



Polymer-Based Systems for Targeted miRNA Delivery

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Abstract

Background: Star copolymers, composed of a combination of hydrophilic and cationic monomers, are particularly promising for these purposes due to their ability to form stable complexes with nucleic acids. This study investigates the synthesis and characterization of novel star copolymers for nucleic acid delivery. **Methods:** Five star copolymers and their corresponding five linear diblock precursors were synthesized via Group Transfer Polymerisation (GTP). The copolymers were composed mainly of DMAEMA (a cationic monomer) and PEGMA (a hydrophilic, biocompatible monomer) in varying proportions. A full characterization of the copolymers was performed using Gel Permeation Chromatography (GPC) and ¹H NMR spectroscopy to determine MM and composition. The thermoresponsive properties of the copolymers were evaluated through Cloud Point determination, pKa measurement, and Dynamic Light Scattering (DLS) to assess size and zeta potential in aqueous solutions. The copolymers were further tested for their ability to form complexes with DNA at an N/P ratio of 15. **Results:** All copolymers exhibited thermoresponsive behavior within a temperature range of 31–43 °C, with a consistent pKa of around 7, independent of MM or composition. The size and zeta potential of the

copolymers in deionized water and PBS at pH 7.4 were determined using DLS. Complexes formed between the polymers and DNA were found to have sizes ranging from 24.4 nm to 78.8 nm, with zeta potentials of approximately +10 mV, which falls within the optimal range for successful nucleic acid delivery. **Conclusion:** The synthesized star copolymers demonstrate promising thermoresponsive behavior and ability to form stable complexes with nucleic acids. Their size and zeta potential indicate potential for use in gene delivery systems, with the observed properties suggesting that the polymers can efficiently encapsulate DNA at an optimal N/P ratio for successful delivery.

Keywords: Star copolymers, Group Transfer Polymerisation, thermoresponsive, pKa, nucleic acid delivery.

1. Introduction

Nucleic acid delivery has been one of the most studied fields in recent decades. The reason for this is the enormous potential of successfully delivering DNA and RNA molecules to cells for the treatment of many diseases. Initially, plasmid DNA or gene delivery was the focus of this research, given the usefulness of modifying the genome of cells for therapeutic purposes. However, gene delivery faces several challenges, particularly because DNA molecules must reach the cell nucleus to exert their desired therapeutic effect. This is a complex task, as delivery vectors must be designed to transport negatively charged nucleic acids across the negatively charged cell membrane and through the nuclear membrane, allowing endosomal escape and nuclear uptake of the DNA (Smith et al., 2010; Johnson & Lee, 2013).

Due to these challenges and the potential of RNA interference (RNAi) to regulate gene expression, interest has shifted

Significance | This study develops thermoresponsive star copolymers with potential for efficient, biocompatible nucleic acid delivery in biomedical applications.

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considerably toward delivering small non-coding oligonucleotides such as small interfering RNA (siRNA) or micro RNA (miRNA). These molecules regulate translation by degrading mRNA in the cytoplasm, so they do not need to enter the nucleus (Brown et al., 2015). Both siRNA and miRNA share similar mechanisms of action: they bind to the RNA-induced silencing complex (RISC), which activates the complex and guides it to specific mRNA molecules, leading to mRNA cleavage (Clark et al., 2016). The key difference between miRNA and siRNA is that miRNA does not need to be perfectly complementary to its target mRNA, enabling it to target multiple mRNA molecules (Clark et al., 2016; Anderson, 2017). This characteristic makes miRNA a powerful tool for treating multigenic diseases that involve the aberrant expression of several genes (Clark et al., 2016; Kumar et al., 2018).

As this study focuses on developing suitable polymeric carriers for miRNA delivery, only a brief introduction to miRNA is provided here (Appendix A: MicroRNA (miRNA) delivery). In nucleic acid delivery, various types of carriers have been proposed and studied. Viral vectors were among the first to be studied due to their natural ability to transfect genes. However, viral carriers have significant drawbacks, including cytotoxicity and the potential for fatal immune responses (Jones et al., 2009; Zhao & Wang, 2012). Consequently, research now focuses on finding safer delivery vectors that are as effective as viral vectors (Figure 1). Polymeric carriers have emerged as promising candidates due to their tailorability and relatively simple production process (Miller & Davis, 2011).

Although cytotoxicity remains an issue with polymeric carriers, it is easier to overcome because synthetic polymers can be easily modified with different monomers and moieties, enabling the creation of various chemical and physical structures (Brown et al., 2015) (Figure 2). Suitable carriers must bind to oligonucleotides to facilitate cellular uptake. For delivery to be successful, the carrier must form a complex with the nucleic acid that is stable enough to reach and penetrate the cell membrane. However, this complex must also dismantle once inside the cell to prevent degradation in the lysosome. This process, known as endosomal escape, is critical for nucleic acid delivery (Clark et al., 2016; Zhao et al., 2013).

One widely used strategy for creating these complexes involves using polycations to neutralize the negative charges on the phosphate groups of nucleic acids (Miller & Davis, 2011). This interaction is strong enough to hold the complex together until it is inside the cell. Additionally, positively charged particles increase cellular uptake, as negatively charged molecules, such as nucleic acids, struggle to cross the negatively charged cell membrane (Brown et al., 2015; Miller & Davis, 2011; Zhao et al., 2013). Among the most studied polycations for gene and oligonucleotide delivery is poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA). Each residue in pDMAEMA contains an amino group, making it

hydrophilic and positively charged at physiological pH. Like other cationic polymers, pDMAEMA has cytotoxic effects but also exhibits high transfection efficiency (Miller & Davis, 2011; Zhao et al., 2013). Its high transfection efficiency may be partly due to its low pKa, close to 7, allowing it to act as a proton sponge that buffers the acidification within endolytic vesicles, destabilizing them and protecting their payload (Jones et al., 2009).

PEGylation of carriers has been extensively used to increase circulation time and biocompatibility (Miller & Davis, 2011; Zhao et al., 2013). Polyethylene glycol (PEG) is known for its hydrophilicity and biocompatibility. For DMAEMA-based polymeric carriers, adding PEG units may also improve transfection efficiency, as observed in gene delivery studies (Miller & Davis, 2011; Brown et al., 2015; Zhao et al., 2013). According to Venkataraman et al. (2010), the benefits of PEGylation may be greater when the polymers are synthesized as block copolymers, where monomers are arranged in homogeneous blocks, rather than as statistical copolymers, where monomers are randomly distributed along the polymer chain.

