UNIVERSITY OF SAO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRAO PRETO

Dodecylated and non-dodecylated poly(succinimide)-based polyplexes with pEGFP-N3 plasmid: polymer synthesis, plasmid transfection and GFP expression assessment

Poliplexos de derivados de poli(succinimida), com/sem grupamento dodecil, e pEGFP-N3: síntese polimérica, transfecção e medida de expressão de GFP

Marcelo Henrique Kravicz

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Thesis presented to the School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, in partial fulfillment of the requirements for the degree of Doctor of Science in the Pharmaceutical Sciences Graduate Program.

Area of Academic/Scientific Training: Pharmaceutics and Cosmetics.

Candidate: Marcelo Henrique Kravicz.

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The undersigned, appointed by the dean of the Pharmaceutical Science Graduate School, have examined the thesis entitled

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presented by **Marcelo Henrique Kravicz**,
a candidate for the degree of Doctor of Science

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Aos meus pais

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RESUMO

KRAVICZ, M. H. **Poliplexos de derivados de poli(succinimida), com/sem grupamento dodecil, e pEGFP-N3: síntese polimérica, transfecção e medida de expressão de GFP**. 2017. 58f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2017.

O uso de genes em terapias é um conceito originado em 1970 em consequência do crescimento exponencial de novas tecnologias para liberação de DNA, também pela capacidade de expressão de genes exógenos em células de mamíferos. Propomos, então, a síntese de polímeros catiônicos, em dois grupos, por meio da aminólise da poli(succinimida) (PSI): grupo $1A_n$, polímeros catiônicos com arcabouço poli (ácido aspártico) com cadeias laterais contendo aminas protonáveis; grupo $2A_n$, polímeros catiônicos anfifílicos, contenho o poli (ácido aspártico) como arcabouço, com aminas protonáveis e cadeias dodecilamina. Estudos de SEC mostraram que derivados dodecilados $2A_n$ tiveram tamanho menor que os polímeros do grupo $1A_n$, não dodecilados. A capacidade tamponante para todos os polímeros sintetizados foi maior que o bPEI 25, e o grupo $2A_n$ apresentou as maiores capacidades tamponantes. Derivados $2A_n$ com as aminas A1 a A4 apresentaram menor CMC do que o grupo $1A_n$. Citotoxicidade dos policátions foi dependente das suas concentrações e, entre todos os polímeros, aqueles com aminas A5 e A6 não foram citotóxicos. A presença da cadeia dodecilamina na PSI não diminuiu a viabilidade celular até 250 µL-1, sugerindo que a porção hidrofóbica não é citotóxica na faixa de concentrações testada. A complexação do pEGFP-N3 com os derivados de PSI foi realizada, bem como a transfecção dos poliplexos em células HeLa. A expressão de GFP dos complexos obtidos com bPEI 25 foi quantificada e comparada com os poliplexos preparados com os derivados da PSI. Ensaios de transfecção mostraram que os derivados dodecilados da PSI apresentaram expressão negligenciável de GFP em células HeLa, sugerindo uma ligação forte entre plasmídeo e os derivados sintetizados e não-liberação do material genético nas células, ou dano celular causado pela cadeia hidrofóbica nas células. Os maiores valores de GFP quantificados foram encontrados nos poliplexos contendo polímeros não-dodecilados e com as aminas A3 e A4, nas razoes N:P 5 a 20 para A3 e N:P 5 para A4. Ambas as estruturas A3 e A4 fazem parte do core do bPEI 25.

Palavras-chave: poli(succinimida), aminolysis, transfecção, sistemas de liberação de genes.

ABSTRACT

KRAVICZ, M. H. **Dodecylated and non-dodecylated poly(succinimide)-based polyplexes with pEGFP-N3 plasmid: polymer synthesis, plasmid transfection and GFP expression**. 2017. 58f. Thesis (Doctoral). School of Pharmaceutical Sciences of Ribeirao Preto – University of Sao Paulo, Ribeirao Preto, 2017.

Genes as drugs for human therapy is a concept originally conceived around 1970, a consequence of the exponential growth in knowledge of human gene function, the more effective technologies for DNA delivery, and the ability to transfer and express exogenous genes in mammalian cells. Here we propose synthesizing two small library groups of cationic polymers via aminolysis of poly(succinimide) (PSI) backbone: group $1A_n$, polycationic polymers with a degradable amide of poly(aspartic) acid backbone, protonable oligoamine side chains into the main polymer structure, and group $2A_n$, amphiphilic cationic polymers with a degradable amide of poly(aspartic) acid backbone, protonable oligoamine side chains into the main polymer structure and dodecyl side chain moieties. SEC showed that dodecylated derivatives $2A_n$ had lower size than $1A_n$ group, non-dodecylated polyelectrolytes. Buffering capacity of all synthesized polymers was higher than the standard bPEI 25, and the dodecylated $2A_n$ group had the highest buffering capacities values. $2A_n$ derivatives with amines A1 to A4 showed lower CMC than their non-dodecylated pairs. Cytotoxicity of all polycations was dependent on the concentrations, and among all polymers, those with amines A5 and A6 had lower cytotoxicity than bPEI 25. Moreover, the presence of the hydrophobic dodecyl side chain in the PSI backbone did not decrease the cell viability until 250 µg mL-1 polymer concentration, thus suggesting the hydrophobic moiety is not cytotoxic in this range. Complexation of pEGFP-N3 plasmid with PSI derivatives grafted with amines A1 to A4 was performed, as well as the transfection of polyplexes into HeLa cells. GFP expression of bPEI25 polyplexes in different complex volumes was quantified and compared with PSI derivatives/pEGFP-N3 polyplexes. Transfection assays showed that dodecylated PSI derivatives had negligible or no GFP expression in HeLa cells, thus suggesting a strong interaction between polycations and pDNA or a cellular damage caused by the hydrophobic moiety, although cytotoxicity assay of polyplexes showed low cytotoxicity of polyplexes. The highest GFP expression values were found for polycations 1A3 and 1A4, both without the dodecylamine side chain, in the N:P ratios 5 to 20 for 1A3, and N:P ratio 5 for 1A4. Both amines A3 and A4 used for the PSI grafting are core structures of bPEI 25.

Keywords: poly(succinimide), aminolysis, transfection, gene delivery systems.

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1. Introduction

1.1. General

European Medicines Agency (EMA) defines gene therapy medicinal products as biological products, which comprise an active substance that contains or consists of recombinant nucleic acid (DNA, RNA). Moreover, this product should have therapeutic, prophylactic or diagnostic effects related directly to the recombinant nucleic acid sequence or to the product obtained from the genetic expression (Wirth *et al.*, 2013).

Genes as drugs for human therapy is a concept originally conceived around 1970 (Couzin-Frankel, 2009; Giacca e Zacchigna, 2012), a consequence of the exponential growth in knowledge of human gene function, the more effective technologies for DNA delivery (Couzin-Frankel, 2009), and the ability to transfer and express exogenous genes in mammalian cells (Kay, 2011; Eltoukhy *et al.*, 2013). After the approval of the first gene therapy agent in 2012 by the EMA, such research field became more interesting (Rinkenauer *et al.*, 2015).

The strategy has already expanded from plasmid DNA (pDNA) to messenger RNA (mRNA), microRNA (miRNA) and small interfering RNA (siRNA) (Yin *et al.*, 2014; Zhang e Wagner, 2017), though different therapeutic pay loads require different tailor-made carriers, thus complicating preclinical development (Wagner, 2014). For example, the large size of pure pDNA (300 to 400 nm of hydrodynamic diameter) is one characteristic that differs the tailoring between it from the siRNA.

Besides the physical difference, the site of action in cells also changes from siRNA to pDNA. In case of siRNA, down regulation of proteins occurs in the cytoplasm, whereas pDNA delivery results in protein production, starting in the cell nuclei with pDNA transcription (Rinkenauer *et al.*, 2015). For an effective pDNA delivery, polycations are good candidates providing pDNA stability and functionality (Itaka e Kataoka, 2011).

1.2. Plasmid DNA (pDNA) and gene therapy

The main challenge for pDNA delivery is the transfer of the nucleic acid into the cell nuclei, thus resulting in protein production, starting from the cell nuclei after pDNA transcription (Rinkenauer *et al.*, 2015). The large size of pDNA and its short half-life in the presence of serum proteins make it necessary to pack this nucleic acid into vesicles or other particles (Rinkenauer *et al.*, 2015; Zhang e Wagner, 2017). Moreover, the easy plasmid unpacking from polyplexes is mandatory, so that DNA is accessible to transcription factors for gene expression (Singh *et al.*, 2015).

1.3. Vectors for gene delivery

Nucleic acids are far larger than conventional drugs and cannot diffuse across lipid membranes into target cells (Lächelt e Wagner, 2015). Since naked DNA can only be successfully delivered when injected into the target cells, transferring vectors have been produced and tailored to protect the nucleic acid and to deliver efficiently the payload directly to the target site (Ruß, 2008). These gene delivery devices can be divided in two groups (i) viral vectors and (ii) non-viral vector (Ruß, 2008).

Viral vectors for gene transfer, such as retroviruses, have raised scepticism about safety, presenting a possible threat to patients (Wirth *et al.*, 2013), though they are the most commonly used gene transfer systems used in clinical trials (Ruß, 2008). They remain by far the most popular approach, having been used in approximately two-thirds of the performed trials (Ginn *et al.*, 2013). However, limitations associated with viral vector regarding their safety, immunogenicity and low loading size have encouraged researchers to develop non-viral carriers (Ruß, 2008; Mintzer e Simanek, 2009) that include cationic lipids, peptide and cationic polymers (Mintzer e Simanek, 2009).

1.4. Polyethyleneimine (PEI) and other cationic polymers

Synthetic cationic polymers appear as new opportunity for better safety and large-scale manufacture, being efficient for spontaneous condensation of large nucleic acids as DNA into nanosized particles, functional polymeric systems called *polyplexes* (Felgner *et al.*, 1997). Therefore, nucleic acid is protected from enzymatic degradation via condensation into the polyplexes, also cell uptake and endolysossomal escape are facilitated (He *et al.*, 2010).

Cationic polymers can be considered perfect noncovalent interaction partners for nucleic acids, providing similar size dimensions but opposite ionic charge (Lächelt e Wagner, 2015). Frequently studied cationic polymers include poly(L-lysine) (PLL), chitosan, dendrimers and polyethyleneimine (PEI) (He *et al.*, 2010; Ulasov *et al.*, 2011).

PEI (Figure 1) is often considered a gold standard for gene transfection and is also the most prominent example of cationic polymers able to transfect (Mintzer e Simanek, 2009). This cationic polymer has been tailored to improve the physicochemical and biological properties of polyplexes, and has several transfection agents commercially available, since first PEI-mediated oligonucleotide transfer was conducted by Boussif *et al.* (1995). Commercially available transfection agents based in the PEI structure include agents ExGen500 and jetPEI, both linear derivatives from PEI (Mintzer e Simanek, 2009).

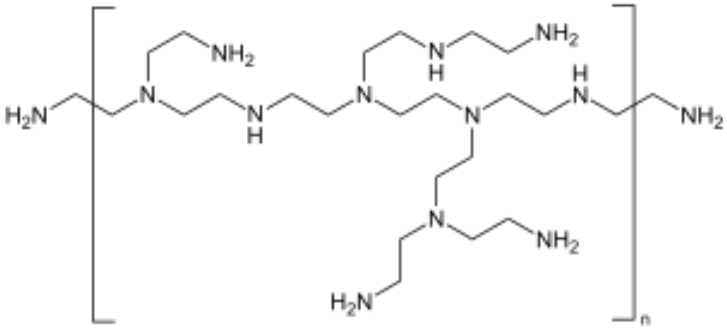

Figure 1 Branched polyethyleneimine 25 kDa (bPEI 25) chemical structure.

Although PEI has proven to be efficient and versatile, and clinical trials suggest PEI-based complexes have good safety profile, this polymer is not degradable (Neuberg e Kichler, 2014) and is significantly toxic in a molecular weight-dependent manner (He *et al.*, 2010; Zhang e Wagner, 2017).

1.5. Poly(succinimide) as biodegradable backbone

Poly(amino acids), polypeptides and their derivatives have been used in re-generative medicine, drug delivery and gene therapy due to their biocompatibility, biomimetic properties and available side groups for functionalization (Gu *et al.*, 2013; Chen *et al.*, 2015; Numata, 2015). Poly(β-alanine) and poly(ε-lysine), structural and

functional proteins, peptides and other polymers derived from amino acids are classified as poly (amino acid)s (Numata, 2015).

Unlike most of poly(amino acid)s prepared by ring-opening polymerization of N-carboxyanhydrides (NCAs), a biodegradable and water soluble poly(aspartic acid) (PASP) can be directly obtained from alkaline hydrolysis of poly(succinimide) (PSI) (Gu *et al.*, 2013), a poly(imide) obtained from thermal polycondensation of aspartic acid (ASP) (Tanimoto *et al.*, 2010; Shen *et al.*, 2013).

Aminolysis of PSI with nucleophilic amino compound is used to form various poly(asparamide)s with side groups like isopropylasparamides segments, thus obtaining a thermoreversible phase transition polymer (Tanimoto *et al.*, 2010), pseudo-branched PEI (Yu *et al.*, 2009), PEI-mimetic polymers (Shen *et al.*, 2013), reversible micelles (Liu *et al.*, 2014) and other biodegradable water-soluble polymeric material (Nakato *et al.*, 2000).

A direction to increase polymer and polyplex biocompatibility is a combination of advantages of the intermediate PSI biodegradable backbone and low molecular weight PEI or oligoamines, thus obtaining a high molecular weight cationic polymer with high transfection efficiency (Zhang *et al.*, 2009; Zhang e Wagner, 2017). The conversion of PSI backbone into poly(aspartamide) after aminolysis increases biocompatibility of cationic polymer and maintain or promote nucleic acid transfection efficiency (Mintzer e Simanek, 2009; Zhang e Wagner, 2017).

Therefore, a variety of cationic polymers with different backbones has been synthesized due to the flexibility in chemical synthesis and the feasibility to obtain virus-inspired polyplexes (He *et al.*, 2010; Lächelt e Wagner, 2015; Zhang e Wagner, 2017).

2. Rational of this work

Gene therapy is a treatment approach that introduces exogenous nucleic acids into specific cells, holding great promise for treatment of several diseases. This therapeutic has already expanded from plasmid DNA (pDNA) to messenger RNA (mRNA), microRNA (miRNA) and small interfering RNA (siRNA) (Yin *et al.*, 2014; Zhang e Wagner, 2017). As these nucleic acids need to be inside the cell for their effective mechanism of action, an appropriated delivery system must be provided.

For instance, our Research Group has already established liquid crystalline nanodispersed delivery systems for siRNA delivery into skin (Vicentini *et al.*, 2013), *in situ* gelling delivery systems as self-assembling systems for local drug delivery (Borgheti-Cardoso *et al.*, 2014; Borgheti-Cardoso *et al.*, 2015) and recently monoolein/oleic acid based hexagonal phase liquid crystal nanoparticles functionalized with membrane transduction peptides (Petrilli *et al.*, 2016). Until now, all studies and projects in our Research Group present the cationic polyethyleneimine (PEI) as an adjuvant for gene complexation and delivery in all formulations.

PEI is widely used as gene carrier due to its ability in promoting in vitro and in vivo transfection. However, it also has some limitation regarding its toxicity and transfection efficiency dependence on polymer structure, such as molecular weight and linear or branched structures (Yin *et al.*, 2014).

A range of PEI modifications for improved polymer stability, biocompatibility and reduced toxicity is reported in literature (Nguyen *et al.*, 2000; Thomas e Klibanov, 2002), also synthetic polymers based in the PEI structure synthesized from biodegradable polymers such as poly(succinimide) (PSI) (Shen *et al.*, 2016), a versatile polymer that can be easily synthesised and modified (Tanimoto *et al.*, 2010).

PEI-mimetic polymers and pseudo-branched PEI are examples of PSI modifications that mimic PEI structure, thus reducing polymer and polyplex cytotoxicity and increasing transfection efficiency (Yu *et al.*, 2009; Shen *et al.*, 2013; Uchida *et al.*, 2014). Moreover, biodegradable amphiphilic copolymers were synthesized by partial aminolysis of PSI with dodecylamine and propylamine for aggregates formation (Chen *et al.*, 2005), hydrophobic nanocrystals with various shape and size were obtained by

aminolysis of PSI with oleylamine (Huang *et al.*, 2013) and PSI- based endosome-escapable particles for diagnosis and therapy (Yang *et al.*, 2011).

Based on these findings, we propose synthesizing two small library groups of cationic polymers via aminolysis of poly(succinimide) (PSI) backbone

- Group $1A_n$, polycationic polymers with a degradable amide of poly(aspartic) acid backbone, protonable oligoamine side chains into the main polymer structure
- Group $2A_n$, amphiphilic cationic polymers with a degradable amide of poly(aspartic) acid backbone, protonable oligoamine side chains into the main polymer structure and dodecyl side chain moieties.

pDNA (pEGFP-N3, plasmid encoding enhanced green fluorescent protein) complexation and transfection into HeLa cells, enhanced green fluorescent protein (GFP-N3) expression and green fluorescent protein (GFP) quantification for transfection efficiency evaluation.

All cationic polymers are expected to complex and transfect pEGFP-N3 plasmid into HeLa cells. However, we hypothesize the presence of dodecylamine as a hydrophobic side chain in the PSI backbone would orient micelle formation, smaller polyplex sizes, increased pEFGP-N3/polymer chemical interactions and increased transfection efficiency measured by GFP quantification.

The use of pEGFP-N3 plasmid instead of siRNA as first trial for all PSI derivatives will also provide an understanding of structure-activity relationship between siRNA and synthesized polymers. Complex formation, transfection and final GFP expression show that the strategy using PSI derivatives would be a promising approach to address our overriding objective of carrier optimization for siRNA.

The findings of this study are providing us insights for the design of new gene delivery systems based in polyplexes formation, lipid and hybrid nanocarriers such as Solid Lipid Nanoparticles (SLN), self-assembling and *in situ* gelling systems based on liquid crystalline nanodispersions.

