABOL gains hydrophilicity upon protonation during the acidification step after synthesis. The polyplexes are prepared in 20 mM HEPES buffer at pH 7.4 and the positive charge might be lost, because the environment is not acidic enough to retain the hydrogen. Consequently, the polymer becomes more hydrophobic and thus crashes out from the aqueous environment, which induces aggregation of the polyplexes.

To confirm this hypothesis, we prepared polyplexes from P1-8d in 20 mM HEPES buffer at pH 6 and measured the size of the polyplexes over time as well (Figure 4). At pH 6, the polymers are able to retain their positive charge better, because the environment is more acidic, and therefore the polyplexes should aggregate to a lesser extent. Accordingly, Figure 4 shows that the size of the polyplexes prepared in pH 6 only increased slightly over time, implicating there is almost no aggregation of the polyplexes. Hence, these results suggest a pH-dependent colloidal stability of the polyplexes.

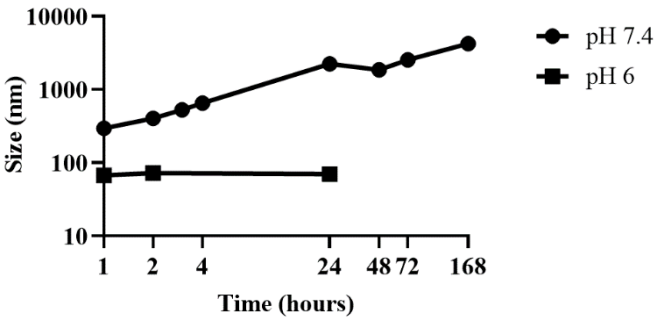


**Figure 4.** The size of polyplexes prepared from P1-8d with an N/P ratio of 37:1 in 20 mM HEPES buffer at pH 7.4 and pH 6 at various time points.

3.3. In vitro and in vivo transfection

The capability of pCBA-ABOL-based polyplexes to functionally deliver mRNA was investigated both in vitro and in vivo (Figure 5a). Initial cell studies showed that only polyplexes prepared from P1-8d with a 37:1 N/P ratio resulted in a high transfection efficiency on HEK293T cells. For this reason, the same formulation was tested on iPS-cardiomyocytes which exhibited a similar efficiency of transfection (Figure 5a). Polyplexes from P1-8d have favorable characteristics for transfection, i.e. a small size and a high zeta potential (Figure 3), but interestingly other polyplexes which have similar characteristics did not show high transfection efficiencies, such as polyplexes made from P1-12d (Figure 3 and 5a).

When testing polyplexes with a higher N/P ratio on cells, a tendency of increasing transfection efficiency was observed. Although P3 with an N/P of 8:1 showed a high transfection efficiency on cells, these results most likely were greatly influenced by the high signal of P1-8d that was detectable in the nearby located wells as well. This was to a lesser extent also the case for the results of P5 with an N/P ratio of 8:1. Thus, the repetition of experiment is required to confirm these previous observations.

In an animal study, the transfection efficiency of polyplexes prepared from P1-8d was tested. Based on our previous cell studies, we selected polyplexes with an N/P ratio of 37:1 as well as a 62:1, since it was observed that a higher N/P ratio resulted in a higher transfection efficiency in vitro. The characteristics of polyplexes prepared from P1-8d with an N/P ratio of 62:1 can be found in the Supporting Information (Figure S2). Mice were given an intramyocardial injection and