In this study, different diblock copolymers based on DMAEMA and PEGMA have been synthesized. In several studies, star-shaped polymers, in which multiple linear polymers are branched, have been crosslinked to form star polymers for gene and oligonucleotide delivery (Green et al., 2012; Venkataraman et al., 2010). Results have shown that star polymers are effective vehicles for nucleic acid delivery, but a full comparison between star polymers and their linear counterparts is often not performed. Such a comparison could provide valuable insights into the mechanisms underlying the enhanced delivery efficiency of star polymers.

In this study, five star copolymers with different compositions and molecular masses (MMs) were synthesized (Table 1). The synthesis of polymers with varying MMs is necessary, as MM is a key factor influencing delivery efficacy and toxicity (Miller & Davis, 2011). For each polymer, only one parameter was altered, allowing a systematic characterization of the effects of MM on delivery performance. Given the widespread use of pDMAEMA in nucleic acid delivery studies and PEG's FDA-approved status, diblock copolymers based on DMAEMA and pentaethylene glycol methacrylate (PEGMA) were synthesized as the arms of the star copolymers. Both star and linear polymers were fully characterized, with measurements of pKa, cloud points (CPs), hydrodynamic sizes, and zeta potential. This characterization will guide the selection of the best-performing polymers for miRNA delivery.

2. Materials and Methods

2.1 Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA, D, 98%), penta(ethylene glycol) methyl ether methacrylate (PEGMA, P, MM=300 g mol⁻¹), ethylene glycol dimethacrylate (EGDMA, 98%)

Table 1. Synthesised polymers: Theoretical polymer structures, target molar masses and compositions. There is no target Molar Mass for the star copolymers as they have been synthesised using the arm-first approach.

Polymer	Theoretical Polymer Structure	Target MM (g/mol)	Target (%)	composition weight)
No.			DMAEMA	PEGMA
P1L P1S	DMAEMAs7-b-PEGMA5 DMAEMA57-b-PEGMA3-S	10100 -	90	10
P2L P2S	DMAEMA91-b-PEGMA2.5 DMAEMA91-b-PEGMA2.5-S	15100 -	95	5
P3L P3S	DMAEMAs6-b-PEGMA5 DMAEMAs6-b-PEGMA5-S	15100 -	90	10
P4L P4S	DMAEMAs1-b-PEGMA75 DMAEMAs1-b-PEGMA7.5-S	15100 -	85	15
P5L P5S	DMAEMA14-b-PEGMA7 DMAEMA14-b-PEGMA7-S	20100 -	90	10

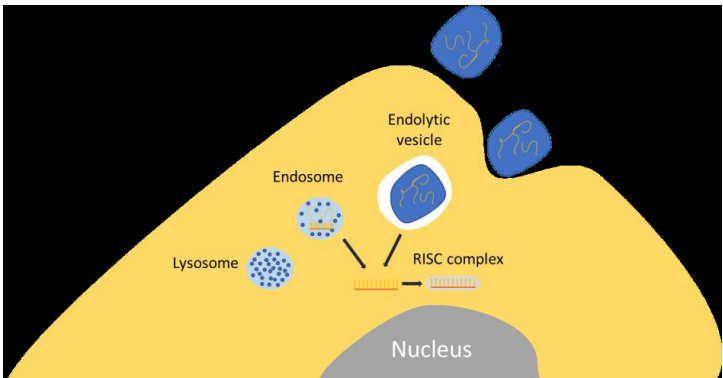


Figure 1. Delivery of a single-stranded miRNA through a polymeric vector to a cell.

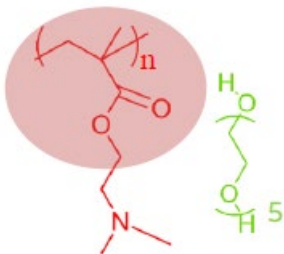


Figure 2. Chemical structure of pDMAEMA (in red) and pentaethylene glycol (PEG, in green). Methyl methacrylate repeating unit appears encircled.

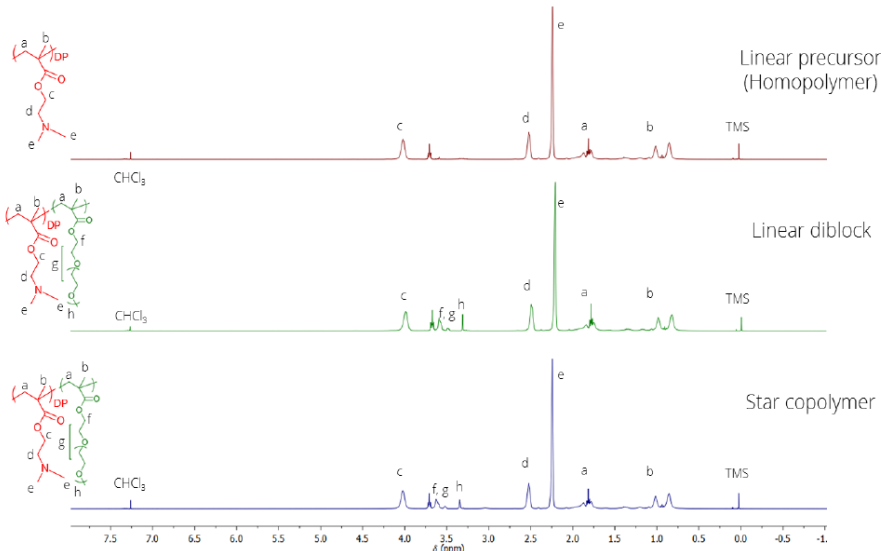


Figure 3. ¹H NMR spectra of polymer P1 linear and star and its pDMAEMA precursor.

2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), calcium hydride (CaH₂, ≥90%), aluminium oxide activated basic (Al₂O₃-KOH), deuterated chloroform (CDCl₃, 99.8%), methyl trimethylsilyl dimethylketene acetal (MTS, 95%), salmon sperm low MM DNA, sodium hydroxide (NaOH, 97%), hydrochloric acid (HCl, ACS reagent, 37%) and tetrahydrofuran (THF, HPLC grade, polymerisation solvent, ≥99.9%) were purchased from Sigma-Aldrich, UK. Phosphate buffered saline (PBS, 10x solution) and tetrahydrofuran (THF, GPC grade, mobile phase in chromatography) were obtained from Fischer Scientific, n-hexane was purchased from VWR chemicals and liquid sachets with pH 7.01 and pH 4.01 solutions were obtained from Hanna Instruments.