3. Purpose

The main subject of the present work is to obtain and to characterize cationic biodegradable polymers to be used as carriers for siRNA and other genes.

To achieve this subject, this study was divided in 3 chapters, as follows:

- Synthesis and characterization of poly(succinimide) derivatives
- HeLa cells transfection with poly(succinimide)-based derivatives: pEGFP-N3 complexation, transfection and GFP quantification
- Poly(succinimide)-based polycations and physicochemical characterization of pEGFP-N3 polyplexes

4. Chapters

4.1. Synthesis and Characterization of poly(succinimide) derivatives

4.1.1. Chemicals and Reagents

L-aspartic acid (ASP), dodecylamine (DDA), *N*-dimethyldipropylenetriamine (DMAPAPA), bis (3-aminopropyl) amine (norspermidine), triethylenetetramine (DEH), tetraethylenepentamine (TEPA), branched polyethyleneimine 25kDa (bPEI 25), 1-(3-aminopropyl)imidazole, 1-(2-aminoethyl)piperazine, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), cell culture media, antibiotics, fetal calf serum (FCS), orthophosphoric acid (H_3PO_4) 85 wt. % in water, deuterated solvents (d_6-DMSO, D_2O), *N*,*N*-dimethylformamide (DMF) and methanol were purchased from Sigma-Aldrich (Steinheim, Germany), unless otherwise indicated. Water was used as purified, deionized water.

4.1.2. Synthesis and Characterization of poly(succinimide) and its derivatives

Synthesis of poly(succinimide), PSI (1)

PSI was obtained according to previously described procedures with modifications (Matsubara *et al.*, 1997; Chen *et al.*, 2005). A thermal polycondensation of L-aspartic acid (ASP) (Figure 2) was performed by mixing ASP (10 g, 75.13 mmol)

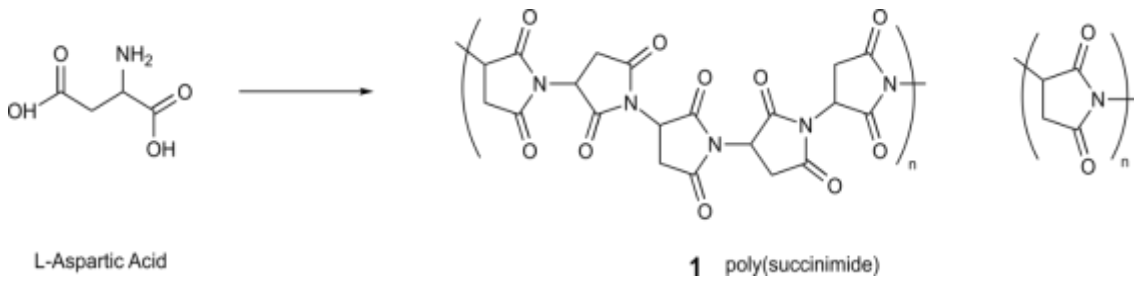

Figure 2 Synthesis of poly(succinimide) (PSI), 1, from thermal polycondensation of L-aspartic acid (ASP).

with 11.67 mol% of orthophosphoric acid 85 wt.% (H_3PO_4) (600 µL, 859 mg, 8.77 mmol) using a mortar and pestle.

The mixture was taken to an oven at 200 °C, under vacuum, for 3 h. The yellowish crude product was dissolved in *N*,*N*-dimethylformamide (DMF), precipitated in methanol, filtered, washed with distilled water, ethanol and dried under vacuum at 40 °C for 24 h.

Synthesis of poly(succinimide)-co-(N-dodecylamide aspartate), PSI-C_{12} (2)

PSI rings can react with alkylamines, alkanolamines and/or oligoamines in a process called aminolysis. Here, 10 mol% of dodecylamine (236 µL, 191 mg, 1.03 mmol) were added to PSI (1000 mg, 10.3 mmol) pre-dissolved in dry DMF (5 mL), at 50 °C, and the reaction kept for 5 h (Figure 3). Then, the reaction mixture was precipitated in distilled water, washed with ethanol and dried under vacuum at 40 °C for 24 h.

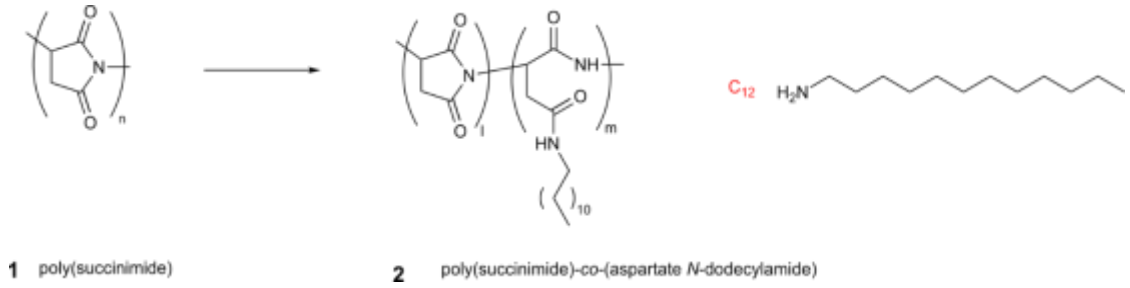

Figure 3 Synthesis of poly(succinimide)-co-(N-dodecylamide aspartate) (PSI-C12), 2, from aminolysis of PSI with dodecylamine (C12).

Synthesis of 1A_n and 2A_n polycations: aminolysis of PSI (1) and PSI-C_{12} (2) with selected amines

For the aminolysis of PSI (1) and PSI-C_{12} (2) (Figure 4), six amines (A1 to A6) (Figure 5) were selected. PSI was previously dissolved in DMF and slowly added with a syringe into the amine solutions previously dissolved in DMF. The reaction was kept for 1 h in ice bath, then dialyzed with dialyzing tubes MWCO 3kDa in water for 48 h and subsequently lyophilized.

4.1.3. Analytical and physicochemical characterization of PSI derivatives

^{1}H NMR

30 mg of PSI (**1**) and PSI-C_{12} (**2**) were dissolved in deuterated DMSO (d_6-DMSO). $1A_n$ an $2A_n$ polycations were dissolved in deuterated water (D_2O). ^1H NMR spectra were recorded using a Bruker AVANCE III 700 (700 MHz) device. Chemical shifts are reported in ppm and refer to the proper solvent as internal standard. Data is reported as follows: *s*, singlet, *d*: doublet, *t*: triplet; *m*: multiplet. Spectra were analyzed using MestreNova by MestreNovaLab Research.

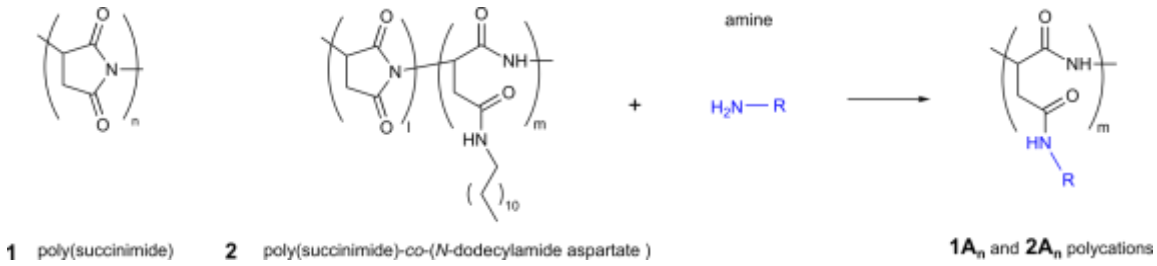

Figure 4 Synthesis of polycationic polymers. Two groups of polycations were obtained: $1A_n$ and $2A_n$, from PSI and PSI-C12 intermediates, respectively, after aminolysis of both polymers with amines.

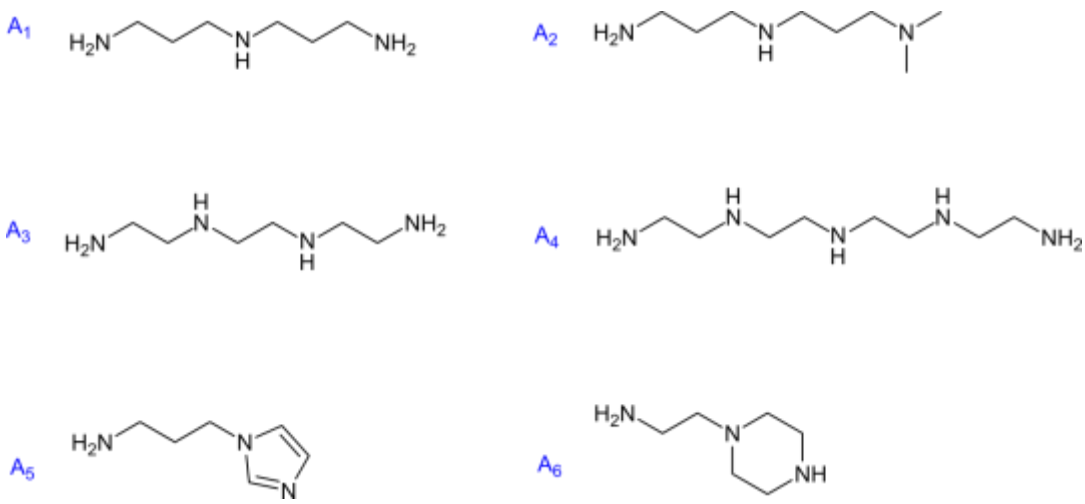

Figure 5 Structures of the ligands conjugated on PSI (1) and PSI-C12 (2). A1 to A6: A1, bis(3-aminopropyl) amine or norspermidine); A2, N,N-dimethyldipropylenetriamine; A3, triethylenetetramine; A4, tetra-ethylenepentamine, A5, 1-(3-Aminopropyl)imidazole and A6, 1-(2-aminoethyl)piperazine.

Size Exclusion Chromatography (SEC)

To determine the size of polymers, size exclusion chromatography (SEC) was performed. The parameters number average molecular weight (M_n), weight average molecular weight (M_w) and higher average molecular weight (M_z) of all polymers were obtained from an UltiMate 3000 LC System (Thermo Scientific) with photodiode array (PDA) - UV detector and Shodex RI - 101 detector (Showa Denko America, Inc.), with

Zenix SEC-100 (Sepax) column. Sodium acetate (NaAc, 0.3 M, pH 4.40) was used as eluent, and flow rate of 0.8 mL min^{-1} and 40 °C were set. The injection volume of the sample was 50 µL. Dextran standards for calibration: M_w 12 kDa, 50 kDa, 80 kDa, 150 kDa and 670 kDa.

Elemental analysis (EA), titration and buffering capacities of polycations

Elemental analysis of polymers was carried out by VARIO ELIII (Germany) elemental analyzer. For the quantification of protonable amine groups, a titration of all polycations was performed. For this, 10 mg of dry material were dissolved in 30mL 150 mM sodium chloride (NaCl) solution. The pH was adjusted to 12 with 100 mM sodium hydroxide (NaOH) and then the polymers were titrated with 100 mM chloridric acid (HCl) to constant pH 2 – 3.

Critical Micellar Concentration (CMC) and pyrene fluorescence

CMC determinations were performed on a Jasco FP-6500 with following settings: λ_{ex} 335 nm, band width 10 nm; λ_{em} 350 – 550 nm, band width 1 nm; data pitch 0.1 nm; data collection speed of 20 nm min^{-1}; 25 °C. A stock solution of pyrene was prepared using the following procedure: 20 µL of a pyrene solution in methanol (3.5 mM) were evaporated in a 100 mL Erlenmeyer flask; 100 mL of ultrapure water were added and the solution was sonicated for 1 h and afterwards filtered through a regenerated cellulose (RC) membrane (0.45 µM). A concentration series of each polymer was prepared using the pyrene stock solution. For each measurement 1.5 mL were filled into a Quartz cuvette. The I_3/I_1-ratio was calculated through the division of the amplitudes at I_3, 385 nm and I_1, 374 nm.

4.1.4. Cytotoxicity of bare cationic polymers

HeLa cells were obtained from American Type Culture Collection (ATCC) and were cultured in in RPMI medium supplemented 10% FCS, 1% antibiotics (penicillin and streptomycin) and 1% non-essential aminoacids (NEAA). Cells were cultured in an incubator at 37 °C and 5% CO_2.

Relative viability of HeLa cells for all bare cationic polymer solutions was obtained after 24 h with MTT method. Briefly, cells were platted in 96-well plate at 1 × 10^4 cells/well, in 200 µL appropriate medium containing 10% FBS. After 24 h, cells

were treated with a serial dilution of polymers for 24 h. Then, medium was replaced with 200 µL of new medium containing 0.5 mg mL^{-1} MTT and cells were incubated for 3 h. Wells were replaced with 100 µL DMSO and the absorbance measured in an Enzyme-Linked Immunosorbent Assay (ELISA) plate reader at 570 nm. The cell growth was calculated according to the equation $[A]_{test}/[A]_{control} \times 100$, in which A is absorbance.

4.1.5.Results and Discussion

Synthesis and Characterization of poly(succinimide) and its derivatives

Poly(succinimide) (PSI) is a poly-imide, a precursor for poly (aspartic acid), a polyamino acid as well as the biodegradable and biocompatible poly(γ-glutamic) acid and poly(ε-lysin). Here, the synthesis strategy of PSI backbone is a thermal polycondensation of aspartic acid (ASP) in the presence of an acidic catalyst.

PSI with different degrees of polymerization can be obtained by changing parameters as time for reaction and presence or not of a catalyst and solvents. In this case, a bulk thermal polycondensation of ASP in the presence of orthophosphoric acid (H_3PO_4) as a catalyst was performed, according to some previous studies (Huang *et al.*, 2009); the authors obtained a PSI with M_n 18,000 and 44,100 g mol^{-1}, with 1.28 and 1.33 of polydispersion index (PDI), respectively. In another study, the obtained polymer had M_w 6,700 g mol^{-1} and no PDI value was showed (Chen *et al.*, 2005). The obtained PSI in this study had M_w 17,100, M_n 11,200 and PDI 1.5, providing a backbone for the following process of aminolysis. However, the parameters for size determination were different from those selected for the size determination of polycations in this study[1].

The aminolysis process of PSI with nucleophilic amino compounds provides a grafting of different moieties in the polymer backbone. This process is used to form various poly(asparamide)s with functional groups in the side chains and increases the hydrophilicity of the polymer by producing two hydrophilic amide bonds after ring opening of the succinimide unit (Tanimoto *et al.*, 2010).

[1] Size parameters for PSI were determined in *N*-Methyl-2-pyrrolidone (NMP) system with lithium bromide (LiBr) and another equipment and column.

In this study, a dodecyl side chain was introduced in the PSI backbone in a feed ratio of 10 mol% and a copolymer poly(succinimide)-*co*-(dodecylamide aspartate) (PSI-C_{12}) was obtained. An aminolysis process with this amine provided injectable hydrogels and *in situ* delivery systems (Takeuchi *et al.*, 2011) and statistical copolymers aggregation (Chen *et al.*, 2005). Then, both PSI and PSI-C_{12} were used in another aminolysis process with six nucleophilic amino compounds (here named as A_n amines) to obtain polycationic polymers for the genetic material complexation, polyplex formation and transfection of HeLa cells with pEFGP-N3 plasmid.

As previously studied, a combination of two amines in the PSI backbone led to polycationic polymers with different molar degrees of substitution and plasmid pEGFP transfection efficiency (Shen *et al.*, 2013), providing polymers with different molar ratios of primary, secondary and tertiary amines, an important parameter for high transfection (Von Harpe *et al.*, 2000). Here, polycationic polymers obtained from the aminolysis of both PSI (**1**) and PSI-C_{12} (**2**) with amines A_1 to A_6 were synthesized, providing two distinct groups: polycationic polymers without a hydrophobic side chain (**1A$_n$** polymers) and those with the dodecyl moiety (**2A$_n$** polymers).

Analytical and physicochemical characterization of PSI derivatives

In this section we present the results of analytical and physicochemical characterization of PSI derivatives: structural characterization by ^{1}H NMR spectra analysis, size determination of all polymers by SEC, quantification of nitrogen and protonable amines by Elemental Analysis and Titration, as well as buffering capacity for all polycationic polymers.

^{1}H NMR

^{1}H NMR spectra of all synthesized PSI and its derivatives are shown in the *Appendix A ^{1}H NMR spectra of all compounds*, and the assignments of all polymers are summarized in Table 1.

PSI synthesis is confirmed with three characteristic peaks in 3.2 and 2.69 ppm (methylene) and 5.25 ppm (methine), related to the succinimide ring (Chen *et al.*, 2005; Tanimoto *et al.*, 2010). End groups, irregular structures and other bioproducts from this thermal polycondensation are not identified and quantified in this study. Moreover, the reaction mechanism of L-aspartic acid to form succinimide units and the polymer itself

is not discussed. Nevertheless, the ring opening of succinimide units to form copolymers and polycations after aminolysis reaction with nucleophilic amino structures (dodecylamine and other amines) was studied.

Table 1 ^{1}H NMR chemical shifts of PSI (1), PSI-C12 (2) and polycationic polymer groups 1A$_n$ and 2An.

Nucleus	^{1}H NMR shift, ppm	Attribution	Nucleus	^{1}H NMR shift, ppm	Attribution
a	5.25	*t*, 1H	j	2.25 – 3.25	-
b	2.69 and 3.2	*s*, 2H	k	1.9	*s*, 2H
c	2.8	-	l	3.85	s, 2H
d	1.5	*s*, 2H	m	7.5	s, 2H
e	1.17	*s*, 18H	n	6.86 – 7.2	-
f	0.86	*s*, 3H	o	6.86 – 7.2	-
g	1.65	*s*, 4H	p	2.74 – 2.25	-
h	2.13	*s*, 6H	q	2.74 – 2.25	-
i	2.17 – 2.25	-			

The feed ratio for the partial aminolysis of PSI units with dodecylamine and PSI-C$_{12}$ (compound 2) was 10 mol%. The ring opening of PSI units would result in a new signal at 4.5 ppm assigned to the methine proton of N-dodecylamide unites, as observed in previous studies with total PSI aminolysis (Chen *et al.*, 2005). However, with a DS 10 mol% no changes in the ^{1}H NMR spectrum can be observed in the poly(succinimide)-*co*-(dodecylamide aspartate) (PSI-C$_{12}$) synthesis (Appendix A).