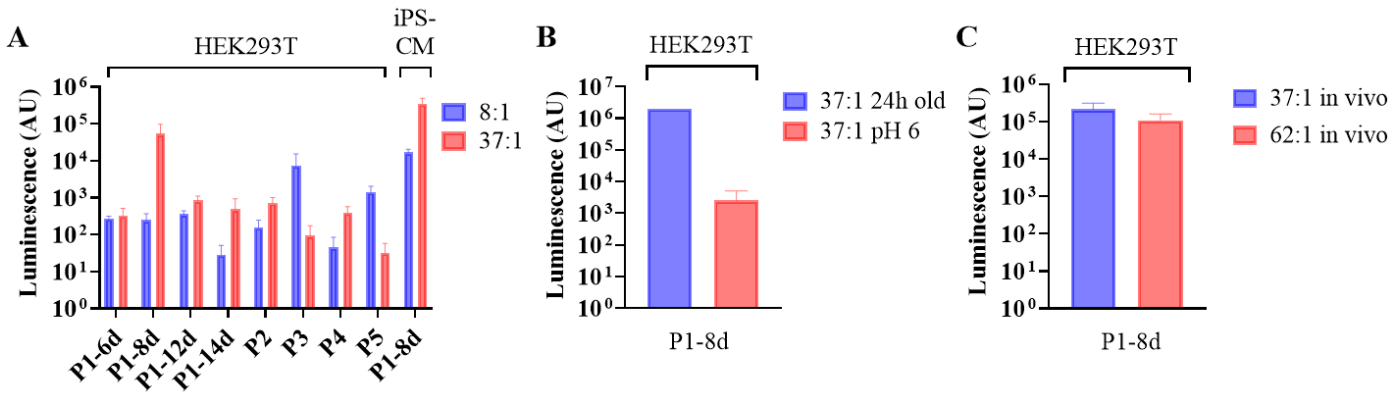


**Figure 5.** (a) In vitro transfection efficiencies in HEK293T cells and iPS-cardiomyocytes of polyplexes prepared from polymers P1-P5 with an N/P ratio of 8:1 and 37:1. (b) In vitro transfection efficiencies in HEK293T cells of aggregated polyplexes (24h old) and stable polyplexes (pH 6). Polyplexes are prepared from polymer P1-8d using an N/P ratio of 37:1. (c) In vitro transfection efficiencies in HEK293T cells for which polyplexes are prepared according to the formulations used in our in vivo study. Polyplexes are prepared from polymer P1-8d using an N/P ratio of 37:1 and 62:1.

subsequently the transfection efficiency was analyzed. Polyplexes prepared from P1-8d were not able to transfect any tissue however.

In general, a poor correlation exists between the in vitro and in vivo transfection of cardiomyocytes. Cardiomyocytes are terminally differentiated cells, which makes these cells hard to transfect[20]. Currently available cardiac cell lines are unable to completely mimic this terminally differentiated state, as well as the rod-shaped morphology and the contractile function of adult cardiomyocytes[21]. The use of isolated adult cardiomyocytes can narrow the gap between in vitro and in vivo transfection results[22].

In addition to this, we hypothesized that the polyplexes prepared from P1-8d aggregate over time during transfection, resulting in a high in vitro transfection efficiency, but not in vivo. Research has shown that the bigger the dimension of polyplexes, the higher and faster their sedimentation and therefore the greater their transfection efficiency in vitro[23]. Physical barriers and the dynamic nature limit the efficacy of large particles in vivo however, and therefore small particles are essential for an efficient in vivo transfection[23]. This therefore also contributes to a poor correlation between in vitro and in vivo outcomes.

We prepared polyplexes 24 hours prior to transfection and this resulted in a very high in vitro transfection efficiency (Figure 5b), which is most likely caused by the aggregation and subsequent sedimentation of the polyplexes. On the other hand, polyplexes prepared using HEPES buffer at pH 6, which were shown to be stable over time (Figure 4), did not show a high transfection efficiency in vitro (Figure 5b). This implicates that pCBA-ABOL-based polyplexes are not suitable for transfection, since only aggregated polyplexes showed a high in vitro transfection efficiency, whereas stable polyplexes were not capable of transfecting the cells with a high efficiency. These findings also suggest that polyplexes which did not show high in vitro transfection efficiencies were stable over time. The colloidal stability of other polyplexes was not investigated however and therefore, to confirm this hypothesis, additional experiments are required.

In our animal study, polyplexes could therefore have either been stable or aggregated. In both cases however, no in vivo transfection should be expected. The formulations that were used in our in vivo study were tested on HEK293T cells. The in vivo formulations consisted of a much smaller volume and were therefore more concentrated, which can stimulate aggregation. Accordingly, high in vitro transfection

efficiencies were found, implicating that the polyplexes were aggregated during in vitro transfection and therefore most likely during in vivo transfection as well (Figure 5c). Hence, this can clarify the discrepancy between our in vitro and in vivo results.

## 4. Conclusion

In this research, the use of pCBA-ABOL-based polyplexes for the transfection of cardiomyocytes was investigated. For this, low molecular weight pCBA-ABOLs were synthesized and polyplexes were prepared from these polymers. A higher N/P ratio resulted in polyplexes with more favorable characteristics for transfection and polyplex characteristics were independent of polymer sizes. Additionally, polyplexes exhibited a pH-dependent colloidal stability, most likely caused by precipitation of the polymer. The transfection efficiency of low molecular weight pCBA-ABOL-based polyplexes was investigated both in vitro and in vivo. In vivo, there was limited uptake of the polyplexes however. A poor correlation between our in vitro and in vivo results was most likely caused by the colloidal instability of the polyplexes, causing sedimentation in cell studies. Research into polyplexes prepared from high molecular weight pCBA-ABOLs is strongly encouraged, with regards to the potential enhancement of RNA delivery. For this, the use of microwave synthesis can be investigated further as a more reproducible method to obtain high molecular weight pCBA-ABOLs.

## Acknowledgements

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**Layman's summary**

Cardiovascular diseases entail complications related to the heart and blood vessels, such as high blood pressure, heart attacks and strokes. In fact, a large group of cardiovascular diseases are caused by the narrowing or blockage of the arteries that supply the heart muscle with blood. The accumulation of cholesterol in the arteries, namely atherosclerosis, is usually the underlying cause of this condition. Consequently, the supply of blood to the heart muscle is reduced and when the cells of the heart muscle do not get enough blood, the cells die and are replaced by scar tissue. Because of this, the heart cannot exert its normal function anymore, leading to heart failure.