2.2 Purification of the monomers and solvent

Prior to polymerisation, the monomers, DMAEMA and PEGMA as well as the cross-linker, EGDMA, were purified in two steps to remove inhibitors, acidic impurities, and humidity. The solvent, THF, was freshly purified to eliminate any moisture using a solvent purification system moisture. DMAEMA and EGDMA were flowed twice in tubes with basic alumina powder to remove inhibitors and neutralise any acids. Due to its high MM and viscosity, PEGMA was diluted by 50% v/v with freshly purified THF and was not treated with alumina. CaH₂ was then added to the monomers and left to stir for three hours to remove humidity. To prevent premature polymerisation, DPPH was added as a free radical inhibitor to both DMAEMA and EGDMA. These monomers were distilled under vacuum prior to polymerisation and were stored refrigerated until use. DPPH was not added to PEGMA, as it cannot be distilled at low temperatures, so the PEGMA solution was filtered through poly tetrafluoroethylene (PTFE) 0.45 µm filters directly into the polymerisation flasks. MTS was used as an initiator and it was distilled and kept refrigerated until polymerisation. Tetrabutylammonium bibenzoate (TBABB) was used as a catalyst. Its synthesis is out of the aim of this project, but it was previously in-house synthesised in the lab by mixing tetrabutyl ammonium hydroxide and benzoic acid. Glassware used in distillations and polymerisations were dried overnight at 140°C and assembled hot under vacuum prior to their use.

2.3 Polymer synthesis

Polymerisation was performed by means of Group Transfer Polymerisation (GTP) in three steps: i) DMAEMA polymerisation, ii) PEGMA polymerisation over the pDMAEMA blocks and iii) cross-linking of the linear copolymers to form the corresponding star copolymers. The synthesis process is **picture** on Figure 4 and is explained in detail in Appendix B: Polymerisation. In Table 2, the polymer P1 synthesis process is shown as an example. For this polymer, the target MM was 10100, so Degrees of Polymerisation (DPs) of 57 and 3 were selected for DMAEMA, and PEGMA, respectively. Monomers and initiator were the same for all

polymers, only the added quantities varied for each polymer in order to obtain polymers with different MMs and compositions.

2.4 Precipitation of the polymers

Firstly, THF volume of the polymer solutions was decreased using a rotary evaporator. Polymers were then precipitated in hexane at room temperature and dried in a vacuum oven at room temperature for several days. The cross-linked copolymers (labelled as PXS, as seen in Table 1) were precipitated three to four times to reduce the proportion of the remaining linear copolymers against the star copolymers. For the third and fourth precipitation, hexane was heated to 50 °C and 60 °C (respectively) using an IKA RCT stirrer hotplate and an IKA ETS-D5 temperature controller.

2.5 Preparation of the complexes

Complexes were prepared for all the diblock and cross-linked copolymers with an N/P ratio of 15. The N/P ratio is the ratio of amino groups of the polymer to phosphate groups of the nucleic acid, so it is essentially the molar ratio of polymer to nucleic acid. Different volumes of 1 g/L polymer solution in PBS were further diluted with the same solvent to a final volume of 3.98 mL. Then, 0.02 mL of DNA solution in PBS at a concentration of 15 mg/mL was added and the solutions were left for the complexes to form for 45 minutes.

2.6 Polymer characterisation Characterisation in organic solvents Gel Permeation Chromatography

Molecular masses (Number Average and Peak) and dispersities (Đ) of all polymers, including the pDMAEMA precursors, were obtained by means of GPC. For the cross-linked polymers, the area of the curves corresponding to the star copolymers and the non-reacted linear ones were obtained to estimate a ratio of star to linear. The equipment used was an Agilent, SECurity GPC system, with a Polymer Standard Service SDV analytical linear M column (SDA083005LIM). The machine was calibrated using six MM linear poly(methyl methacrylate) (PMMA) standard samples (2000, 4000, 8000, 20000, 50000, 100000 g/mol) purchased from Sigma-Aldrich, UK. A solution of THF with a 5 volume % of triethylamine was used as eluent or mobile phase. Prior to the measurements, samples were diluted in the eluent at a ~10 mg/mL concentration and filtered using Millex-LCR, non-sterile, HPCL certified 0.45 µm filters purchased from Milipore. The pump used was a “1260 ISO” isocratic pump and the flow rate was set at 1mL/min. Refractive index (RI) signal was measured using an Agilent 1260 RI detector.

Proton Nuclear Magnetic Resonance Spectroscopy

All polymers and precursors were characterised by means by means of ¹H NMR spectroscopy to confirm chemical structure and composition (Figure 3). Samples were dissolved in deuterated chloroform CHCl₃ and analysed using a 400 MHz Avance Bruker NMR spectrometer instrument.

Characterisation in aqueous solutions

Table 2. Steps in the synthesis of polymers DMAEMA57-b-PEGMA3 and DMAEMA57-b-PEGMA3-s (P1L and P1S) with a target MM of 10100 g/mol.

Synthesis step	Substance	Target % weight	Volume added (mL)	Polymer collected
Step 1 - DMAEMA polymerisation	Initiator - MTS		0.47	~0.01mL
Step 2 - PEGMA polymerisation	Monomer 1 - DMAEMA	90	22.32	pDMAEMA®
3	Monomer 2 - PEGMA	10	4.41	10 mL diblock
Step - Cross-linking	Remaining initiator - MTS	----	0.37	no 17 mL
	Cross-linker - EGDMA	----	1.37	star

Table 3. Results of GPC and H1 NMR. There were no target MMs for the star copolymers because they were synthesised following an arm-first approach.

Polymer No.	Theoretical Polymer Structure	Molecular	Mass	GPC (g/mol)	Average No. of arms	D	H1	NMR	weight)
		Target	Mn	Mp			Target	Experimental	
P1L	DMAEMAs7-b-PEGMA _s	10100	10700	11900	---	1.11		87	13
P1S	DMAEMAs7-b-PEGMA _s -S	-	118400	127700	11	1.25	90	10	13
P2L	DMAEMAen-b-PEGMA _{2s}	15100	15500	17700	---	1.15		93	7
P2S	DMAEMAen-b-PEGMA _{2s} -S	-	222000	245600	14	1.29	95	5	7
P3L	DMAEMAs6-b-PEGMA _s	15100	17100	18600	---	1.12		87	13
P3S	DMAEMAs6-b-PEGMA _s -S	-	187900	209600	11	1.21	90	10	13
P4L	DMAEMAs1-b-PEGMA _{7s}	15100	16800	20200	---	1.19	85	81	19
P4S	DMAEMAs1-b-PEGMA _{7.5} -S	-	199000	226000	12	1.32		15	16
P5L	DMAEMA14-b-PEGMA	20100	31200	41200	---	1.3		87	13
P5Sb	DMAEMA114-b-PEGMA-S	-	589500	638700	19	1.36	90	10	13