The PSI grafting with this alkylamine was obtained from the integrate area ratio of the signals at 0.89 and 5.25 ppm, assigned to the methyl of dodecylamine and the methine of succinimide unit, respectively. For this study, the actual feed ratio found was ~12 mol%.

Both PSI and its copolymer PSI-C$_{12}$ grafting with selected amines A$_n$ resulted in a total aminolysis and ring opening of PSI backbone, as can be observed with the chemical shift of methine protons signal at 5.25 ppm to a new signal at 4.5 ppm. As a partial or complete racemization is expected to occur during the PSI synthesis, grafted PSI shows the split NMR signals due to the stereoregularity differences (Matsubara *et al.*, 1998).

The disappearance of peak at 5.25 ppm and two chemical shifts values of methine protons at 4.5 were caused by a α- and β-ring opening (Matsubara *et al.*, 1998).

Table 2 α- and β-opening of succinimide rings of PSI and PSI-C12 in the polycation groups 1An and 2An after total aminolysis process.

	$1A_n$ group						$2A_n$ group					
	$1A_1$	**$1A_1$**	**$1A_3$**	**$1A_4$**	**$1A_5$**	**$1A_6$**	**$2A_1$**	**$2A_2$**	**$2A_3$**	**$2A_4$**	**$2A_5$**	**$2A_6$**
α	0.8	0.8	0.67	0.7	0.78	0.7	0.68	0.70	0.47	0.54	0.60	0.54
β	0.17	0.19	0.33	0.3	0.35	0.24	0.28	0.28	0.50	0.42	0.35	0.43
α:β	4.7:1	4:2:1	2:1	2.3:1	2.2:1	2.9:1	2.4:1	2.5:1	0.94:1	1.28:1	1.71:1	1.25:1

High grafting ratios were successfully achieved with aminolysis of PSI and PSI-C_{12} and the ring opening α:β ratio was calculated from the integral area ratios of methine peaks at 4.65 (α-CH) to 4.48 ppm (β-CH) (Yu *et al.*, 2009). Table 2 shows the different ratios for all the PSI derivatives and the α-opening predominance, as observed in previous studies with PSI grafting with bPEI (Yu *et al.*, 2009). Mole ratios were calculated by integrating the peaks 4.65 and 4.48 using the equations in the *Appendix B Equations*.

Table 3 presents data related to the total ring opening after aminolysis of PSI with dodecylamine. As PSI had already some rings opened in the polycondensation synthesis (about 10%), the actual values for DS mol% of PSI-C_{12} in the $2A_n$ group was calculated 11.67 (SD 2.73).

Table 3 Feed ratio (Fr) mol% of dodecylamine (C12) and cationic moieties in $2A_n$ PSI derivatives. 1DSC12, mol% calculated from obtained feed ratio after total aminolysis of PSI-C12. Value based on 1H NMR analysis. 2Feed ratio of dodecylamine (theoretical). 3Actual feed ratio of dodecylamine. Value based on 1H NMR analysis. 4Actual feed ratio of grafted amines An in $2A_n$ polycations. Values based on 1H NMR analysis.

Polycation	**$2A_1$**	**$2A_2$**	**$2A_3$**	**$2A_4$**	**$2A_5$**	**$2A_6$**
F_r, mol% x (C_{12}): y (A_n)	0.22:1	0.24:1	0.33:1	0.29:1	0.34:1	0.29:1
DS_{C12} mol%[1]	18	19	24	22	25	22
PSI[2] (opened rings), mol%	10					
DS_{C12}[3] mol%	8	9	14	12	15	12
DS_A[4] mol%	82	81	76	88	75	88

Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) analysis was used to determine the molecular weights of the polymers. As polymers consist of repeating units called monomers, chemically bonded into chains, the latter are expressed in terms of molecular weight of the chain, relative molecular mass of monomers and the number of connected units in the chain. Moreover, synthetic polymers contain chains of unequal length, and the molecular weight is not a single value.

Table 4 SEC Determined by GPC. PDI = weight-average molecular weight/number-average molecular weight, or Mw/Mn

$1A_n$	M_w	M_n	M_z	PDI	$2A_n$	M_w	M_n	M_z	PDI
$1A_1$	16.1	7.4	46.4	2.2	$2A_1$	8.6	2.7	19.8	3
$1A_2$	19.6	9.7	34.5	2	$2A_2$	5.4	2.2	10.8	2.4
$1A_3$	17.3	7.2	35.6	2.4	$2A_3$	5.5	2.2	12.3	2.5
$1A_4$	25.7	9.4	57.5	2.7	$2A_4$	8.1	1.9	11.4	4.3
$1A_5$	18.1	9.3	31.4	2	$2A_5$	4.7	1.3	14.7	3.6
$1A_6$	25.7	11.4	50.9	2.2	$2A_6$	9.7	2.8	23.3	3.5

Here, polycations of group $1A_n$ have higher Mw values than $2A_n$ (Table 4). As SEC analysis was performed according to a previous study (Shen *et al.*, 2013), in which authors synthesized polycations without a hydrophobic side chain, different solubilities between both groups could explain this behaviour. Moreover, all PSI derivatives without dodecylamine side chain (polycations group $1A_n$), PDI values did not vary according to the selected amine used for the PSI grafting. The same behaviour was not found for the $2A_n$ group, in which a distribution of PDI values was found, from 2 to 4. Moreover, the PDI values increased with the presence of the alkyl chain.

Elemental analysis (EA), titration and buffering capacities of polycations

Determination of nitrogen content of polymers is necessary due to the subsequent complexation step using pEGFP-N3 plasmid. In that step, plasmid is complexed with synthesized polymer according to a ratio, with numerous ways for describing complexes composition: DNA/cationic lipid (or cationic polymer), cationic lipid (or cationic polymer)/DNA, all them expressed either as wt:wt, mol:wt, wt:mol, or charge:charge (here called N:P ratio, nitrogen positive charges equivalent and phosphate negative charges) (Felgner *et al.*, 1997).

Elemental analysis (EA) was performed for PSI (1), PSI-C_{12} (2) and their derivatives ($1A_n$ and $2A_n$) to determine the nitrogen composition for further complexation studies, in which the number of amines is necessary. A complete EA determination, also for carbon and hydrogen quantification, is detailed in the *Appendix C Elemental Analysis of all compounds*.

In the case in which positively charged moieties were derived from titratable groups within the useful pH range, and it is difficult to access the exact amount of positive charges contributed by cationic moiety, the maximum possible charge would be used (Felgner *et al.*, 1997). From this information, the nitrogen amount used for

complexation and transfection of pEFGP-N3 was established as the one obtained from titration.

Buffering capacity profile of all PSI derivatives was also obtained by titration of polymers solution with HCl 100 mM (Figure 6), and bPEI was used as control. All polymers had a buffering capacity and no abrupt pH changes occurred with HCl addition and protonation of amines. Curves were PEI-like, especially for $2A_n$ group, in which dodecyl side chain is grafted.

Table 5 shows buffering capacities (β) values of all polycations and the percentage β of amine groups that become protonated from pH 7.4 to 5.1 can be calculated from the equation buffering capacity (β, %)

$$\beta = \frac{\Delta(HCl) \times C(HCl)}{N} \times 100$$

in which ∆(HCl) is the volume of acid with concentration *C* used for the pH change in that range (Ou *et al.*, 2008).

According to the proton sponge hypothesis, having a high buffering capacity enables polycations to increase gene transfection efficiency by facilitating endosomal escape, releasing the protected DNA after endocytosis, helping polyplexes to escape from endosomes/lysosomes (Ou *et al.*, 2008).

Dodecylated $2A_n$ group had the highest values, except $2A_3$. However, PSI derivatives $1A_n$ without the dodecylamine chain had higher values than bPEI 25. Here, PEI had about 24 % protonated amines, the same value previously observed (Lin *et al.*, 2007).

A high amount of HCl was required to change the pH of a solution indicates this polymer has strong buffering capacity (Singh *et al.*, 2015). A lower extent of protonation is related to lower buffering capacity, lower amount of protonable amines in the polymer and no abrupt titration curve observed.

Previous studies related endosomal escape of polyplexes to the buffering effect associated to the amino groups in the constituent polycations and proton sponge hypothesis (Uchida *et al.*, 2011). Moreover, it is documented that bPEI induces endosomal escape because of its buffering capacity between pH inside the endosomal and physiological (Singh *et al.*, 2015).

Pumping of protons into the endosome, along with chloride ions influx to maintain charge neutrality, increases ionic strength inside the endosome, osmotic swelling and physical rupture of the endosome, resulting in the escape of the vector from degradation in the lysosomal pathway (Akinc *et al.*, 2005).

Table 5 Buffering Capacity (β, %) of PSI polycations and bPEI 25 in the pH range 7.4 to 5.1

	A_1	**A_2**	**A_3**	**A_4**	**A_5**	**A_6**	bPEI 25
$1A_n$	39,30	32,92	66,96	40,20	36,31	13,64	23,85
$2A_n$	41,28	49,12	49,96	60,43	60,26	40,62	

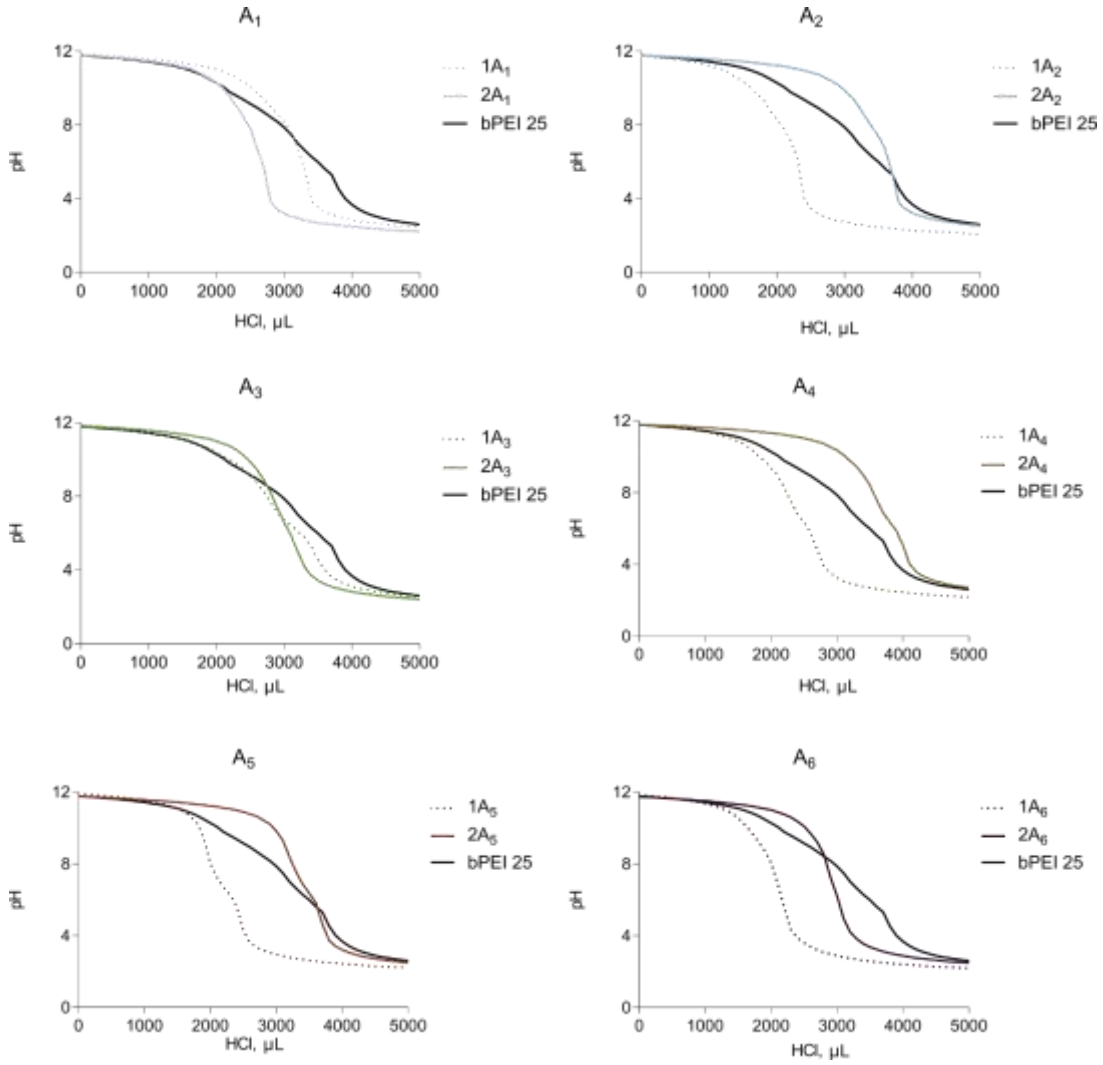

Figure 6 Acid-base titration profiles of PSI derivatives. $1A_n$ and $2A_n$ groups, in 150 mM NaCl solution. The difference between the groups is the presence of a dodecylamine side chain in (DS 10 mol%) the PSI backbone in polycations of $2A_n$ group. bPEI was used as a control.

According to the proton sponge hypothesis, such buffering effect increases osmotic pressure inside endosomes, disrupting the endosomal membrane, thus releasing the nucleic acid into the cytoplasm (Behr, 1997). Most of the modifications in

the bPEI structure reduces its gene delivery activity (Thomas e Klibanov, 2002), so it is recommended that the buffering capacity be balanced with all properties of polymers (Singh *et al.*, 2015).

Critical Micellar Concentration (CMC) and pyrene fluorescence

Kalyanasundaram e Thomas (1977) proved the dependence that fluorescence vibrational structure of pyrene could be used in the determination of CMC of surfactant solutions, the 1:3 ratio method. This method has been used in the determination of parameters in micellar and mixed-surfactant systems, and micellar properties of ionic and nonionic surfactants (Aguiar *et al.*, 2003). Here, the CMC of all polycations $1A_n$ and $2A_n$ was determined according to Dominguez *et al.* (1997) and Qiu e Bae (2007). For convenience, we describe the 1:3 ratio as I_3/I_1 ratio versus *log* of polymer concentration (Figure 7).

It is known that increasing the hydrophobicity of a polymer chain to a certain degree would facilitate micellization of amphiphilic polymers. Table 6 shows CMC values for all PSI derivatives and bPEI 25. Minimal hydrophobicity is indicated for bPEI 25, as previous observed (Sawant *et al.*, 2012).

Table 6 CMC (mg mL^{-1}) of PSI derivatives $1A_n$ and $2A_n$

$1A_1$	**$1A_1$**	**$1A_3$**	**$1A_4$**	**$1A_5$**	**$1A_6$**	**bPEI 25**
0.96	0.8	0.63	0.51	0.3	0.35	-
$2A_1$	**$2A_2$**	**$2A_3$**	**$2A_4$**	**$2A_5$**	**$2A_6$**	
0.5	0.27	0.23	0.12	0.2	0.3	

Plots of the pyrene 1:3 ratio as a function surfactant concentration (Figure 7) show a typical intersection of straight lines drawn through the experimental data, here the CMC; as surfactant concentration increases, pyrene 1:3 ratio decreases, thus indicating pyrene is sensing a more hydrophobic environment until the incorporation of such probe into the formed micelles (Kalyanasundaram e Thomas, 1977; Aguiar *et al.*, 2003). In the absence of micelles, that is, below CMC, pyrene senses the polar environment of water molecules (Dominguez *et al.*, 1997).

Non-dodecylated polycation $1A_5$ had no change in the CMC value when compared to its pair 2A5. As both cationic polymers contain amine A_5 (1-(3-aminopropyl) imidazole) in the PSI backbone, hydrophobic de-ionized interactions of unprotonated imidazole rings and micelle-like aggregation could be occurring, as observed in previous studies of Seo e Kim (2006) with imidazole-grafted-poly(aspartamide) with DS above 82%.

