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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

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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.

Uma das estratégias promissoras da Terapia Gênica no tratamento e prevenção de doenças adquiridas e herdadas é a utilização de small interfering RNA (siRNA), molécula efetora no processo de interferência de RNA (RNAi). Para que o siRNA, uma molécula negativamente carregada, seja internalizada nas células, esteja disponível no citoplasma e tenha sua efetiva ação, é necessária a sua complexação com polímeros que possuam carga residual positiva, como por exemplo, a polietilenoimina (PEI). Entretanto, mesmo a PEI sendo amplamente utilizada como carreador, esse polímero não é biodegradável e possui elevada toxicidade em modelos celulares. Assim, este trabalho teve como objetivo a síntese de polímeros anfifílicos catiônicos biodegradáveis para a formação de poliplexos com siRNA e avaliação in vitro da transfecção e silenciamento gênico. Para isso, foi sintetizada a poli (succinimida) (PSI), polímero obtido via policondensação do ácido L-aspártico (ASP). Para a inserção de porções anfifílicas e catiônicas, foram utilizadas alquilaminas e oligoaminas, necessárias para complexação, transfecção e liberação do siRNA dentro das células Por fim, foi selecionado o policátion PSI-C12- NN11 para a avaliação da viabilidade celular frente células de melanoma B16F10, em 4 e 24 h; comparados os resultados de viabilidade celular desse polímero com seus precursores PSI-C12 e PSI, foi observada que a aminólise diminui a viabilidade celular para a linhagem utilizada, sendo a menor viabilidade aquela associada à presença da oligoamina NN 1 do policátion, em acordo com dados da literatura. Estudos de transfecção e silenciamento gênico in vitro não foram realizados nessa etapa do trabalho.

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. 000f. 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 1An, polycationic polymers with a degradable amide of poly(aspartic) acid backbone, protonable oligoamine side chains into the main polymer structure, and group 2An, 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. In this study, PSI was synthesized and non-dodecylated and dodecylated cationic copolymers were obtained, here named in a small library 1An and 2An, respectively. SEC showed that dodecylated derivatives 1An had lower size than 2An group, dodecylated polyelectrolytes. Buffering capacity of all synthesized polymers was higher than the standard bPEI 25, and the dodecylated 2An group had the highest buffering capacities values. 2An 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 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. The highest GFP expression values were found for polycations 1A3 and 1A4, both without the dodecylamine side chain, for 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.

1. Introduction

1.1. General

Europe 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 (1).

Genes as drugs for human therapy is a concept originally conceived around 1970 (2, 3), a consequence of the exponential growth in knowledge of human gene function, the more effective technologies for DNA delivery (2), and the ability to transfer and express exogenous genes in mammalian cells (4, 5). After the approval of the first gene therapy agent in 2012 by the EMA, such research field became more interesting (6).

The strategy has already expanded from plasmid DNA (pDNA) to messenger RNA (mRNA), microRNA (miRNA) and small interfering RNA (siRNA) (7, 8), though different therapeutic pay loads require different tailor-made carriers, thus complicating preclinical development (9). 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 (6). For an effective pDNA delivery, polycations are good candidates providing pDNA stability and functionality (10).

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 (6). 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 (6,

8). Moreover, the easy plasmid unpacking from polyplexes is mandatory, so that DNA is accessible to transcription factors for gene expression (11).

1.3. Vectors for gene delivery

Nucleic acids are far larger than conventional drugs and cannot diffuse across lipid membranes into target cells (12). 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 (13). These gene delivery devices can be divided in two groups (i) viral vectors and (ii) non-viral vector (13).

Viral vectors for gene transfer, such as retroviruses, have raised scepticism about safety, presenting a possible threat to patients (1), though they are the most commonly used gene transfer systems used in clinical trials (13). They remain by far the most popular approach, having been used in approximately two-thirds of the performed trials (14). However, limitations associated with viral vector regarding their safety, immunogenicity and low loading size have been encouraged researchers to develop non-viral carriers (13, 15) that include cationic lipids, peptide and cationic polymers (15).

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* (16). Therefore, nucleic acid is protected from enzymatic degradation via condensation into the polyplexes, also cell uptake and endolysosomal escape are facilitated (17).

Cationic polymers can be considered perfect noncovalent interaction partners for nucleic acids, providing similar size dimensions but opposite ionic charge (12). Frequently studied cationic polymers include poly(L-lysine) (PLL), chitosan, dendrimers and polyethyleneimine (PEI) (17, 18).

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 (15). 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 (19). Commercially available transfection agents based in the PEI structure include agents ExGen500 and jetPEI, both linear derivatives from PEI (15).

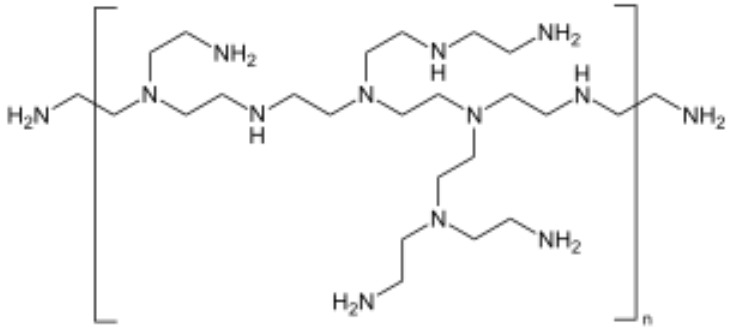

Figure 1 Branched polyethyleneimine 25 kDa (bPEI 25) chemical structure.

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 (20) and is significantly toxic in a molecular weight-dependent manner (8, 17).

1.5. Poly(succinimide) as biodegradable backbone

Poly(amino acids), polypeptides and their derivatives have been used in regenerative medicine, drug delivery and gene therapy due to their biocompatibility, biomimetic properties and available side groups for functionalization (21-23). 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 (23).

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) (21), a poly(imide) obtained from thermal polycondensation of aspartic acid (ASP) (24, 25).

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 (24), pseudo-branched PEI (26), PEI-mimetic polymers (25), reversible micelles (27) and other biodegradable water-soluble polymeric material (28).

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 (8, 29). The conversion of PSI backbone into poly(aspartamide) after aminolysis increases biocompatibility of cationic polymer and maintain or promote nucleic acid transfection efficiency (8, 15).

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 (8, 12, 17).

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 1An and 2An, respectively. SEC showed that dodecylated derivatives 1An had lower size than 2An group, dodecylated polyelectrolytes. Buffering capacity of all synthesized polymers was higher than the standard bPEI 25, and the dodecylated 2An group had the highest buffering capacities values.

2An 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 2An 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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