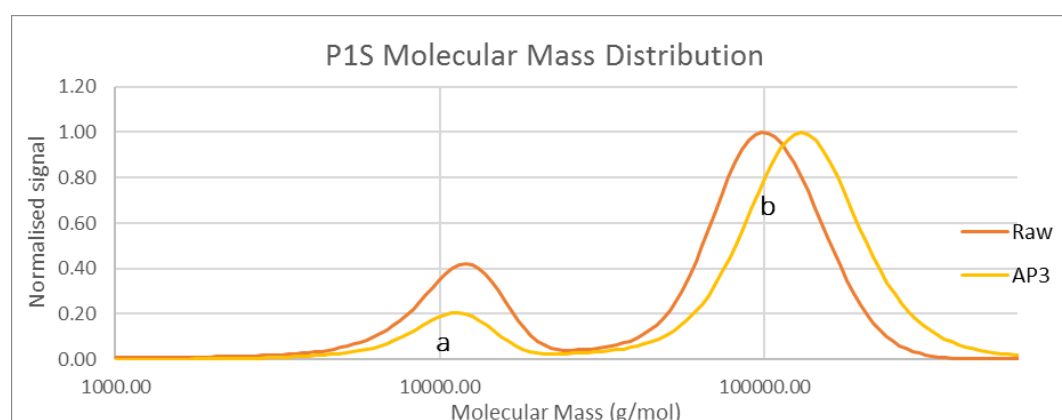


Figure 4. Molecular Mass distribution of one of the star copolymers, P1S. (a) The peak corresponding to the linear precursor was decreased after the third precipitation. (b) All GPC parameters for the star copolymers were obtained from this peak.

All the linear diblocks and the star copolymers were dissolved in deionised water (DI) at a 1% w/w. These solutions were used to estimate the cloud point, the effective dissociation constant (pKa) and the hydrodynamic diameter of each polymer. The complexes solutions along with polymers solutions and raw DNA in PBS were also used to obtain the zeta potential and the hydrodynamic radius in a physiological medium. The polymer solutions had a concentration of 0.75 mg/mL and the DNA solution had a concentration of 0.07 mg/mL. These values were selected so they were comparable to the concentrations of the polymer and the DNA in the complex solutions.

Cloud points

The Cloud points, or the temperature at which the polymers precipitated in aqueous solutions [23], were measured using a temperature controller and a hot plate to monitor and control the temperature of a water bath in which the samples were immersed. The solutions were not protonated, and all of them presented a pH between 9 and 10. The temperature was increased from room temperature to a maximum of 45°C and visual observations were carried out every one degree. No observations were obtained above 45 °C as all the polymer solutions presented thermoresponse within this temperature range. Stirring was maintained during the whole duration of the experiments to ensure homogeneous heat transfer.

Potentiometric titrations

The pKa of each polymer was measured through potentiometric titrations. Portable HI98103 pH checkers from Hanna instruments were used. Calibration was carried out using stock pH 4.01 and pH 7.01 solutions. Samples were initially set at pH 2 by phased additions of 10-50 mL HCl 1M. Then, pH was increased slowly with additions of 10 µL NaOH 1M, waiting a few minutes after each addition for the pH to stabilise and noting down the value. These concentration and volume of the basic solution added were selected to obtain an optimal number of points in the titration (from 30 to 50). The effective pKa, defined as the pH at which the DMAEMA units were at 50% deprotonation, was calculated as the average between the pH values at the two Equivalence Points.

Dynamic Light Scattering

All aqueous solutions of the polymers and complexes were characterised by means of DLS to obtain the hydrodynamic diameters in DI water and/or PBS. Zeta potential was also measured for the PBS solutions (polymers, DNA and complexes). All samples were adjusted to a physiological pH (7.4) by adding appropriate amounts of HCl 0.25 M. DI water solutions were also filtered with nylon 0.45µm syringe filter NSTR purchased from Fisher Scientific. PBS solutions were not filtered so the proportion of polymer to DNA of the complexes was not affected (and neither were the polymer PBS solutions for comparability reasons).

The equipment used was a Zetasizer Nano ZSP (Malvern Instruments Ltd). All measurements were performed at 25°C and

using a backscatter angle of 173°. Each measurement was repeated three times with a maximum of 100 reruns per measurement (for zeta potential measurement the number of reruns was modified automatically according to the results). Z-potential measurement were repeated three times, with a variable number of reruns that was automatically selected by the software. Results for the size tests were compared with the theoretical extended and folded lengths of the polymers considered as upper and lower size limits, respectively. These were calculated, as seen in Equation 1 and 2, assuming a length of 0.254 nm for each methacrylate residue for the fully extended chains and a random coil configuration with a carbon-carbon bond distance of 0.154 nm for the folded chains (Ward & Georgiou, 2013), (Constantinou et al., 2016). *r_{min}* is obtained from the root mean square of the end-to-end distance of a random coil. Theoretical diameters (both maximum and minimum) of the stars are assumed to be twice those of the linear chains.

$$r_{max}=0.254 \cdot (DPDMAEMA+DPPEGMA) \quad \text{Equation 1}$$

$$r_{min}=\sqrt{dg2}=2 \cdot 0.154 \sqrt{2.2 \cdot 2 \cdot DPDMAEMA+DPPEGMA3} \quad \text{Equation 2}$$

Where,

r_{max} is the theoretical extended length of one chain.

r_{min} is the theoretical folded length of one chain.

0.254 nm is the assumed length of the monomers.

0.154 nm is the length of a carbon-carbon bond.

DP is the degree of polymerisation obtained by GPC and NMR.

Zeta potential measurements were performed using two DTS1070 Folded Capillary Zeta Cells purchased from Malvern Instruments. The same cells were used for the size characterisation of the polymers and complexes in PBS. The size of the polymers in water was measured using Fisherbrand™ disposable cuvettes purchased from Fisher Scientific.

3. Results and discussion

Polymers of three different target MMs and three different target compositions were synthesized and characterised. Ten linear diblock copolymers and ten star copolymers (cross-linked diblocks with EGDMA) were obtained.

3.1 Molecular Masses and composition

GPC characterisation results, including number average and peak MMs (M_n and M_p), dispersions indices (Đ) and compositions, are shown in Table 3. Stars copolymers had always some percentage of linear precursor (shown in Table 3). For these polymers, dispersion and experimental MM values shown are those obtained after the third precipitation for the peak corresponding to that of the star (Figure 6b). All results for the linear copolymers and H1 NMR results for the star copolymers were taken from the initial measurements where no precipitation had taken place.