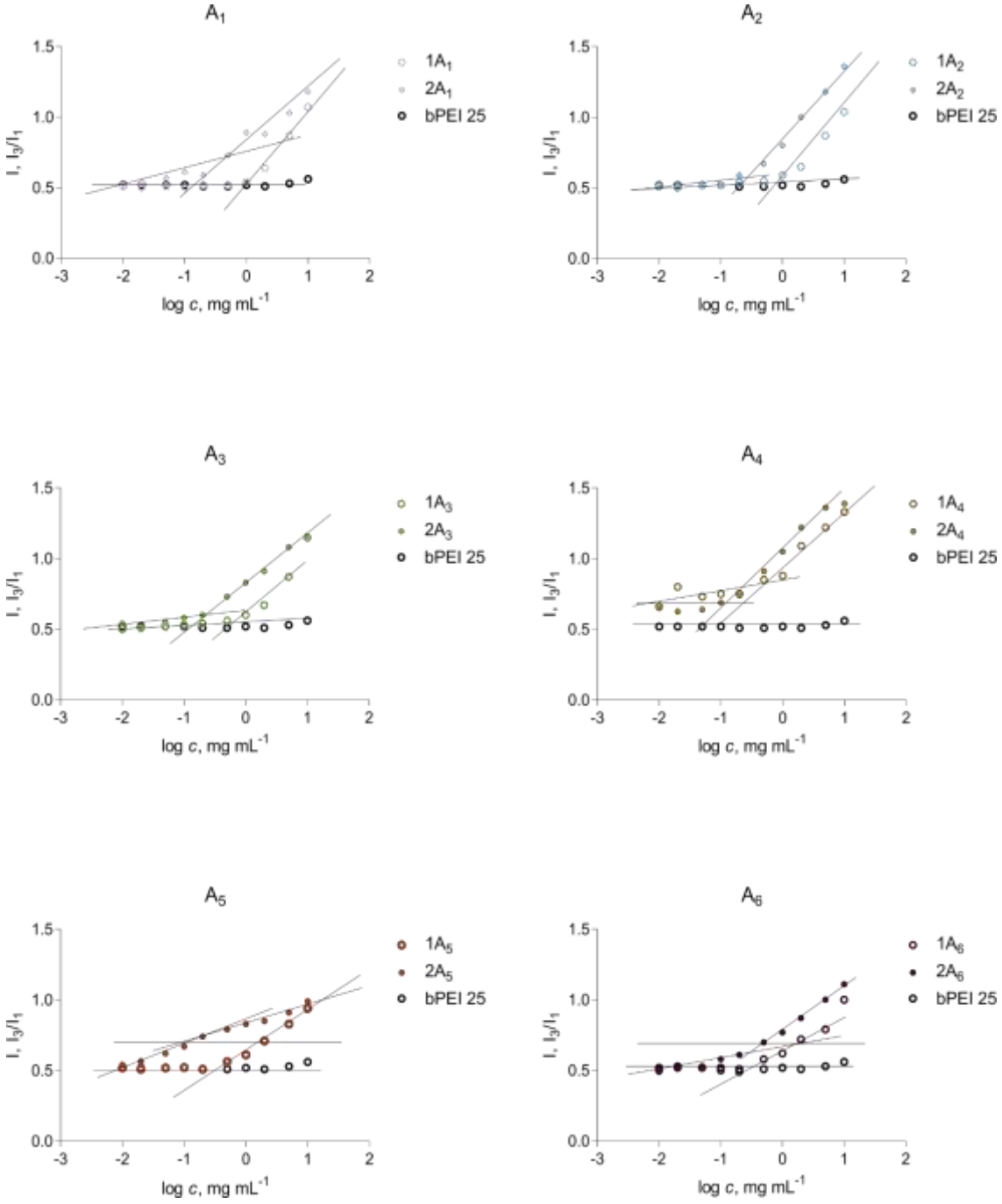

Figure 7 CMC of PSI derivatives

No changes in the CMC values were also observed in PSI derivatives $1A_6$ and $2A_6$, both PSI grafted with a piperazine side chain. This moiety is found in polymerizable piperazine cationic surfactants and is a component of pH-responsive nanoporous hydrogels (Roshan Deen *et al.*, 2004).

Polycations of $2A_n$ group had CMC values comparable to the polymers $1A_5$ and $2A_5$, thus indicating that these polymers have polymer-polymer interactions and can form micelle-like structures as polycations $1A_5$, $1A_6$ and $2A_6$.

Micellar formation occurs in lower polymer concentrations in the case of $2A_n$ group, as observed from the intersection point of two lines (Figure 7), one drawn though lowest polycation concentrations and another along the rising part of the plot. bPEI 25 was used as a control and its solution had a minimal hydrophobicity or could not be detected, as mentioned before (Roesler *et al.*, 2011; Sawant *et al.*, 2012).

Increasing hydrophobicity of PSI backbone with alkyl segments to a certain degree did facilitate micellization of amphiphilic polymers. Additional interactions between polyplexes and cell membranes is facilitated by hydrophobic modification including cholesterol, oleic acid, stearic acid (Sawant *et al.*, 2012), cyclododecyl and dodecyl (Liu *et al.*, 2014; Shen *et al.*, 2016).

Cytotoxicity of bare cationic polymers

A challenge for pDNA delivery is to combine high transfection, gene expression and negligible polycation cytotoxicity. A great concern still remains for the PEI-based polyplexes availability in the translational research due to PEI toxicity (Miyata *et al.*, 2008), although its polyplexes have high complexation and transfection efficiency both *in vitro* and *in vivo* (Zhang e Wagner, 2017).

In vitro cytotoxicity test (MTT assay) was performed in HeLa cells. Relative cell viability (%) was compared with control cells that were not treated with PSI derivatives groups $1A_n$ and $2A_n$, in concentration range from 0.025 to 250 μg mL^{-1} (Figure 8). The same analysis was carried out for bPEI 25. HeLa cell viability decreased about 50% with bPEI 25 treatment at 2.5 μg mL^{-1} polymer concentration.

Cytotoxicity of all polycations was dependent on the concentrations. Among all polymers, those with amines A_5 and A_6 had lower cytotoxicity than bPEI 25. A5 amine is an imidazole moiety in both $1A_5$ and $2A_5$ polymers, and biocompatible polymers were

already obtained with this grafting process (Seo e Kim, 2006). Here, the presence of the hydrophobic dodecyl side chain in the PSI backbone did not decrease the cell viability till 250 µg mL^{-1} polymer concentration, thus suggesting the hydrophobic moiety is not toxic in the range of polymer concentrations. The same behaviour was found for groups $1A_6$ and $2A_6$ with amine A_6, a piperazine group from 0.025 to 25 µg mL^{-1}, range in which polycations showed more than 60% HeLa cell viability, and dodecyl side chain did not change the toxicity profile.

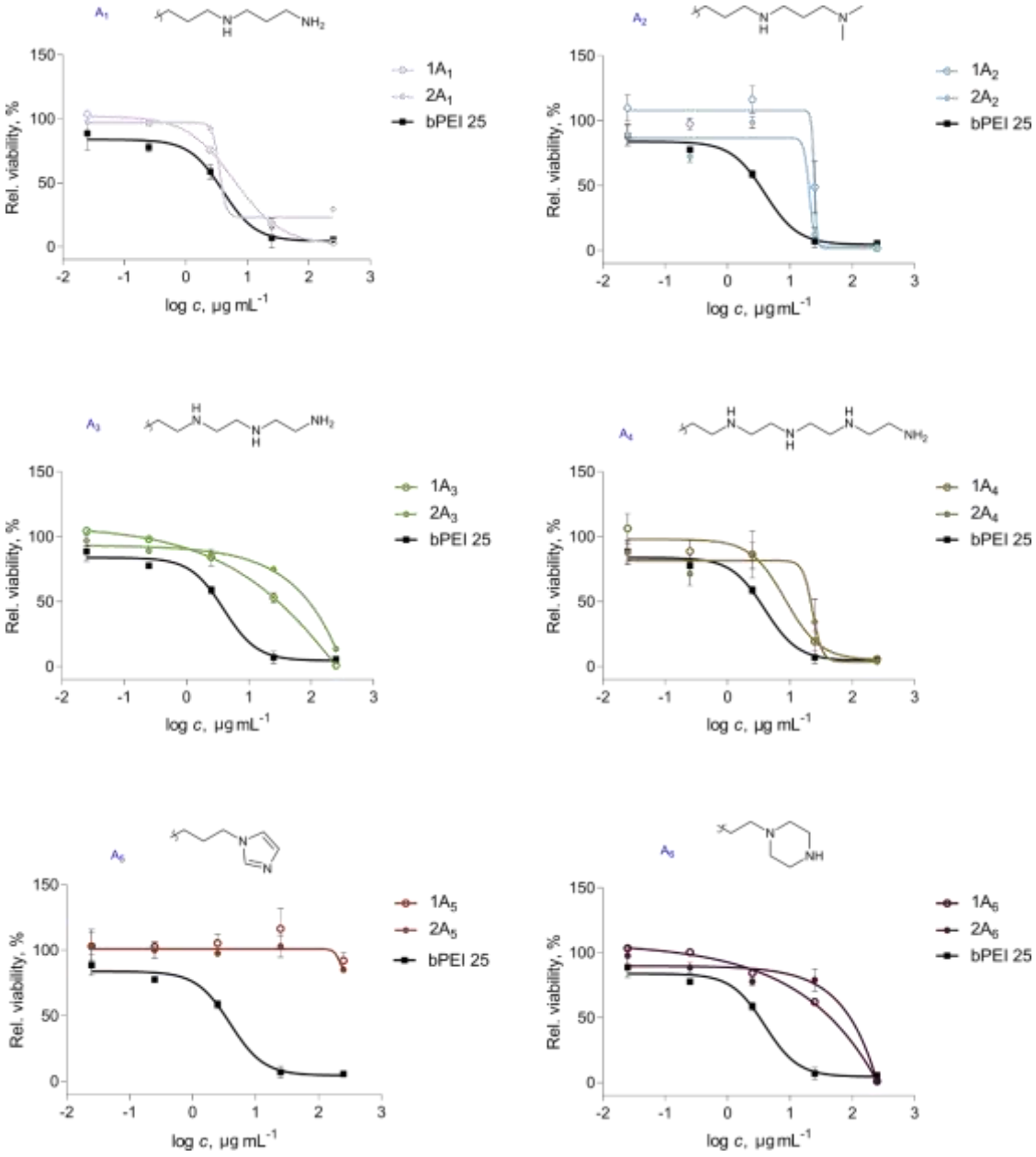

Figure 8 Cytotoxicity of bare polymers measured by MTT methods.

For polycations of A_3 and A_4 group, an increased toxicity was observed, clearly dependent on the increase of polymer concentration. Both amines are ethyleneimine-based structures, PEI components, though PSI derivatives with those amines had lower toxicity than bPEI in the concentration range from 0.025 to 25 μg mL^{-1}, and from 0.025 to 2.5 μg mL^{-1} for A_3 and A_4 grafted PSI, respectively.

4.2. HeLa cells transfection with poly(succinimide)-based derivatives: pEGFP-N3 complexation, transfection and GFP quantification

4.2.1. Chemicals and Reagents

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), 1-(3-aminopropyl)imidazole, 1-(2-aminoethyl)piperazine, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). Cell culture media, antibiotics, Fetal Calf Serum (FCS) Water was used as purified, deionized water.

4.2.2. Cell culture

HeLa cells were obtained from American Type Culture Collection (ATCC) and were cultured in in RPMI medium supplemented 10% FCS, 1% antibiotics (penicillin and streptomycin) and 1% non-essential aminoacids (NEAA). Cells were cultured in an incubator at 37 °C and 5% CO2.

4.2.3. Polyplex formation

Polyplex formation occurs due to electrostatic interactions between the positively charged amino groups of the polymers and the negatively charged phosphate groups of DNA. (Petrov et al., 2013).

Polyplexes were prepared in 350 µL final volume using fixed amount of pEGFP-N3 (1µg DNA, 3 nmol phosphate) and different N:P ratios (0.5, 1, 2, 5, 10 and 20). N:P value means the ratio between the number of amines of the cationic polymer and the phosphates of the plasmid. Briefly, 131.25 µL of a 0.133µg μL^{-1} pEGFP-N3 solution was prepared with HEPES buffer (20 mM, pH 7.4) in a 500 µL vial. Each N:P ratio had the same volume and pEGFP-N3 plasmid weight, 17,5 µg of pEGFP-N3 per vial.

4.2.4. *In vitro* transfection efficiency assay of pEGFP-N3/1A_n and pEGFP-N3/2A_n polyplexes

pEGFP-N3 plasmid encodes a red-shifted variant of wild-type green fluorescent protein (GFP) which has higher expression in mammalian cells. Here, HeLa cells were platted in 24-well plate at 5 × 10^4 cells/well, in 500 µL complete RPMI medium containing 10% FCS, 1% antibiotics and 1% non-essential aminoacids (NEAA). After 24 h, medium was changed to a fresh one without FCS, 500 µL final volume.

Then, cells were treated with 25, 50, 75 and 100 µL of previously prepared polyplexes, 500 µL final volume, for 6 h incubation (37 °C and 5% CO_2.). Medium was changed to a fresh complete one. After 48 h, cells were washed with PBS and detached with trypsin/EDTA solution. Detached cells were transferred to a flow cytometry tube, centrifuged for 5 min at 140g and resuspended in PBS. An Accuri C6 (Becton Dickinson, Heidelberg, Germany) and BD Accuri C6 Software were used for the data acquisition. GFP expression was measured and data was analysed by FlowJo® V10 flow cytometer analysis software. All experiments were performed in triplicates.

4.2.5. Cytotoxicity of polyplexes

Relative viability of HeLa cells for all polyplexes was obtained after 6 h with MTT method. Briefly, cells were platted in 24-well plate at 5 × 10^4 cells/well, in 500 µL appropriate medium containing 10% FBS. After 24 h, cells were treated with 25 to 100 µL of polyplexes N:P 0.5 to 20 for 6 h. Then, medium was replaced with 500 µL of new complete medium containing 0.5mg mL^{-1} MTT and cells were incubated for 4 hours. Wells were replaced with 200 µL DMSO and the absorbance measured in an ELISA plate reader at 570 nm. The cell growth was calculated according to the equation $[A]_{test}/[A]_{control} \times 100$, in which A is absorbance.

4.2.6. Results and Discussion

Transfection efficiency of cationic polymers varies among cell lines and is highly dependent on the conditions and environment in which complexes are formed. Factors requiring optimization include salt concentration, volume, incubation time, mixing order and ratio of polymer to DNA (Hsu e Uludag, 2012).

***In vitro* transfection efficiency assay of pEGFP-N3/1A$_n$ and pEGFP-N3/2A$_n$ polyplexes**

To optimize the pEGFP-N3 transfection and GFP expression in Hela cells, a series of pEGFP-N3/bPEI 25 polyplexes in different ratios and volumes was prepared (Figure 9). 25 µL of pEGFP-N3/bPEI 25 N:P 5 and 10 were considered optimal transfection parameters for HeLa cells transfection and GFP expression. Here, HeLa cells were treated with polyplexes in a serum-free medium for 6 h and then left for more 42 h for GFP expression and quantification

Transfection ability of polycation 1A$_1$ (Figure 10) was conducted and compared with bPEI 25. GFP expression was maximal with N:P ratio 5 and 10, as observed in bPEI 25 polyplexes. As the volume increases, GFP expression decreases, for all N:P ratios, and 25 µL is the optimal polyplex volume. GFP expression was not achieved with polyplexes prepared with polycation 2A$_1$.

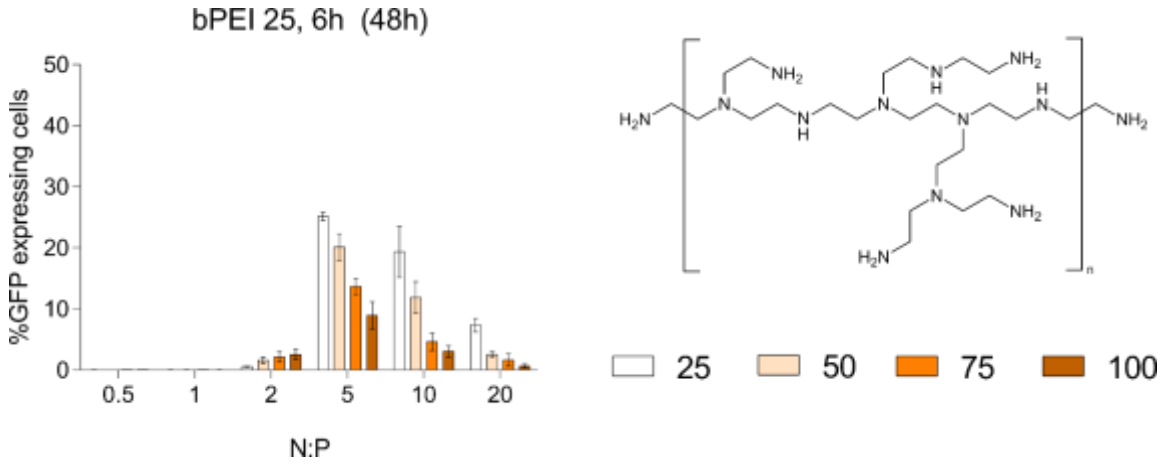

Figure 9 Screening of in vitro of pEGFP-N3/bPEI 25 polyplex transfection in HeLa cells and GFP expression quantification after 48 hours. Polyplexes in different N:P ratios and volumes (25, 50, 75 and 100 µL) were used. (n = 3)

As shown on (Figure 11), GFP expression for polycations 1A$_2$ and 2A$_2$ was lower than expression with using bPEI 25 polyplexes. However, the presence of dodecylamine side chain in the PSI backbone of polycation 2A$_2$ led to an increased GFP transfection, the opposite behaviour of A$_1$-grafted PSI polycation. The PSI derivative had a maximal GFP expression with N:P 5 ratio.