Currently, the treatments for these type of cardiovascular diseases are invasive procedures, like a bypass surgery, and the focus is merely on restoring the blood flow to the heart muscle. However, when the cardiac tissue has been damaged, it is no longer possible to be repaired by the human body, and for this reason, current treatments are not able to halt disease progression.

In this study a new treatment is proposed with the aim to restore cardiac muscle upon injury by replacing it with newly formed tissue. The treatment is comprised of small particles (carrier) and a drug (containing a specific code) which is delivered to the heart muscle cells. This carrier is necessary to functionally deliver the code to the cells, as the drug itself is not capable to enter into the cells. When the small particles have entered the cells, the code containing specific instructions is then read by the heart cells, stimulating them to multiply themselves. In this way, the damaged heart muscle tissue would be repopulated with live cells.

In this project, it was investigated whether the particles can stimulate the heart muscle cells to execute what is stated in the code. In previous research it was found that the particles are more effective when very large carriers are used. This previous study was focused on muscle cells, but not heart muscle cells specifically. In this research, only small carriers were used to build the particles and it was found that such particles were not able to stimulate the heart cells. Therefore, it is encouraged to perform further research into the use of large carriers to stimulate the heart cells.

## Supporting Information

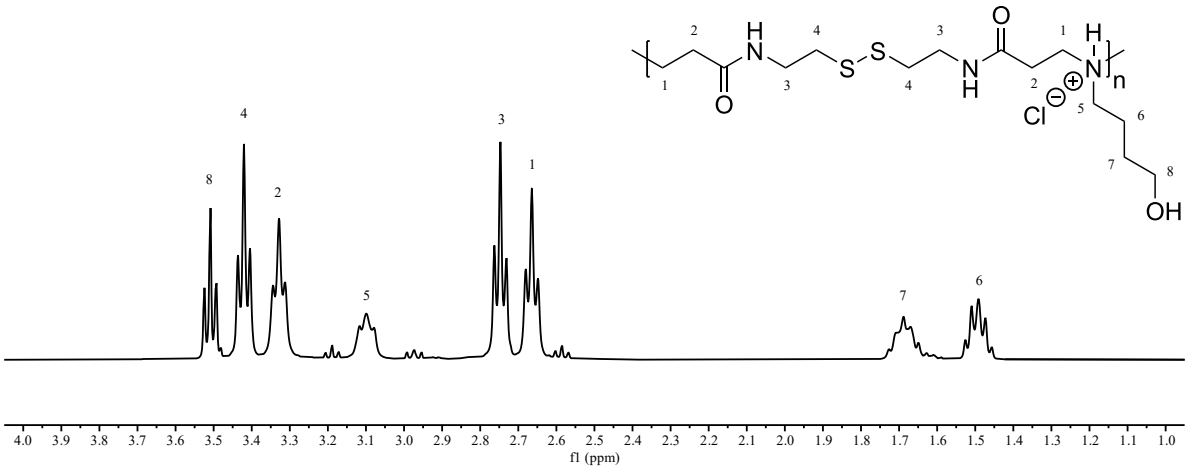


**Figure S1.** $^{1}$H NMR spectrum of purified pCBA-ABOL in $D_2O$ with assigned peaks.

$^{1}$H NMR ($D_2O$, 400 MHz): δ = 1.49 (p, 2H, C<u>H</u>$_2$CH$_2$CH$_2$OH from ABOL), 1.68 (p, 2H, C<u>H</u>$_2$CH$_2$OH from ABOL), 2.66 (t, 4H, NC<u>H</u>$_2$CH$_2$CO from CBA), 2.75 (t, 4H, NHC<u>H</u>$_2$CH$_2$S from CBA), 3.10 (t, 2H, C<u>H</u>$_2$N from ABOL), 3.33 (t, 4H, NCH$_2$C<u>H</u>$_2$CO from CBA), 3.42 (t, 4H, NHCH$_2$C<u>H</u>$_2$S from CBA), 3.51 (t, 2H, C<u>H</u>$_2$OH from ABOL)

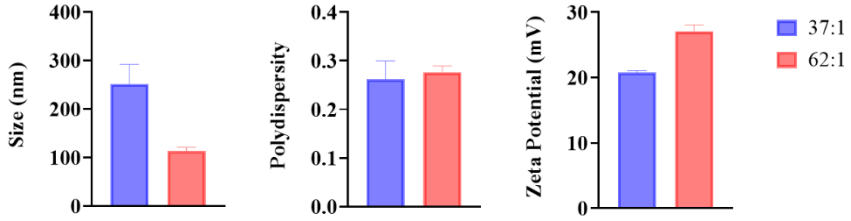


**Figure S2.** Size, polydispersity and zeta potential of polyplexes prepared from P1-8d using an N/P ratio of 37:1 and 62:1.