Together with the NMR spectra, GPC results confirmed the formation of the polymers. In the MM distribution obtained for the linear diblocks by GPC, only one peak was detected, which was slightly shifted to the right compared to that of the homopolymer precursor was found. This indicates that no precursor was present in the diblocks and that the diblock had successfully synthesised. MMs were higher than targeted, which suggests that the initiator was partially deactivated, as seen in other studies where GTP was used (Constantinou et al., 2016). The use of PMMA standards for the calibration of the GPC can also be responsible for an apparent increase of the MM. Low MM linear copolymers (P1L-P4L) presented a low dispersion, below 1.2, while for P5L it was of 1.3.

Dispersity values were higher for the star copolymers, as it is common in stars synthesised via an arm-first approach, and they ranged from 1.21 to 1.29. The average number of arms, calculated as the quotient between the Mn values of the star and those of the corresponding linear polymers, was between 11 and 19. Both the high number of arms and dispersity for the polymer P5S (and the high $\bar{D}$ of P5L) may be explained by an increase in the degree of overlap between the molecules of the copolymers because of their longer chains.

Composition of the polymers was confirmed by ^1H NMR, as seen in Figure 5. In the upper spectrum, several peaks corresponding to DMAEMA homopolymer are present. The peak at 2.25 ppm is the most distinctive, and it is caused by the protons bonded to the terminal carbons of the side chain, labelled as “e”. The addition of PEGMA can be observed in the second spectrum, as much fainter peaks corresponding to the protons “f”, “g” and “h” appear between 3.65 ppm and 3.3 ppm. Experimental compositions of the polymers, determined through the integration of the peaks “e” and “h” for DMAEMA and PEGMA (respectively), were all close to the target ones.

Precipitations

Precipitations in hexane were carried out to collect the polymers and to decrease the proportion of linear precursors in the star copolymers. The first and second precipitation were performed at room temperature but for the third and fourth precipitations hexane was heated to 50°C.

After the first precipitation, the percentage of star was well over 50% for all the polymers except for P5S, probably due to star-star coupling (Appendix C: Precipitations). Purity of the star shows a trend with MM: the purity decreases as the MM increases. Increased PEGMA content also appears to be related to a higher difficulty in eliminating the linear precursor through precipitation. An example of one of the precipitations can be found on Figure 6.

3.2 Cloud points

CPs were determined visually in 1% w/w solutions in water. At room temperature, all polymers were soluble and all solutions were clear. CPs were close to each other for the linear and the star

copolymers, ranging from 37°C to 43°C for the linear polymers and from 31°C to 34°C for the stars. In Table 4 the values of the CPs are shown for each polymer, together with their Mn and composition, as the CP is strongly dependent on these parameters (Constantinou et al., 2016).

All polymers presented thermoresponsive behaviour, as it has been observed in other studies for DMAEMA/PEG-based polymers (Constantinou et al., 2016). An average decrease of 8 °C is observed in the CPs of the stars compared to those of linear polymers, possibly caused by the hydrophobic nature of the EGDMA, used as a cross-linker. The much weaker interactions of the core of the stars with water, compared to those of the linear polymers, can also be responsible for the thermoresponsive behaviour of the stars at lower temperatures.

3.3 Determination of the pKa

The monomers in all the polymers were always PEGMA and DMAEMA, however, the only ionisable unit is DMAEMA. Titration curves showed two equivalence points corresponding to the starting and final point of the titration of the amino groups, as seen in Figure 5. Effective pKa values are listed in Table 5. All linear copolymer solutions were clear at all pH values. Three of the star copolymers, P2S, P3S and P5S, precipitated abruptly at alkaline pH values, the lowest being at 10.2. Pieces of precipitated polymers were visible in the solution, which went from cloudy to slightly cloudy in a few seconds.

Results show that the pKa is around 7 for all the polymers, in the optimal range for delivery as reported in other studies for pDMAEMA based polymers (Ward & Georgiou, 2013). Neither MM nor composition appeared to have any effect on the values obtained. No significant differences between most of the stars and their linear counterparts were found, in agreement with other studies which reported no effect of this type of polymer architecture in the pKa (Ward & Georgiou, 2013).

For P5, however, there is a difference in the pKa between the star and the linear of 0.5, which might be caused by the increased hydrophobicity of P5S due to the star-star coupling occurred mostly after the fourth precipitation (much larger molecules). The precipitation in P2S and P3S might be explained by the content of PEGMA. At a percentage of this monomer of up to 13% and at a Mn between 15000 and 16000 the polymers appear to be hydrophobic enough to precipitate at high pH values (11 and 10.5). However, at a similar MM but higher PEGMA content of 16%, P4S does not precipitate, probably because it is more hydrophilic.

3.4 Size and z-potential

DLS was used to obtain size and z-potential of polymers and complexes. From the several peaks present in each intensity Particle Size Distribution (PSD) obtained, those which fell inside the theoretical size range were selected, as seen in Figure 6. The occasional peaks corresponding to sizes outside of the theoretical

Table 4. Cloud points of the linear and star copolymers compared to Mn and composition.

Polymer	M _n	Composition (weight)		Cloud point + ©
No.	lower size	DMAEMA	PEGMA	
P1L	10700	87	13	43
P1S	118400	87	13	32
P2L	15500	93	7	37
P2S	222000	93	7	31
P3L	17100	87	13	41
P3S	187900	87	13	32
P4L	16800	81	19	43
P4S	199000	84	16	33
P5L	31200	87	13	37
P5S	589500	87	13	34

Table 5. Titration results

Polymer	Effective pK	Observations	
No.	(+0.1)	Opacity	Precipitation
P1L	7.0	Clear	None
P1S	7.0	Clear	None
P2L	7.2	Clear	None
P2S	7.2	11.0 Slightly cloudy	11.0 - Sudden precipitation
P3L	7.1	Clear	None
P3S	7.1	10.5 Slightly cloudy	10.5 - Sudden precipitation
P4L	6.9	Clear	None
P4S	6.8	Clear	None
P5L	7.3	Clear	None
P5S	6.8	10.2 Slightly cloudy	10.2 - Sudden precipitation

Table 6. Size and z-potential of polymers and complexes obtained via DLS.