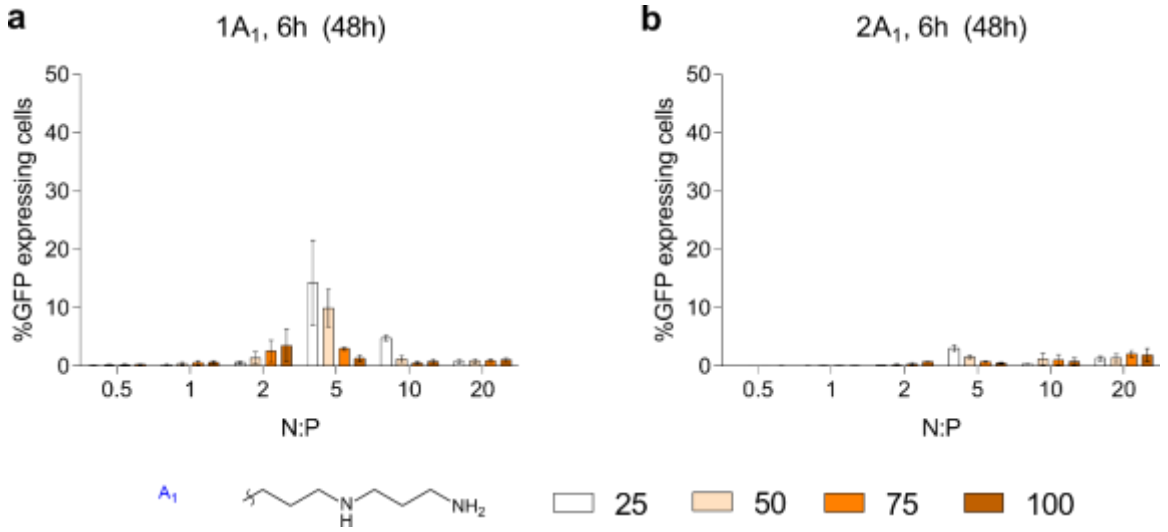

Figure 11 In vitro pEGFP-N3 transfection and GFP expression quantification after 48 h incubation in HeLa cells. PSI derivatives (a) 1A1 and (b) 2A1 were used as a vector for pEGFP-N3 plasmid transfection. Protein expression measured after 48 hours in a flow cytometer. Polyplexes in different N:P ratios and volumes (25, 50, 75 and 100 µL) were used. (n = 3)

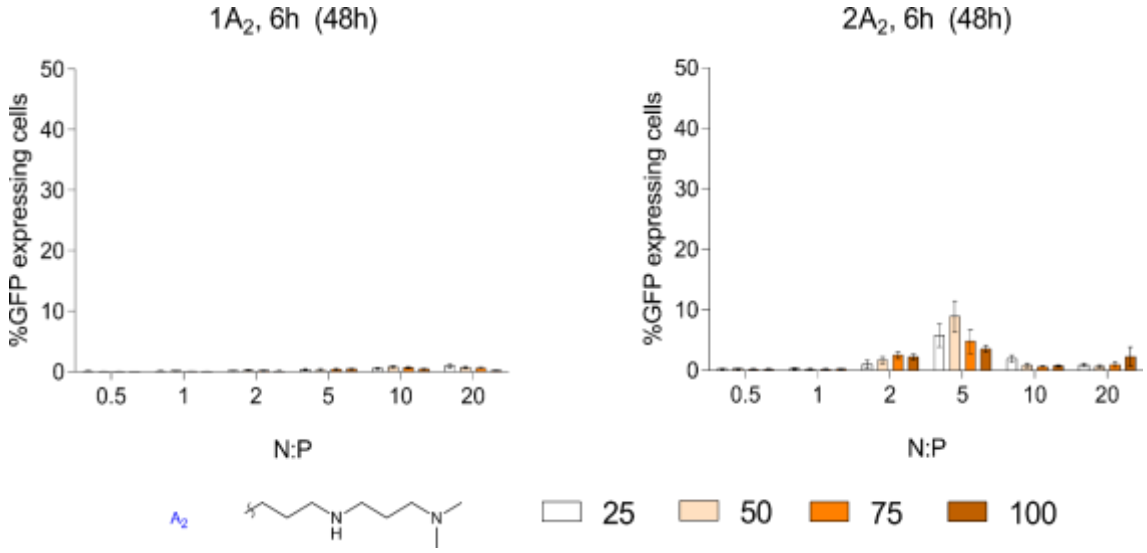

Figure 10 In vitro pEGFP-N3 transfection and GFP expression quantification after 48 h incubation in HeLa cells. PSI derivatives (a) 1A2 and (b) 2A2 were used as a vector for pEGFP-N3 plasmid transfection. Protein expression measured after 48 hours in a flow cytometer. Polyplexes in different N:P ratios and volumes (25, 50, 75 and 100 µL) were used. (n = 3)

Polycation $1A_1$ and $1A_2$, grafted PSI with different ratios of A_1 and A_2, were also obtained and used as vectors for pEGFP plasmid transfection in a study with murine melanoma cell lines B16-BL6 (Shen *et al.*, 2013). The authors found with a specific ratio of both amines an optimal transfection and GFP expression in this cell line. In that case, the presence of a tertiary amine (A_2) played a role in the GFP expression,

increasing plasmid transfection and GFP expression in a combined effect with amine A_1.

The highest GFP expression values were found for polycations $1A_3$ and $1A_4$, both without the dodecylamine side chain, for N:P ratios 5 to 20 for $1A_3$, and N:P ratio 5 for $1A_4$ (Figure 12). Both amines A_3 and A_4 used for the PSI grafting are core structures of BPEI 25. The only difference between both amines is the presence of one more ethyleneimine group in $1A_4$. Also inspired by natural polyamines including spermine and spermidine, Uchida *et al.* (2016) prepared cationic N-substituted polyaspartamides with varying aminoethylene units in the side chain of PSI.

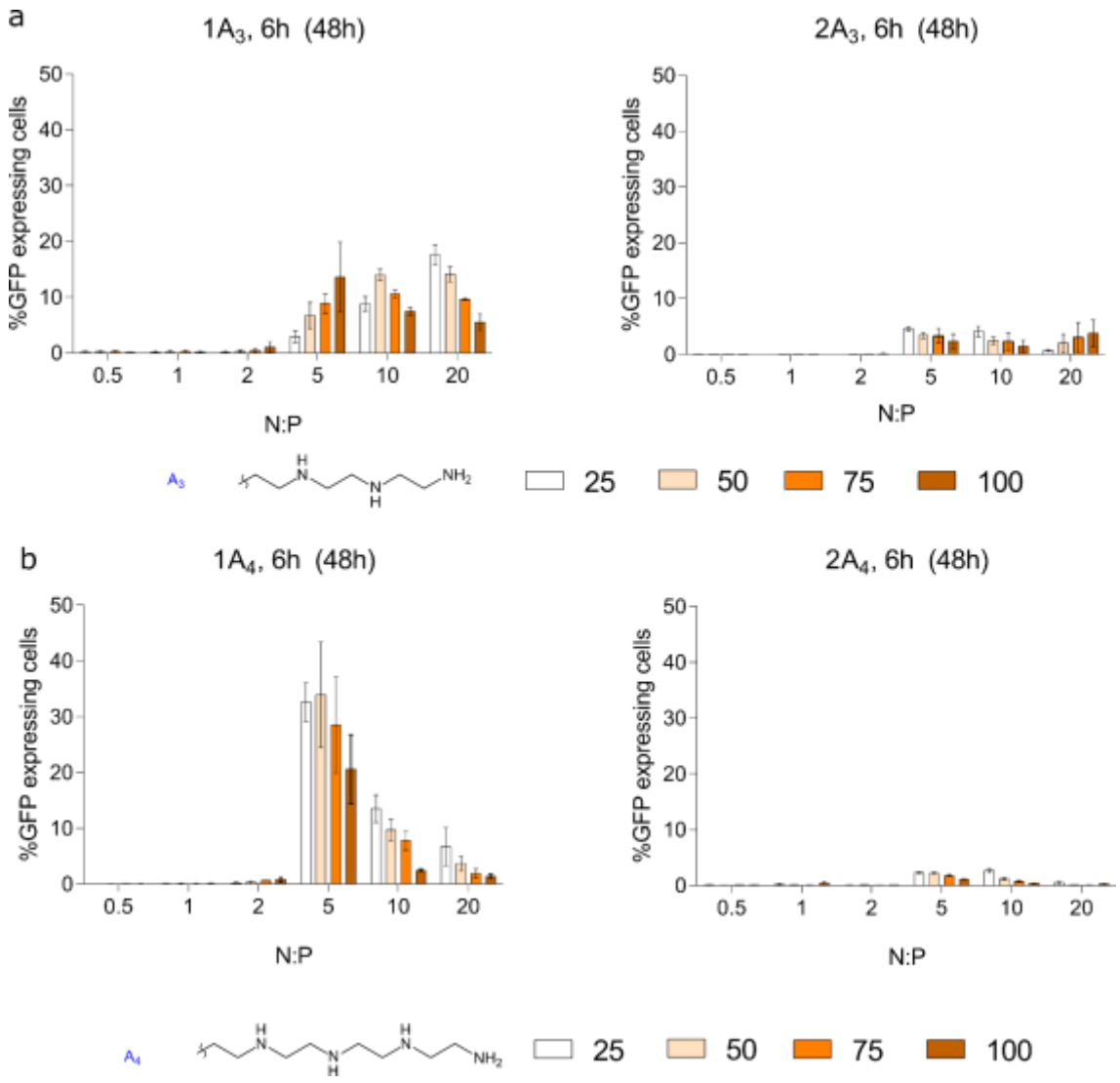

Figure 12 In vitro pEGFP-N3 transfection and GFP expression quantification after 48 h incubation in HeLa cells. PSI derivatives (a) 1A3 and 2A3, (b) 1A4 and 2A4 were used as a vectors for pEGFP-N3 plasmid transfection. Protein expression measured after 48 hours in a flow cytometer. Polyplexes in different N:P ratios and volumes (25, 50, 75 and 100 µL) were used. (n = 3).

Cell viability of polyplexes

In vitro pEGFP-N3 transfection and GFP expression assays results previously showed an optimal N:P ratio for transfection for all synthesized polycations. For all polymers, a N:P 5 was chosen for the cell viability of polyplexes assessment with MTT method, even for the groups $1A_3$ and $2A_3$, in which the optimal ratio was N:P 10 (Figure 13). Polyplexes volume range was from 25 to 100 µL, the same volumes used for the transfection assay.

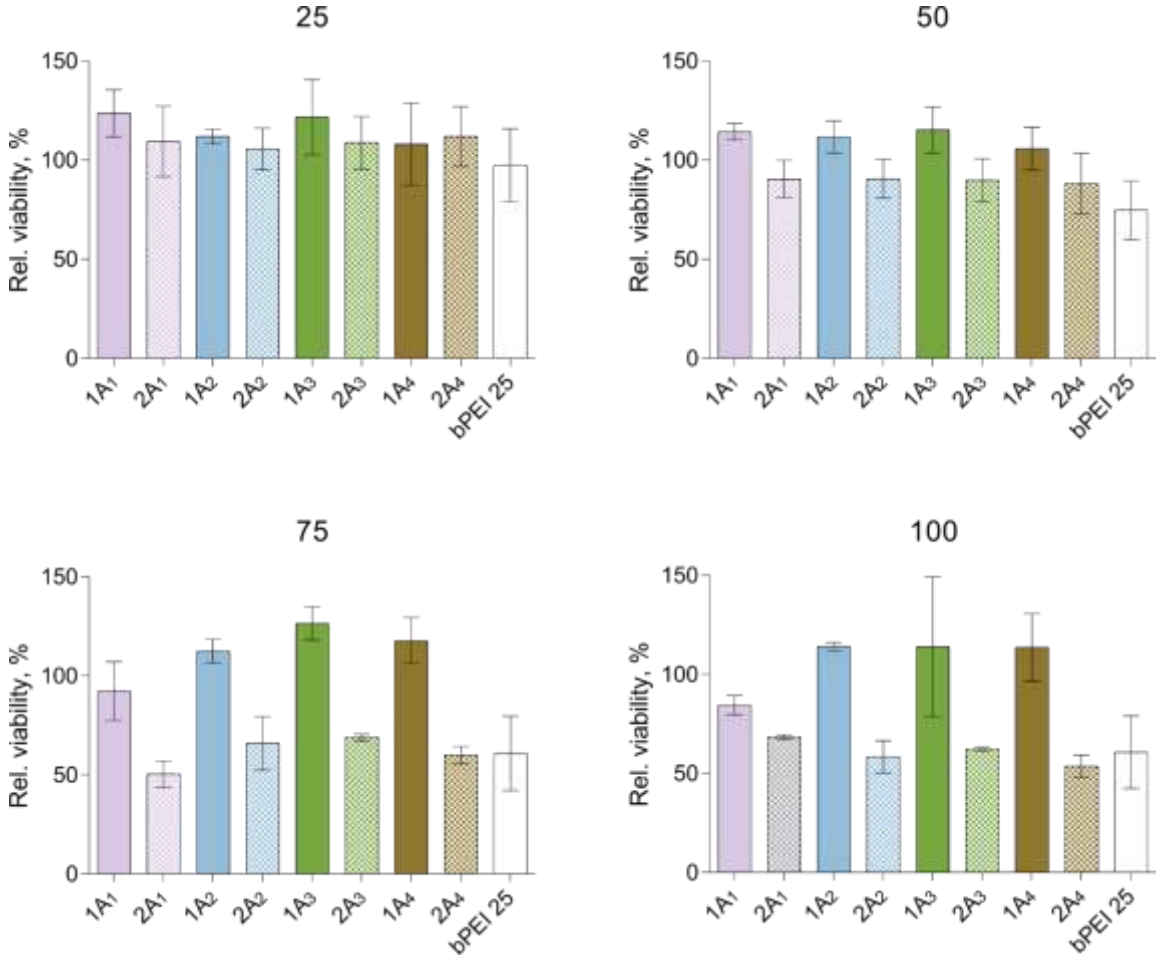

Figure 13 MTT assay and relative viability of HeLa cells after incubation with polycations 1A1, 1A2, 1A3 and 1A4, also their polycations with dodecylamine side chain 2A1, 2A2, 2A3 and 2A4. For this assay a N:P ratio 5 and volumes 25, 50, 75 and 100 µL were used. The time for incubation was the same used for the transfection, 6 h. Results are expressed as mean ± SEM, n = 3.

As expected, lower polyplexes volumes 25 and 50 µL provided no change in the cell viability in all polyplexes with synthesized polymers or bPEI 25. Cell toxicity is achieved for higher volumes, 75 and 100 µL of polyplexes and even GFP expression was lower for these volumes. The main hypothesis for this is the high concentration of polyplexes and the high number of free amines that can interact with cell membrane.

4.3. Poly(succinimide)-based polycations and physicochemical characterization of pEGFP-N3 polyplexes

This chapter shows the results regarding physicochemical properties of polyplexes pEFGP-N3/1A_n and pEFGP-N3/2A_n. Polyplex formation was performed (procedure previously described in Chapter 4.2) and here only cationic polymers grafted with oligoamines A_1 to A_4 were tested, due to their *in vitro* transfection profile. Polycations obtained from PSI grafting with A_5 and A_6, imidazole group and piperazine respectively, showed low cytotoxicity and a micelle-like behaviour (Chapter 4.1) although their polyplexes did have negligible GFP expression in HeLa cells.

When aqueous solutions of polycations PSI derivatives 1A_n and 2A_n and the plasmid DNA are mixed, polyelectrolyte complexes are formed and are usually insoluble in water, separating out as a new concentrated polymer phase, a complex coacervate (Gucht *et al.*, 2011).

Moreover, self-assembled amphiphilic macromolecules such as the dodecylated 2A_n PSI derivatives can form nanoscale micelles in appropriate liquid media and are useful as nanomicellar carriers prodrugs, drug polymer conjugates, new polymers with low critical micellar concentration and layer-by-layer assemblies (Trivedi e Kompella, 2010).

Here we propose a study of some physicochemical properties of these polyelectrolyte complexes by accessing pEGFP-N3 binding with polycations, and some polyplex parameters are obtained, such as particle size, residual charge and relative stability by pEGFP-N3 releasing in the presence of a competing polyanion, heparin (Shatsberg *et al.*, 2016).

4.3.1. Electrophoresis mobility shift assay – pEGFP-N3 binding

For the pEGFP-N3 gel shift assay, agarose gel was prepared by dissolving 1.2 g of agarose in 20 g of buffer, and heated up to 100 °C. After addition of 5 µL of Midori Green Advanced DNA stain for the detection of the nucleic acid, the agarose solution was poured into the electrophoresis chamber. 10 µL of previously prepared polyplexes

were mixed with 5 µL loading buffer and then the mixture added to the gel. Electrophoresis was performed at 120 V for 30 min.

4.3.2. Dynamic Light Scattering (DLS) measurements – Particle Size and Zeta Potential

350 µL of polyplexes were prepared and the size measurement was performed on a Malvern Zetasizer ZS in disposable micro UV-cuvettes at 25 °C. Measurements were carried out in the backscatter mode at 25 °C and using a Nd:YAG laser with 532 nm emission wavelength. Samples were prepared with HEPES buffer 20 mM pH 7.4. All samples were measured three times and the size distribution was plotted by intensity distribution. For zeta potential, 66uL of NaCl 150mM + 933ul HEPES, final 150 mM NaCl final concenctration.

4.3.3. Polyanion competition assay

Relative stability in the presence of heparin as competing agent was performed according to Shatsberg *et al.* (2016). Previously prepared polyplexes were incubated in the presence of increasing amount of heparin (0, 0.2, 0.4, 0.6, 0.8 and 1 IU per 50 µL final volume) and incubated for 1 h. Then, 10 µL of polyplexes + heparin were mixed with 5 µL loading buffer and mixture added to the gel. Electrophoresis was performed at 120 V for 30 min.

4.3.4. Results and Discussion

Electrophoresis mobility shift assay – pEGFP-N3 binding

Two groups of PSI derivatives named $1A_n$ and $2A_n$ containing the amines A_1 to A_4 were previously synthesized and complexed with pEGFP-N3 plasmid that encode a GFP expression. This binding process of pEGFP-N3 with a polycation is described as a coacervate, a complex of polyelectrolytes (Gucht *et al.*, 2011). Here, all synthesized polycations containing amines A_1 to A_4 complexed the selected plasmid DNA from N:P ratios 5 to 20, as observed in the Figure 14.

Polycations grafted with amine A1 complexed the plasmid DNA at N:P 5, for both $1A_1$ and $2A_1$ groups. The presence of the alkyl chain did not change the minimal N:P ratio for complexation (Figure 14).

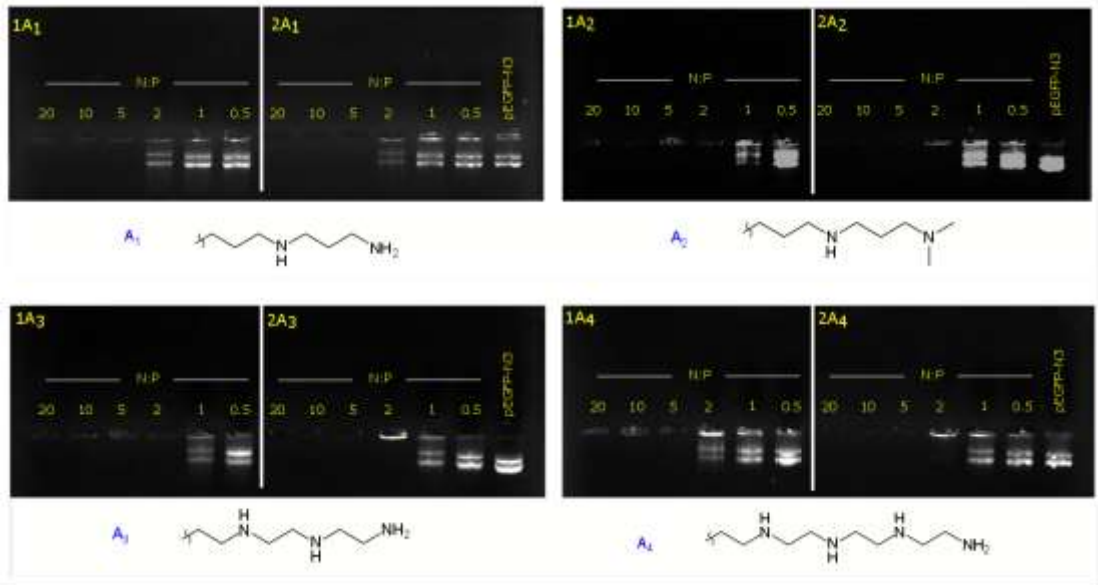

Figure 14 Electrophoresis mobility shift assay of PSI derivatives complexed with pEGFP-N3. Migration of pEGFP-N3 polyplexes at different N:P ratios.

Polycations with amines A_2 differ from those grafted with A_1 on two methyl end groups in the A_2 moiety. $1A_2$ and $2A_2$ had minimal binding ratio N:P 2, lower ratio than their pairs without the methyl groups. This suggest stronger interaction between polymer and the DNA. Moreover, N:P ratio for both $1A_2$ and $2A_2$ were the same, N:P 2.

Amine A_3 and A_4 share the same chemical structure and had different binding behaviour in both dodecylated and non-dodecylated groups. Here, for both $1A_3$ and $2A_3$, the minimal complexation occurred at N:P 2, the same as for A_2 grafted PSI derivatives. However, for the amine A_4, the minimal complexation was obtained at N:P 5, with the best N:P ratio for GFP expression.

Dynamic Light Scattering (DLS) measurements – Particle Size and Zeta Potential

Size and surface charge of polyplexes were determined by DLS and zeta potential measurements. As a control for size and residual charge, measurements of bPEI 25 polyplexes were performed Figure 15. As observed, size for all N:P ratios is around 100 nm, even though for the polydispersion index (PdI) is clear the optimal N:P 5 for these polyplexes.

Zeta potential values of pEGFP-N3/bPEI25 polyplexes (Figure 15), pEGFP-N3/1An and pEGFP-N3/$2A_n$ polyplexes have a charge inversion between N:P ratios

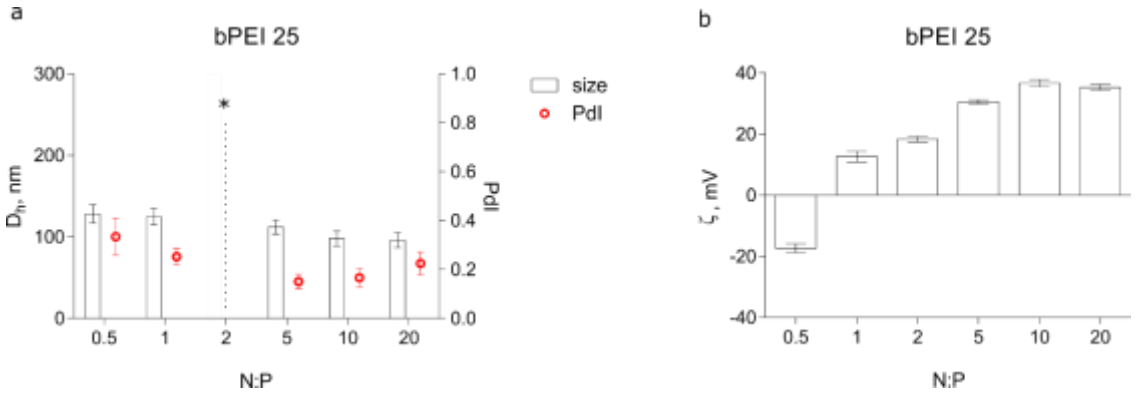

Figure 15 bPEI 25 polyplexes: (a) hydrodynamic diameter Dh (nm) and PdI and (b) zeta potential (mV).

0.5 and 1, from a negatively to a positively charged polyplex. It is considered that particles with a zeta potential higher than 30 mV are physically more stable due to the presence of electrostatic repulsion (Shatsberg *et al.*, 2016). Here, obtained pEGFP-N3/1A_4 polyplexes at N:P ratio 5 that had high GFP expression also had positive zeta potential values.

The presence of the dodecyl side chain in the PSI derivatives did not change the size of polyplexes obtained from PSI grafting with amine A_1 and A_2 (Figure 16). Both groups present similar values for size and the same PdI profile with an optimal

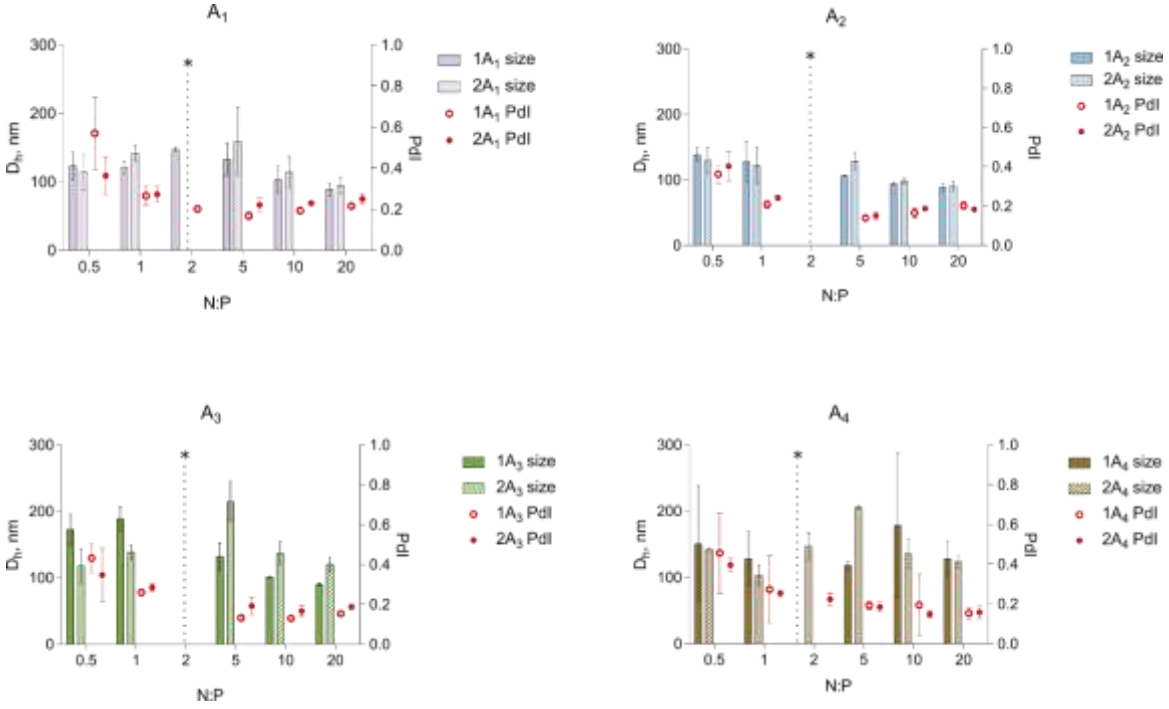

Figure 16 1A_n and 2A_n (amines A1 to A4) polyplexes with pEGFP-N3: hydrodynamic diameter Dh (nm) and PdI.

N:P ratio 5. However, for the A_3 grafted PSI, sizes of $2A_n$ group were smaller than those from $1A_n$, from N:P ratio 0.5 to 1. The opposite behaviour was observed for N:P ratios higher than 2, in which pEGFP-N3/$2A_3$ polyplexes had higher sizes than those

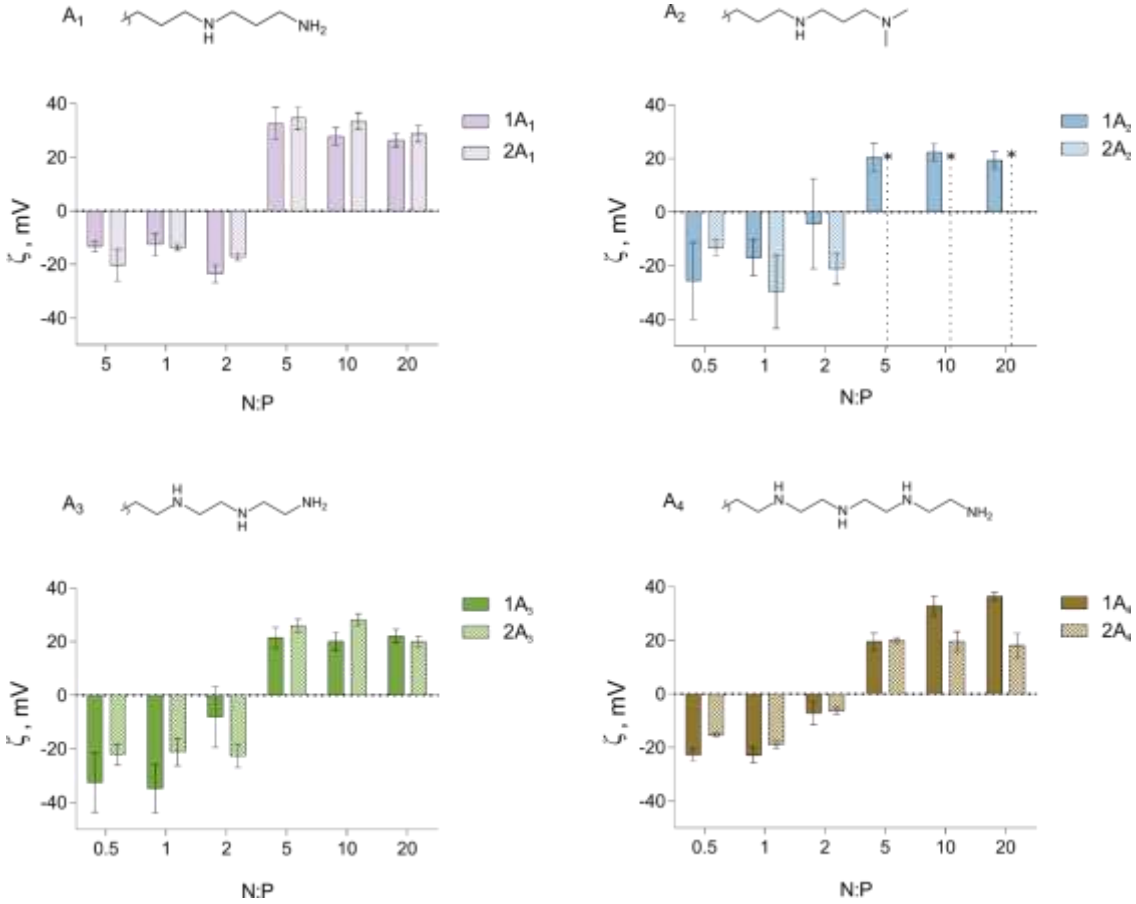

Figure 17 Zeta Potential.

pEGFP-N3/$1A_3$ polyplexes. PdI had also an optimal value at N:P ratios 5 and 10.

pEGFP-N3/$2A_4$ polyplexes had lower size than pEGFP-N3/$1A_4$ in the N:P ratios 0.5 and 1 with high PdI. From N:P 5, this behaviour changes and pEGFP-N3/$1A_4$ particles had smaller sizes than pEGFP-N3/$2A_4$. PdI values for these particles are reduced as N:P ratio increases.

Dodecylamine side chain into PSI derivatives seems to have no influence on sizes of A1 and A2 grafted PSI. However, for PSI derivatives with A3 and A4 side chains, for lower N:P, dodecylated derivatives had smaller sizes than non-dodecylated at N:P ratio 0.5 and 1. PdI values for these particles are high.

Another behaviour observed was a trend in all polyplexes at N:P ratio 2, in the polyplexes containing PSI derivatives and bPEI 25. Artefacts appeared and may be

caused by neutralization of charges, thus not providing an accurate DLS value at this N:P ratio.

Polyanion competition assay

In these studies, we further examined polyplex integrity in the presence of heparin, a competing polyanion (Figure 18). DNA plasmid has been shown to be released from polyplexes after heparin competition using a commonly described method (Gwak *et al.*, 2017).

Polyplexes pEGFP-N_3/1A_n and pEGFP-N_3/2A_n with amines A_1 to A_4 and bPEI 25/1A_n at N:P 5 were mixed with different concentrations of heparin, from 0 to 1 UI per sample, 50 µL total volume.

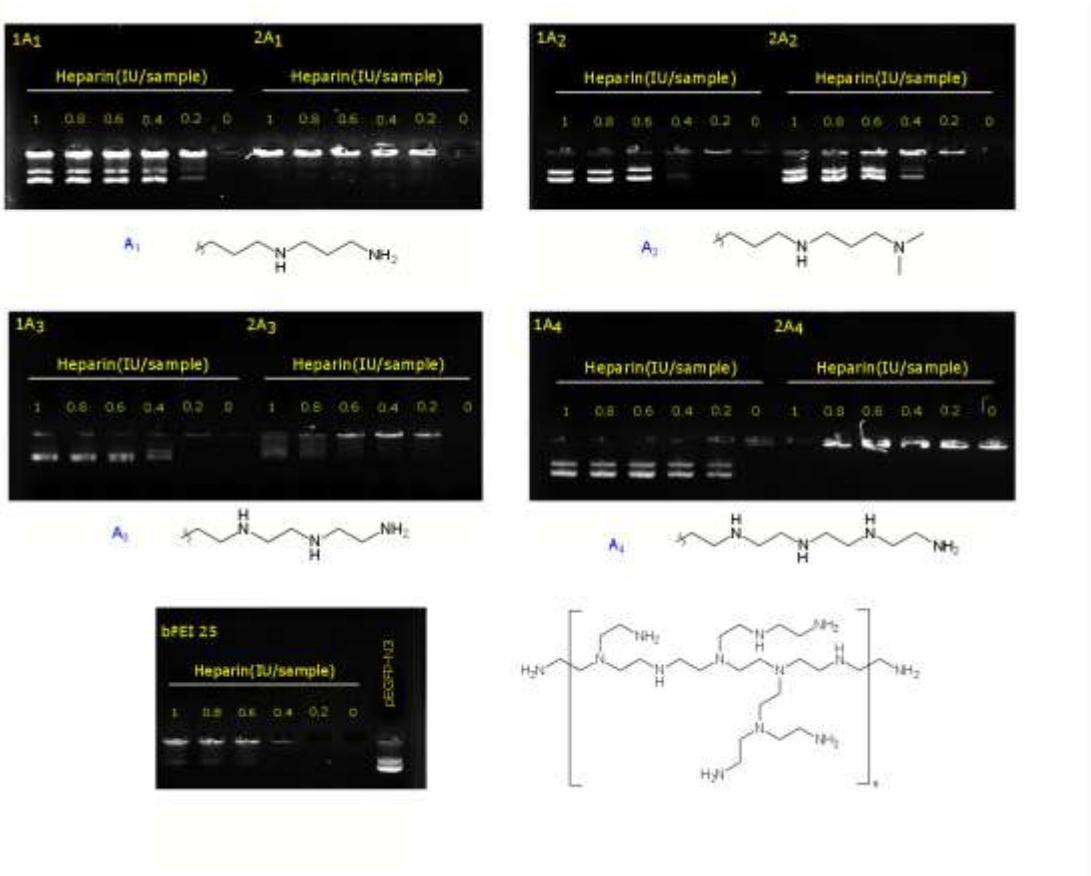

Figure 18 Heparin competition assay of pEGFP-N3/1A_n and pEGFP-N3/2A_n polyplexes, with amines A1 to A4, and pEGFP-N3/bPEI 25 polyplexes. All polyplexes were prepared at N/P 5.

Polyplexes prepared with bPEI 25 dissociated with 0.6 to 1 UI heparin, thus suggesting a decomplexation and release of the DNA. For polyplexes pEGFP-N_3/1A_n, the amount of heparin to compete and release the DNA was lower than 0.6UI, except for pEGFP-N_3/1A_2 polyplexes.

Dodecylated PSI derivatives in the polyplexes pEGFP-N_3/$2A_n$ showed stronger binding between the nucleic acid and the polymers, except for pEGFP-N_3/$2A_2$ that had shower the release of DNA with 0.2 to 1 UI of heparin per sample, concentration of heparin that decreased the integrity of polyplex and released the DNA. No release of the DNA from the polyplexes were observed in the range of heparin concentration for all other polyplexes.

Although an efficient gene delivery can be observed in polyplexes with strong pDNA binding via hydrogen binding (Gwak *et al.*, 2017), here we observe that the presence of a alkyl chain can modulate the integrity of polyplexes by hydrophobic interactions, increasing the binding between polymer and the DNA.

5. Final Considerations

Aminolysis process of poly(succinimide) with nucleophilic amino compounds provides a grafting of different moieties in the polymer backbone and this process is useful to form various poly(asparamide)s with functional groups.

In this study, PSI was synthesized and non-dodecylated and dodecylated cationic copolymers were obtained, here named in a small library $1A_n$ and $2A_n$, respectively. SEC showed that dodecylated derivatives $1A_n$ had lower size than $2A_n$ group, dodecylated polyelectrolytes. Buffering capacity of all synthesized polymers was higher than the standard bPEI 25, and the dodecylated $2A_n$ group had the highest buffering capacities values.

$2A_n$ derivatives with amines A1 to A4 showed lower CMC than their non-dodecylated pairs. However, no changes in the CMC values were also observed in PSI derivatives 1A6 and 2A6, both PSI grafted with a piperazine side chain. Polycations of $2A_n$ group had CMC values comparable to the polymers 1A5 and 2A5, thus indicating that these polymers are able have polymer-polymer interactions and to form micelle-like structures as polycations 1A5, 2A5, 1A6 and 2A6.

Cytotoxicity of all polycations was dependent on the concentrations, and among all polymers, those with amines A5 and A6 had lower cytotoxicity than bPEI 25. Moreover, the presence of the hydrophobic dodecyl side chain in the PSI backbone did not decrease the cell viability untill 250 µg mL-1 polymer concentration, thus suggesting the hydrophobic moiety is not cytotoxic in the range of polymer concentrations.