Polymer	Theoretical	Theoretical		Experimental	size	Zeta	potential
No.	lower size	upper size	In water5	In PBS	Complexes	In PBS	Complexes
P1L	3.0	16.3	18.2	6.5	615°	+5.1	+2.32
P1S	6.0	32.6	28.2	24.4	24.4	+11.1	+8.83
P2L	4.0	28.7	32.7	8.7	10.1/615	+9.53	+4.36
P2S	7.9	57.3	43.8	43.8	32.7	+7.47	+10.5
P3L	3.9	28.3	21	10.1	5.61/531	+7.02	+7.82
P3S	7.9	56.7	37.8	58.8	32.7	+6.09	+10.7
P4L	3.6	23.9	15.7	10.1	8.72/615	+6.58	+6.29
P4S	7.2	47.8	50.7	43.8	32.7/712	+10.3	+11.3
P5L	5.1	47.5	18.2	13.5	11.7/712	+5.13	+5.49
P5S	10.2	94.9	37.8/295	78.8	78.8	+12.5	+10.0
LMM DNA	----		----	615	----	-5.31	----

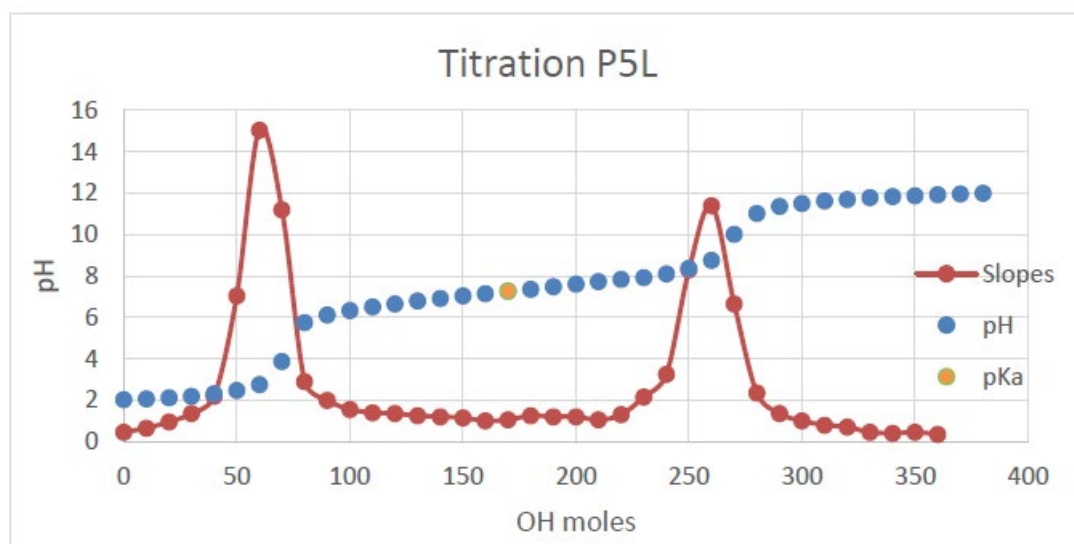


Figure 5. Evolution of pH and its slope with the addition of NaOH during the titration of P5L. The pH corresponding to the pKa is pictured in yellow.

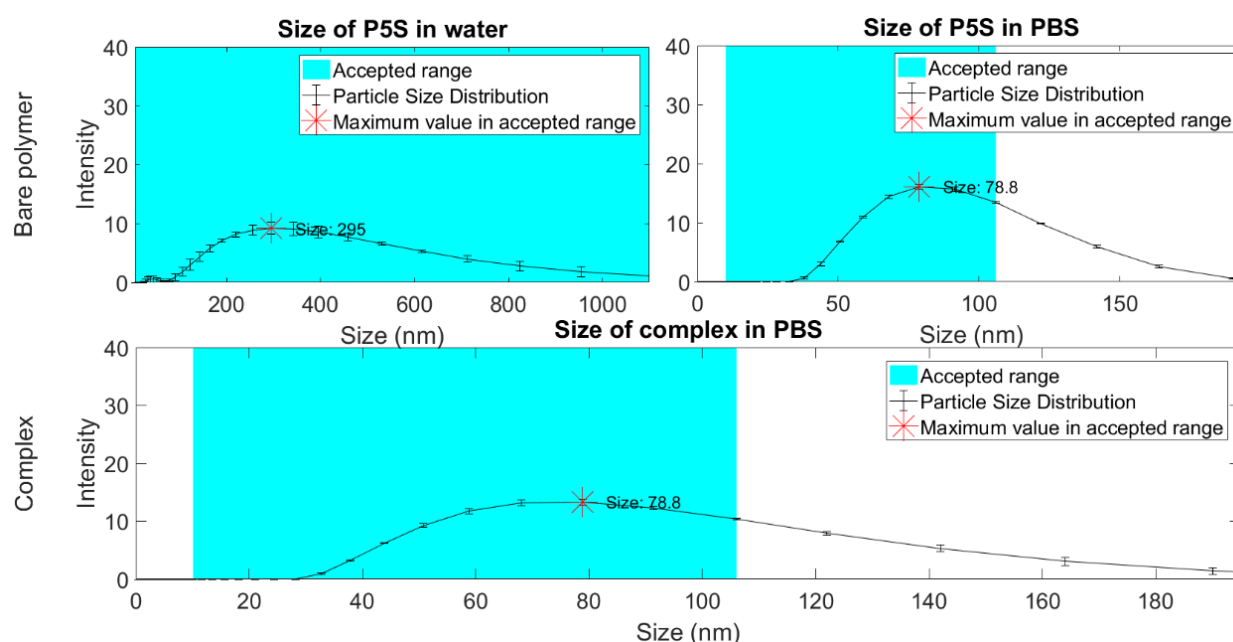


Figure 6. PSDs of P5S in water (a) and PBS (b). In (c) the PSD of the complex is plotted. Only the regions of interest (where peaks are found) are shown. Theoretical range is in blue and the maximum of that range in red. A broad peak of intensity can be observed in each PSD, especially in a), and is caused by aggregations of particles and star-star coupling, characteristic of this polymer. The error bars show the standard deviation of the three measurements performed.

range, either smaller or bigger, were assumed to be caused by contamination or aggregations of particles, respectively. The maximum of each peak inside the theoretical range of the intensity PSD, has been regarded as the size of the corresponding polymer (Figure 6) as the maximum is related to the most common size for the size range covered by the peak [32]. Aggregation were observed in all measurements of the linear copolymers. These aggregations were less frequent in the measurements for the star copolymers which is likely caused by the improved steric stabilisation of the stars. Especially for the linear polymers, the several peaks found implied that noisy PSDs and z-potential distributions were obtained, which have diffculted the comparison of the results with those found in the literature. However, some trends and relationships between the polymers in the different media have been observed and can be found in Appendix E: Analysis of size .

Polymer sizes (shown in Table 6) do not show a trend with neither MM nor composition. In water, sizes of the star copolymers are in average 1.9 higher to sizes of the linear copolymers, in accordance to the assumption of the sizes of the stars being twice the size of the linear polymers, but in PBS this the stars are almost 5 times bigger than the linear polymers. This is explained by the low sizes of the linear polymers in PBS compared to the sizes in water (which are more than twice as big), while the sizes of the stars do not systematically vary between the two media.