Complexation of pEGFP-N3 plasmid with PSI derivatives grafted with amines A1 to A4 was performed, as well as the transfection of polyplexes into HeLa cells. GFP expression of bPEI25 polyplexes in different complex volumes was quantified and compared with PSI derivatives/pEGFP-N3 polyplexes. Transfection assays showed that dodecylated PSI derivatives had negligible or no GFP expression in HeLa cells, thus suggesting a strong interaction between polycations and pDNA or cellular damage caused by the hydrophobic moiety. However, MTT of polyplexes showed low cytotoxicity of polyplexes.

Transfection ability of polycation $1A_1$ was conducted and GFP expression was maximal with N:P ratio 5 and 10, as observed in bPEI 25 polyplexes. Also, as the polyplex volume increases, GFP expression decreases, for all N:P ratios, with 25 µL as optimal polyplex volume.

GFP expression for polycations $1A_2$ and $2A_2$ was lower than expression with using bPEI 25 polyplexes. However, the presence of dodecylamine side chain in the PSI backbone of polycation $2A_2$ led to an increased GFP transfection, the opposite behaviour of A_1-grafted PSI polycation

The highest GFP expression values were found for polycations $1A_3$ and $1A_4$, both without the dodecylamine side chain, for N:P ratios 5 to 20 for $1A_3$, and N:P ratio 5 for $1A_4$. Both amines A_3 and A_4 used for the PSI grafting are core structures of bPEI 25.

Lower polyplexes volumes 25 and 50 µL provided no change in the cell viability in all polyplexes with synthesized polymers or bPEI 25. Cell toxicity is achieved for higher volumes, 75 and 100 µL of polyplexes and even GFP expression was lower for these volumes.

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Appendices

Appendix A. ^{1}H NMR spectra of all compounds

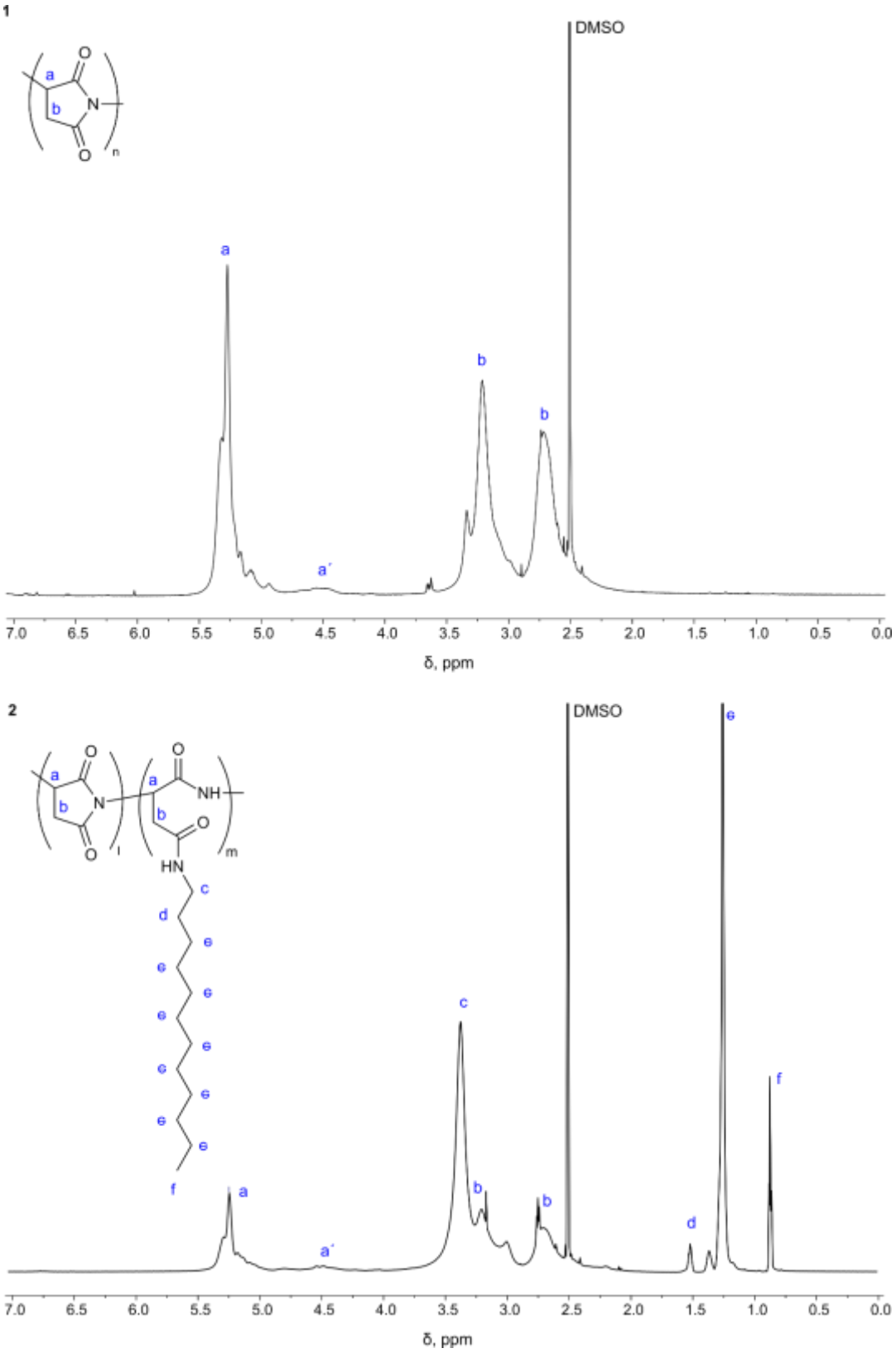

Figure 19 1H NMR spectra of poly(succinimide) (PSI) (1) and poly(succinimide)-co-(dodecylamide aspartate) (PSI-C12) (2) in d6-DMSO. a δ 5.5 and a´ δ 4.5 peaks related to closed and opened ring after aminolysis of PSI.

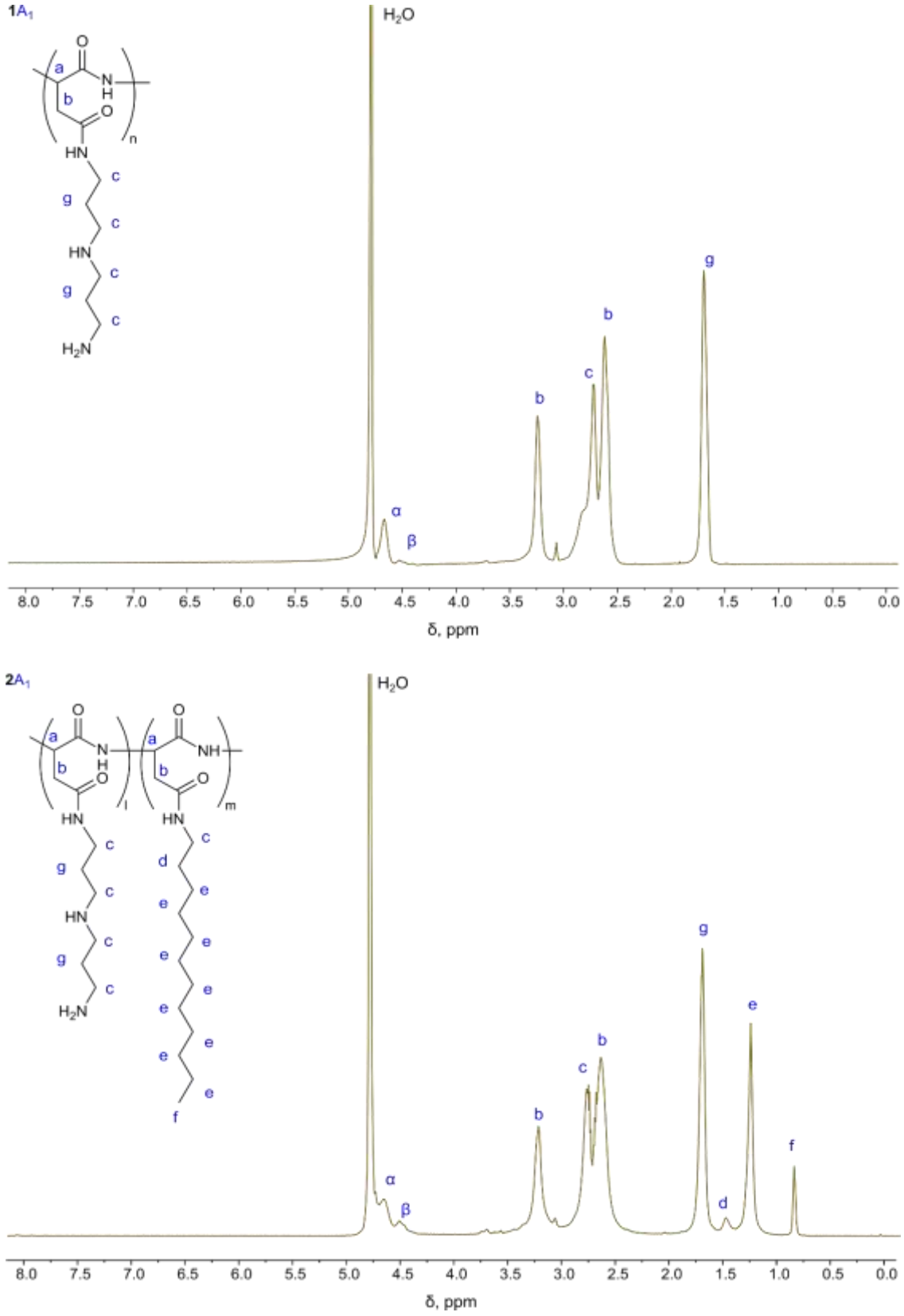

Figure 201H NMR spectra of compounds 1A1 and 2A1 in D2O. α and β represent PSI ring opening chemical shifts after total aminolysis

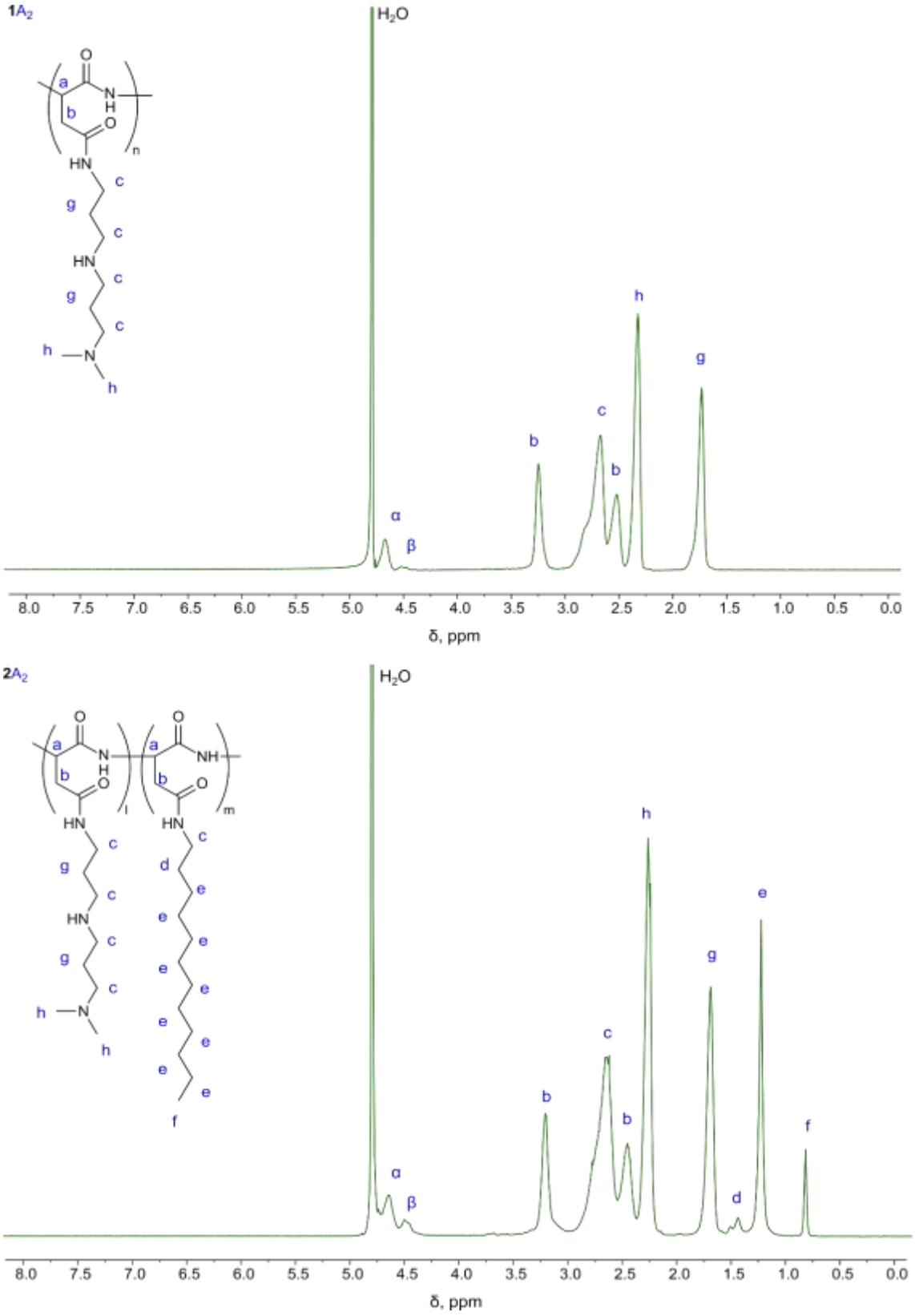

Figure 21 1H NMR spectra of compounds 1A2 and 2A2 in D2O. α and β represent PSI ring opening chemical shifts after total aminolysis.

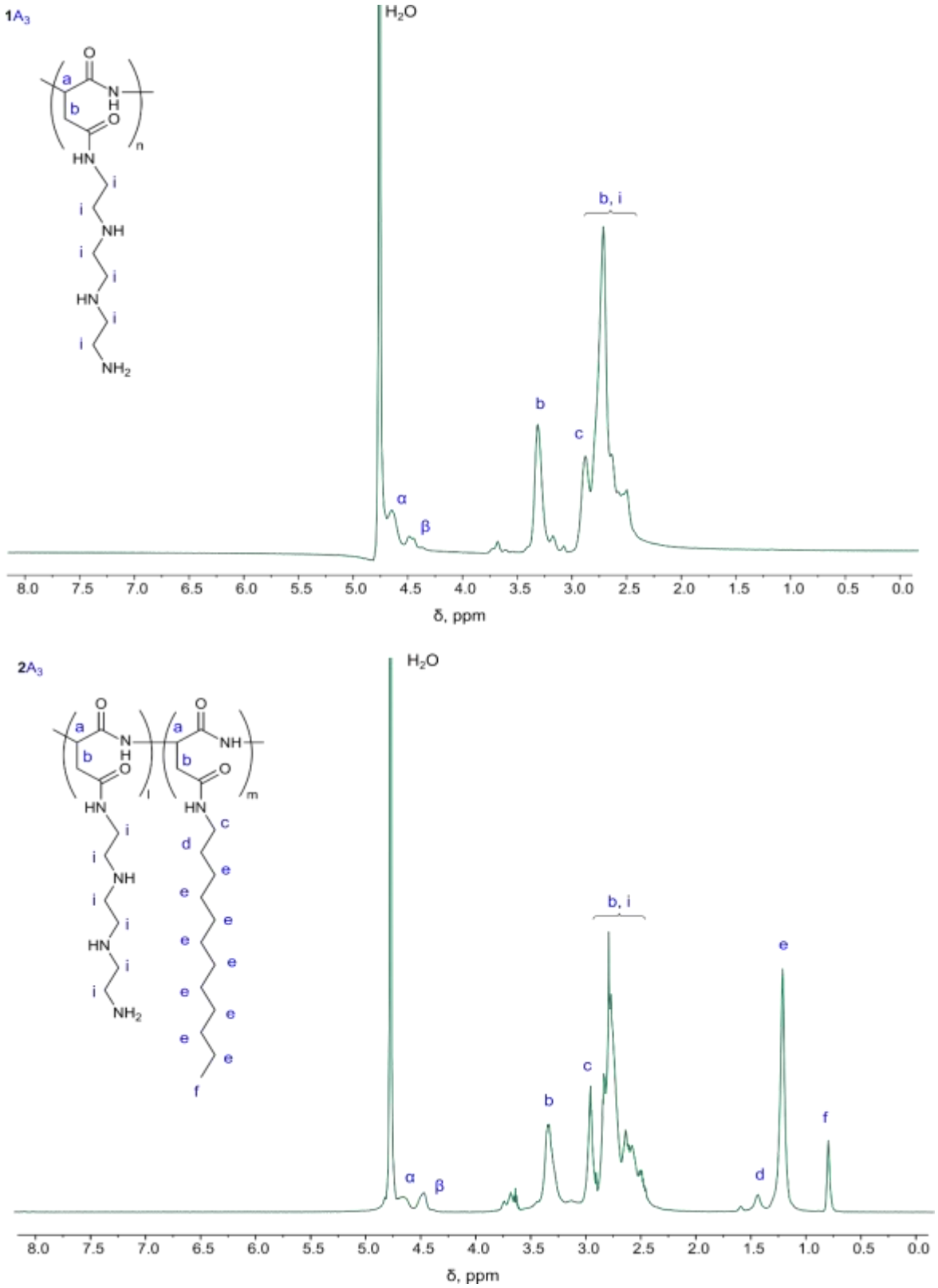

Figure 22 1H NMR spectra of compounds 1A3 and 2A3 in D2O. α and β represent PSI ring opening chemical shifts after total aminolysis.

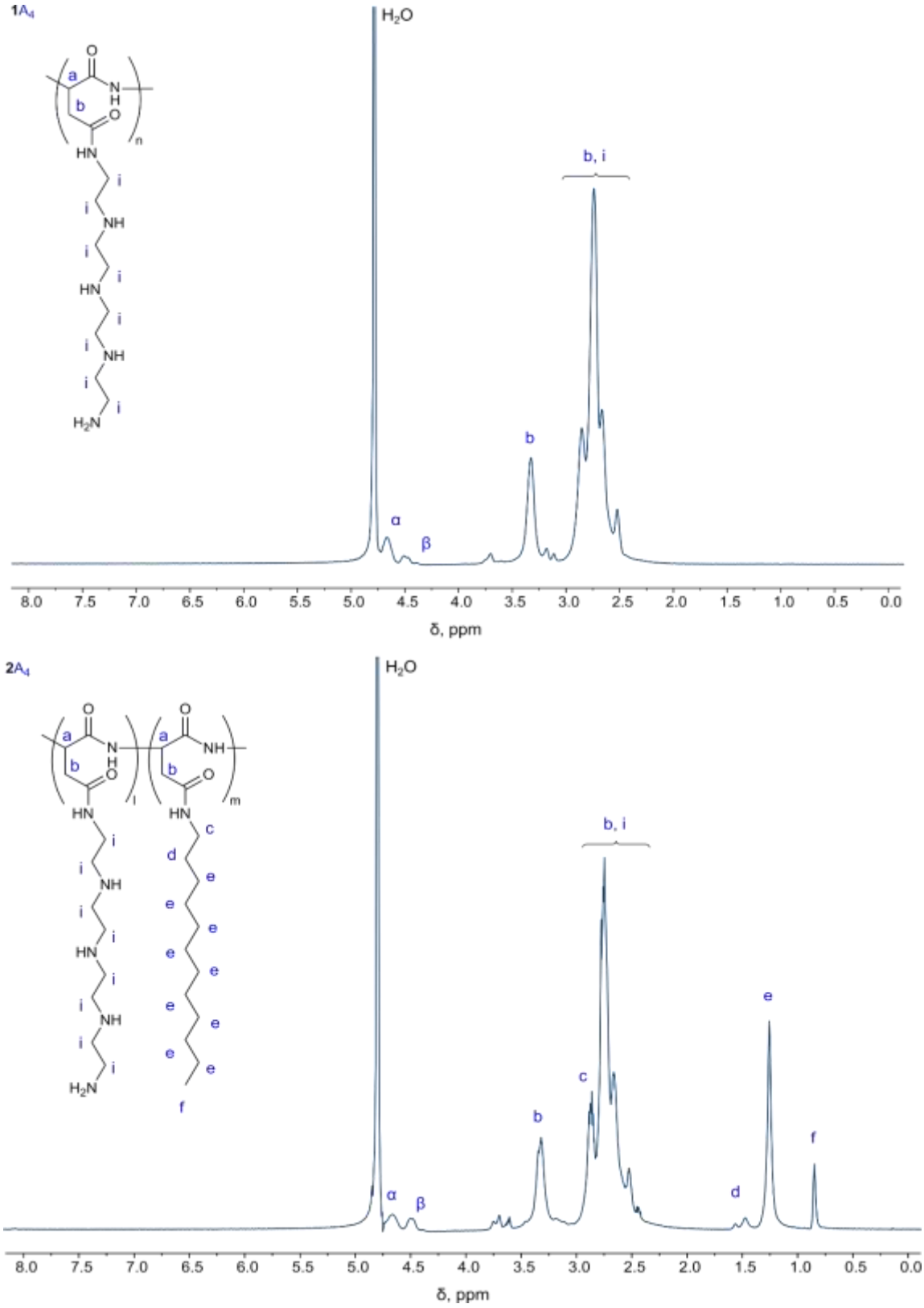

Figure 23 1H NMR spectra of compounds 1A4 and 2A4 in D2O. α and β represent PSI ring opening chemical shifts after total aminolysis.

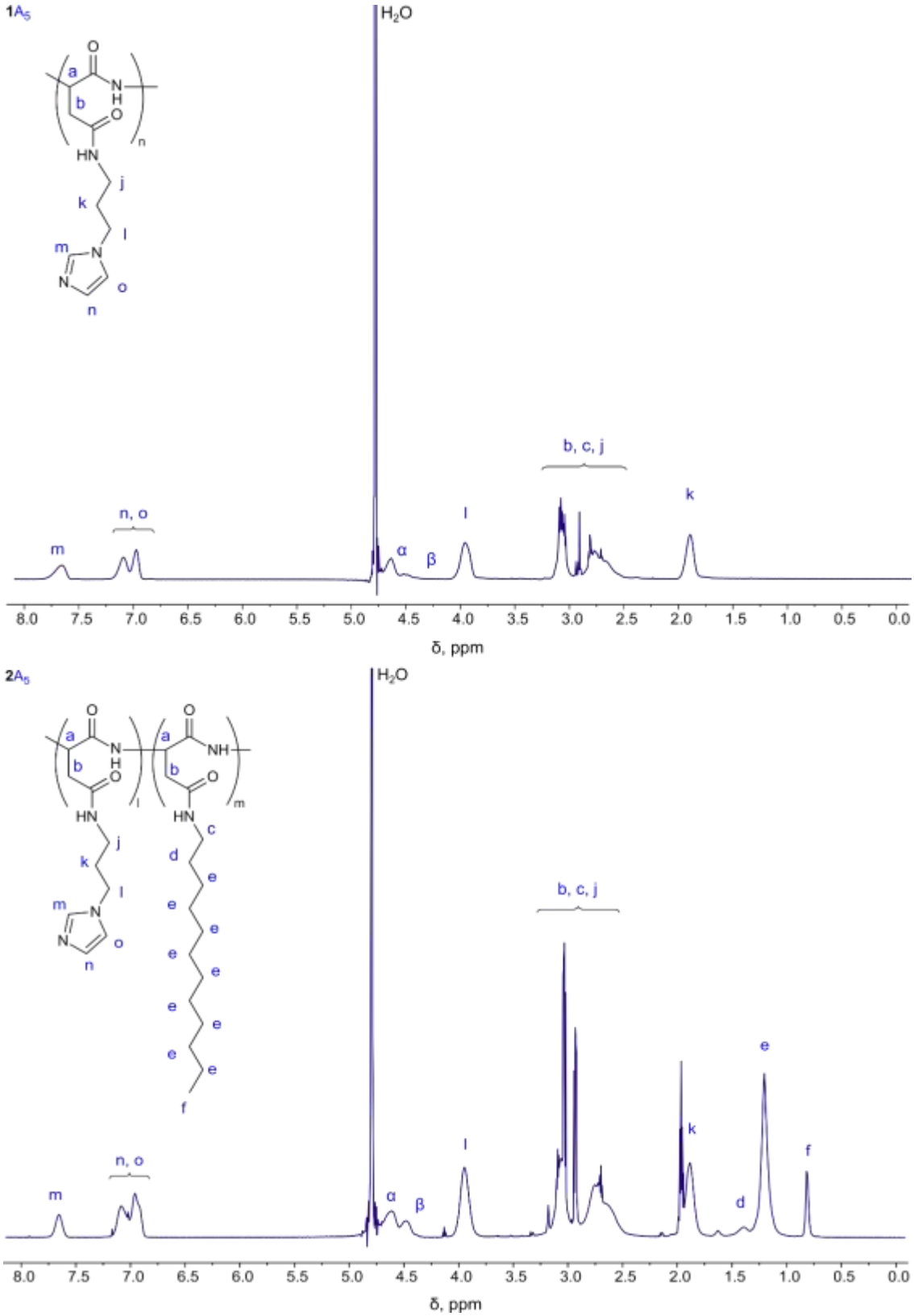

Figure 24 1H NMR spectra of compounds 1A5 and 2A5 in D2O. α and β represent PSI ring opening chemical shifts after total aminolysis.

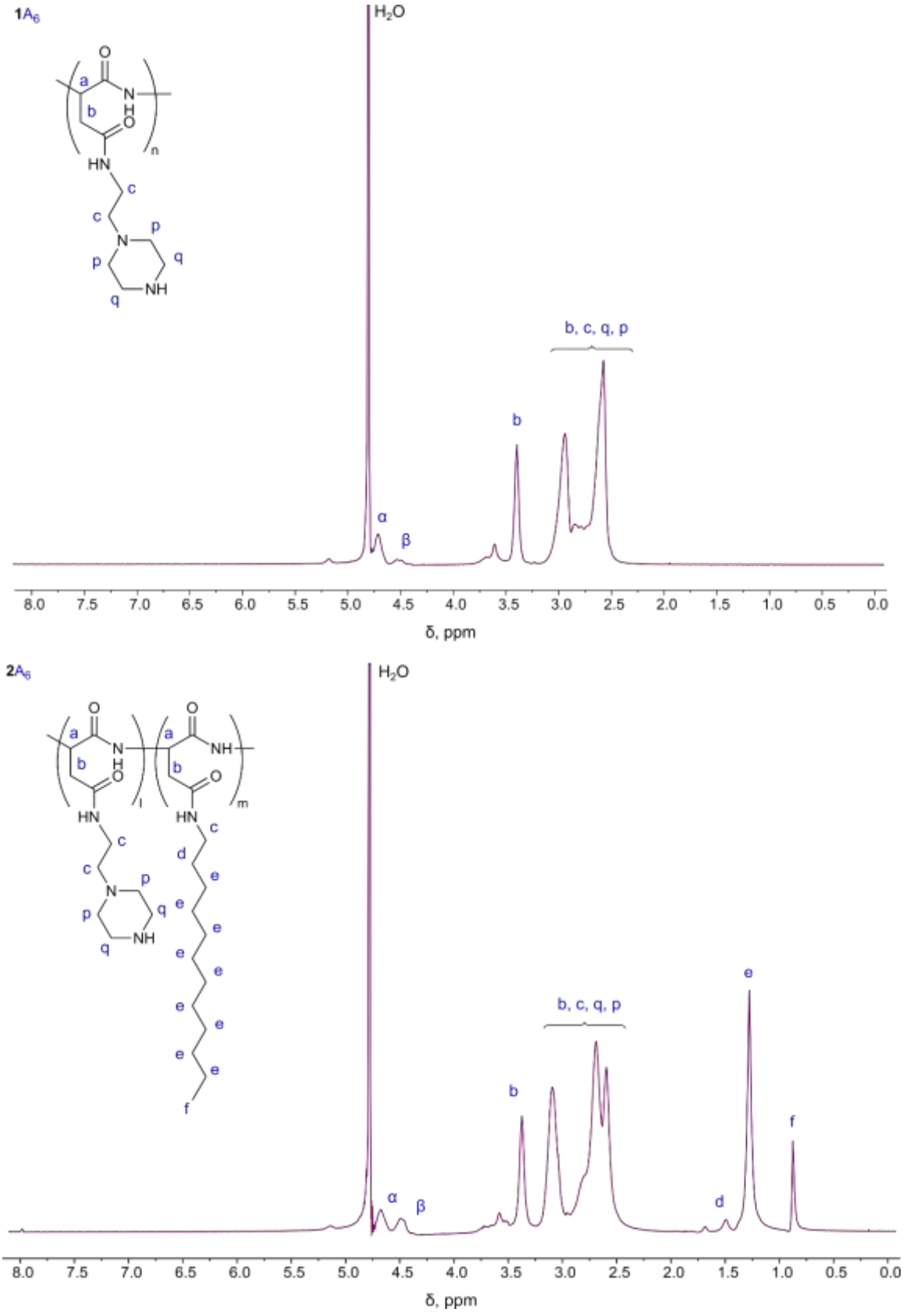

Figure 25 1H NMR spectra of compounds 1A6 and 2A6 in D2O. α and β represent PSI ring opening chemical shifts after total aminolysis.

Appendix B. Equations

$$\frac{x}{y} = \frac{\frac{4}{3}\int f}{\int g - \frac{4}{3}\int f}$$

Equation 1 Molar ratio and degree of substitution (DS) mol% between dodecylamine (C_{12}) and amine A_1 in compound $2A_1$.

$$\frac{x}{y} = \frac{\frac{4}{3}\int f}{\int g - \frac{4}{3}\int f}$$

Equation 2 Molar ratio and degree of substitution (DS) mol% between dodecylamine (C_{12}) and amine A_2 in compound $2A_2$.

$$\frac{x}{y} = \frac{\frac{12}{23}\int d, e, f}{\left(\int b, c, i - \int b - \int c\right) - \frac{12}{23}\int f}$$

Equation 3 Molar ratio and degree of substitution (DS) mol% between dodecylamine (C_{12}) and amine A_3 in compound $2A_3$.

$$\frac{x}{y} = \frac{\frac{16}{23}\int d, e, f}{\left(\int b, c, i - \int b - \int c\right) - \frac{4}{3}\int f}$$

Equation 4 Molar ratio and degree of substitution (DS) mol% between dodecylamine (C_{12}) and amine A_4 in compound $2A_4$.

$$\frac{x}{y} = \frac{\int f}{\int m, n, o - \int f}$$

Equation 5 Molar ratio and degree of substitution (DS) mol% between dodecylamine (C_{12}) and amine A_5 in compound $2A_5$.

$$\frac{\mathrm{x}}{\mathrm{y}} = \frac{\frac{16}{23}\int \mathrm{f}}{\int \mathrm{b, c, p, q} - \frac{16}{23}\int \mathrm{f}}$$

Equation 6 Molar ratio and degree of substitution (DS) mol% between dodecylamine (C_{12}) and amine A_6 in compound $2A_6$.

Appendix C. Elemental Analysis of all compounds

Table 7 Elemental Analysis of poly(succinimide) (PSI) and poly(succinimide)-co-(dodecylamide aspartate) (PSI-C12).

1, PSI

Run	**17**	**18**	**19**	**20**	**Avr.**	**SD**
%C	44,16	44,29	44,49	44,19	44,28	0,13
%N	12,52	12,46	11,99	12,43	**12,35**	0,21
%H	4,047	3,96	4,04	4,00	4,01	0,03
%O	39,27	39,29	39,48	39,38	39,36	0,08

2, PSI-C_{12}

Run	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**Avr.**	**SD**
%C	50,18	50,40	50,50	50,53	50,25	50,08	50,48	50,65	49,31	49,10	50,15	0,50
%N	12,11	12,03	12,09	12,11	12,15	12,01	11,88	12,12	11,66	11,74	**11,99**	0,16
%H	5,748	5,688	5,562	5,536	5,597	5,464	5,570	5,538	5,242	5,302	5,52	0,15
%O	31,96	31,88	31,85	31,82	32,00	32,45	32,07	31,69	33,79	33,86	32,34	0,77

Table 8 Elemental Analysis of all polycations $1A_n$.

$1A_1$

Run	30	31	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	50,02	50,09	50,06	0,04	5,77	0,58	48,06
%N	22,16	22,19	22,18	0,02	9,83	**0,98**	**70,15**
%H	8,330	8,463	8,40	0,07	4,39	0,44	435,14

$1A_2$

Run	32	33	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	52,87	52,75	52,81	0,06	8,53	0,85	71,00
%N	20,09	20,00	20,05	0,04	7,70	**0,77**	**54,94**
%H	9,117	9,155	9,14	0,02	5,13	0,51	508,51

$1A_3$

Run	34	35	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	46,04	46,16	46,10	0,06	1,82	0,18	15,13
%N	23,87	23,90	23,89	0,01	11,54	**1,15**	**82,35**
%H	8,180	8,126	8,15	0,03	4,14	0,41	410,99

$1A_4$

Run	36	37	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	47,68	47,73	47,71	0,02	3,42	0,34	28,50
%N	24,99	24,92	24,96	0,03	12,61	**1,26**	**89,99**
%H	8,625	8,665	8,65	0,02	4,63	0,46	459,80

$1A_5$

Run	38	39	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	49,89	49,95	49,92	0,03	5,64	0,56	46,94
%N	22,98	22,99	22,99	0,00	10,64	**1,06**	**75,93**
%H	6,786	6,699	6,74	0,04	2,73	0,27	271,06

$1A_6$

Run	40	41	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	48,16	48,45	48,31	0,15	4,02	0,40	33,49
%N	21,73	21,85	21,79	0,06	9,44	**0,94**	**67,40**
%H	8,081	8,060	8,07	0,01	4,06	0,41	402,80

Table 9 Elemental Analysis of all polycations $2A_n$.

$2A_1$

Run	18	19	20	21	22	23	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	49,68	49,67	49,37	49,41	49,46	49,74	49,56	0,15	-0,59	-0,06	-4,94
%N	18,41	18,22	18,11	18,11	17,98	17,96	18,13	0,15	6,14	**0,61**	**43,85**
%H	8,270	8,275	8,409	8,415	8,286	8,116	8,30	0,10	2,77	0,28	274,85

$2A_2$

Run	26	27	Avr.	SD					Δ(%CNH)	w(mg)	n(mmol)
%C	52,62	52,77	52,70	0,08					2,55	0,25	21,21
%N	17,19	17,11	17,15	0,04					5,16	**0,52**	**36,84**
%H	8,821	8,745	8,78	0,04					3,26	0,33	323,24

$2A_3$

Run	28	29	30	31	32	33	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	46,50	46,62	47,77	47,92	48,01	47,87	47,45	0,63	-2,70	-0,27	-22,48
%N	19,66	19,76	19,52	19,46	19,54	19,65	19,60	0,10	7,61	**0,76**	**54,32**
%H	8,066	8,156	8,171	8,249	8,201	8,190	8,17	0,06	2,65	0,26	262,65

$2A_4$

Run	34	35	Avr.	SD					Δ(%CNHO)	w(mg)	n(mmol)
%C	47,87	50,69	49,28	1,41					-0,87	-0,09	-7,23
%N	19,65	21,11	20,38	0,73					8,39	**0,84**	**59,90**
%H	8,190	9,087	8,64	0,45					3,11	0,31	308,91

$2A_5$

Run	39	40	Avr.	SD					Δ(%CNHO)	w(mg)	n(mmol)
%C	50,30	50,03	50,17	0,13					0,02	0,00	0,14
%N	18,28	18,31	18,30	0,01					6,31	**0,63**	**45,01**
%H	7,199	7,086	7,14	0,06					1,62	0,16	160,50

$2A_6$

Run	41	42	43	44	45	46	Avr.	SD	Δ(%CNH)	w(mg)	n(mmol)
%C	47,99	61,53	49,35	49,51	49,38	49,11	51,15	4,67	1,00	0,10	8,30
%N	17,32	21,99	17,37	17,59	17,51	17,61	18,23	1,68	6,24	**0,62**	**44,56**
%H	7,634	9,852	7,817	7,762	7,963	7,862	8,15	0,77	2,62	0,26	260,28