This means that star polymers and linear polymers in water are, according to the results, not fully extended nor fully coiled, as they have an intermediate size between the lower and upper theoretical size limits. Linear polymers in PBS, however, may be forming a folded coil, which would explain their lower size. This small size might be caused by the charged particles that are found in the medium, as the negative ions of the PBS matrix interact with the polymer structure (Nimesh et al., 2010). This interaction would decrease the repulsion between the positively charged amino groups of the polymer, allowing the chains to fold and thus decreasing its size. This effect of the medium is not so noticeable for the star copolymers probably due to the chains being less exposed to the media.

Z-potential results are shown in Table 6. The electrodes of the DTS1070 cells used were darkened after two or three uses. This is due to the thigh conductivity of the dispersant, PBS, and it might have affected the measurements (Nobmann, 2017). Results do not show any trend or relationship of the z-potential with the MM or composition nor any systematic variation after the formation of the complexes. The z-potential of the stars did increase a 42% compared to those of the linear polymers, which can be explained by the fact that several chains of the cationic polymer form the stars. It is difficult to compare results as not many studies regarding z-potential characterisation in PBS at 7.4 have been found, and this parameter is very dependent on pH (Samsonova et al., 2011),

(Zhang et al., 2012). Nevertheless, the values appear to be in the optimal range for delivery, as the complexes are slightly positively charged, which allows the interaction with the membrane and thus the uptake (Zhang et al., 2012).

4 Conclusion

For this project, five different star copolymers and their five linear diblock precursors were successfully synthesised through GTP. Copolymers were mainly composed of DMAEMA, a cationic and hydrophilic monomer, and had different contents of PEGMA, very hydrophilic and biocompatible. A full and systematic characterisation, analysing the effects of changes in composition and molecular mass (both together and individually) in the properties of the polymers has been carried out. The linear diblock precursor of the star copolymers was not completely removed through precipitation, although the purity of the polymers did increase after three precipitations. Cloud points displayed no changes caused by MM or composition of the linear chains, but an average decrease of 8 °C of the CPs was observed for the star copolymers compared to their linear counterparts. Effective pKa values were all around 7.0, and during the titrations, a higher PEGMA content appeared to be responsible for an increase of hydrophilicity, avoiding precipitation at high pH values. The sizes of the linear copolymers were greatly affected by the medium, as they were almost fully coiled in PBS while very extended in water. Size of the complexes are in the expected range and z-potential values of the complexes in PBS at pH 7.4 are close to +10 mV, which may facilitate the uptake of the complexes by the cell membrane.

Author contributions

O.K. contributed to the conceptualization, methodology development, data analysis, and manuscript preparation.

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Competing financial interests

The authors have no conflict of interest.

References

- Bader, A. G., Brown, D., Stoudemire, J., & Lammers, P. (2011). Developing therapeutic microRNAs for cancer. *Gene Therapy*, 18(12), 1121–1126. <https://doi.org/10.1038/gt.2011.45>
- Czech, B., & Hannon, G. J. (2011). Small RNA sorting: Matchmaking for Argonautes. *Nature Reviews Genetics*, 12(1), 19–31. <https://doi.org/10.1038/nrg2935>
- Friedländer, M. R., et al. (2014). Evidence for the biogenesis of more than 1,000 novel human microRNAs. *Genome Biology*, 15(4), R57. <https://doi.org/10.1186/gb-2014-15-4-r57>

- Georgiou, T. K., Vamvakaki, A. M., Patrickios, C. S., And, E. N. Y., & Phylactou, L. A. (2004). Nanoscopic cationic methacrylate star homopolymers: Synthesis by group transfer polymerization, characterization and evaluation as transfection reagents. *Biomacromolecules*, 5(6), 2037–2045. <https://doi.org/10.1021/bm049903e>
- Guo, S., & Huang, L. (2011). Nanoparticles escaping RES and endosome: Challenges for siRNA delivery for cancer therapy. *Journal of Nanomaterials*, 2011, 1–12. <https://doi.org/10.1155/2011/782593>
- Hayes, J., Peruzzi, P. P., & Lawler, S. (2014). MicroRNAs in cancer: Biomarkers, functions and therapy. *Trends in Molecular Medicine*, 20(8), 460–469. <https://doi.org/10.1016/j.molmed.2014.06.003>
- Iyer, A. K., Singh, S., Ganta, S., & Amiji, M. M. (2013). Role of integrated cancer nanomedicine in overcoming drug resistance. *Advanced Drug Delivery Reviews*, 65(13), 1784–1802. <https://doi.org/10.1016/j.addr.2013.05.003>
- Jain, A., & Jain, S. (2008). PEGylation: An approach for drug delivery. A review. *Critical Reviews in Therapeutic Drug Carrier Systems*, 25(5), 403–447. <https://doi.org/10.1615/CritRevTherDrugCarrSyst.v25.i5.30>
- Karamanos, N. K., & Neophytou, C. M. (2008). Novel polymers for delivery of nucleic acids. *Current Drug Delivery*, 5(1), 33–47. <https://doi.org/10.2174/156720108784772832>
- Kim, S. Y., & Lee, J. H. (2008). Biodegradable poly(ethylene glycol) and poly(β -amino ester) for delivery of nucleic acids. *Macromolecular Research*, 16(10), 918–926. <https://doi.org/10.1007/BF03259229>
- Kuo, J., & Liu, Y. (2011). pH-Responsive poly(β -amino ester) for delivery of siRNA. *Journal of the American Chemical Society*, 133(34), 13468–13471. <https://doi.org/10.1021/ja2056573>
- Lam, J. K. W., Chow, M. Y. T., Zhang, Y., & Leung, S. W. S. (2015). siRNA versus miRNA as therapeutics for gene silencing. *Molecular Therapy - Nucleic Acids*, 4. <https://doi.org/10.1038/mtna.2015.22>
- Lee, H. J., & Jeong, J. H. (2011). Development of a polymeric carrier for the simultaneous delivery of multiple siRNAs. *Molecular Therapy*, 19(9), 1554–1560. <https://doi.org/10.1038/mt.2011.141>
- Lee, J., et al. (2012). Biodegradable polymeric nanoparticles for siRNA delivery: A review. *Journal of Controlled Release*, 161(3), 429–438. <https://doi.org/10.1016/j.jconrel.2012.03.028>
- Lim, D. W., & Yeom, Y. I., & Park, T. G. (2000). Poly(DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. *Biomaterials*, 21(9), 901–910. [https://doi.org/10.1016/S0142-9612\(99\)00262-7](https://doi.org/10.1016/S0142-9612(99)00262-7)
- Mastrobattista, E., Hennink, W. E., & Schiffelers, R. M. (2007). Delivery of nucleic acids. *Pharmaceutical Research*, 24, 1561–1563.
- Mout, R., et al. (2013). An efficient method for the synthesis of surface-modified silica nanoparticles for gene delivery. *Biomaterials*, 34(26), 6363–6373. <https://doi.org/10.1016/j.biomaterials.2013.05.018>
- Namvar, A., Bolhassani, A., Khairkhan, N., & Motevalli, F. (2015). Physicochemical properties of polymers: An important system to overcome the cell barriers in gene transfection. *Biopolymers*, 103(7), 363–375. <https://doi.org/10.1002/bip.22560>
- Pafiti, K. S., Patrickios, C. S., Georgiou, T. K., Yamasaki, E. N., Mastroyiannopoulos, N. P., & Phylactou, L. A. (2012). Cationic star polymer siRNA transfectants interconnected with a piperazine-based cationic cross-linker. *European Polymer Journal*, 48(8), 1422–1430. <https://doi.org/10.1016/j.eurpolymj.2012.03.021>
- Peng, B., Chen, Y., & Leong, K. W. (2015). MicroRNA delivery for regenerative medicine. *Advanced Drug Delivery Reviews*, 88, 108–122. <https://doi.org/10.1016/j.addr.2015.05.001>
- Qian, X., et al. (2014). Star-branched amphiphilic PLA-b-PDMAEMA copolymers for co-delivery of miR-21 inhibitor and doxorubicin to treat glioma. *Biomaterials*, 35(7), 2322–2335. <https://doi.org/10.1016/j.biomaterials.2013.11.039>
- Robbins, P. D., & Ghivizzani, S. C. (1998). Viral vectors for gene therapy. *Pharmacology & Therapeutics*, 80(1), 35–47. [https://doi.org/10.1016/S0163-7258\(98\)00006-6](https://doi.org/10.1016/S0163-7258(98)00006-6)
- Rupaimoole, R., & Slack, F. J. (2017). MicroRNA therapeutics: Towards a new era for the management of cancer and other diseases. *Nature Publishing Group*, 16. <https://doi.org/10.1038/nrd.2016.183>
- Silva, A. C., Lopes, C. M., Sousa Lobo, J. M., & Amaral, M. H. (2015). Nucleic acids delivery systems: A challenge for pharmaceutical technologists. *Current Drug Metabolism*, 16(1), 3–16.
- Tan, Y. L., Shum, W. C., & Chan, J. W. (2013). N-acetylgalactosamine-conjugated polypeptide-based siRNA delivery systems for liver cancer therapy. *Biomaterials*, 34(19), 4623–4632. <https://doi.org/10.1016/j.biomaterials.2013.03.016>
- Thomas, C. E., Ehrhardt, A., & Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics*, 4, 346. <https://doi.org/10.1038/nrg1073>
- Uprichard, S. L. (2005). The therapeutic potential of RNA interference. *FEBS Letters*, 579(26), 5996–6007. <https://doi.org/10.1016/j.febslet.2005.09.057>
- van de Wetering, P., Cherng, J.-Y., Talsma, H., Crommelin, D. J. A., & Hennink, W. (1998). 2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. *Journal of Controlled Release*, 53(1–3), 145–153. [https://doi.org/10.1016/S0168-3659\(97\)00116-4](https://doi.org/10.1016/S0168-3659(97)00116-4)
- van de Wetering, P., Moret, E. E., Schuurmans-Nieuwenbroek, N. M. E., van Steenberghe, M. J., & Hennink, W. E. (1999). Structure–activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for nonviral gene delivery. *Biomacromolecules*, 1(4), 628–633. <https://doi.org/10.1021/bm9900059>
- Venkataraman, S., Ong, W. L., Ong, Z. Y., Joachim Loo, S. C., Rachel Ee, P. L., & Yang, Y. Y. (2011). The role of PEG architecture and molecular weight in the gene transfection performance of PEGylated poly(dimethylaminoethyl methacrylate) based cationic polymers. *Biomaterials*, 32(9), 2369–2378. <https://doi.org/10.1016/j.biomaterials.2010.12.016>
- Wang, C., & Li, X. (2015). Nanocarriers for delivery of siRNA in cancer therapy: Current challenges and future directions. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 11(8), 1787–1798. <https://doi.org/10.1016/j.nano.2015.05.008>
- Wang, C., & Liu, Y. (2014). Cationic polymeric nanoparticles as gene delivery vectors. *Materials Today*, 17(9), 484–488. <https://doi.org/10.1016/j.mattod.2014.07.004>
- Wang, C., & Zhang, X. (2007). Synthesis and characterization of amphiphilic graft copolymers based on poly(L-lactide) and poly(ethylene glycol). *Macromolecular Rapid Communications*, 28(10), 1040–1046. <https://doi.org/10.1002/marc.200700015>

- Ward, M. A., & Georgiou, T. K. (2010). Thermoresponsive terpolymers based on methacrylate monomers: Effect of architecture and composition. *Journal of Polymer Science Part A: Polymer Chemistry*, 48(22), 5092–5102. <https://doi.org/10.1002/pola.24293>
- Wibowo, D., & Sykes, D. M. (2012). Nucleic acid delivery using polymer-based nanoparticles. *Advances in Polymer Science*, 247, 1–40. https://doi.org/10.1007/12_2012_15
- Yang, C., Li, H., Goh, S. H., & Li, J. (2007). Cationic star polymers consisting of α -cyclodextrin core and oligoethylenimine arms as nonviral gene delivery vectors. *Biomaterials*, 28(21), 3245–3254. <https://doi.org/10.1016/j.biomaterials.2007.04.013>
- Yao, Q., et al. (2015). PEG–poly(β -amino ester) micelles for siRNA delivery to lung cancer. *Molecular Pharmaceutics*, 12(9), 3308–3318. <https://doi.org/10.1021/acs.molpharmaceut.5b00402>
- Zhang, Q., Zhang, Y., Wang, J., & Yu, S. (2012). Polymeric nanoparticles as a versatile delivery platform for drugs, proteins, and nucleic acids. *Drug Development and Industrial Pharmacy*, 38(3), 287–295. <https://doi.org/10.3109/03639045.2011.577528>
- Zhang, Y., et al. (2011). pH-sensitive poly(β -amino ester)-based nanoparticles for delivery of nucleic acids. *Biomaterials*, 32(29), 7380–7390. <https://doi.org/10.1016/j.biomaterials.2011.06.005>
- Zhang, Y., Wang, Z., & Gemeinhart, R. A. (2013). Progress in microRNA delivery. *Journal of Controlled Release*, 172(3), 962–974. <https://doi.org/10.1016/j.jconrel.2013.09.017>