Journal of Drug Delivery Science and Technology 37 (2017) 115–122

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

RGD-derivatized PEI-PEG copolymers: Influence of the degree of substitution on the targeting behavior

Daniele Pezzoli [a], Paolo Tarsini [b], Lucio Melone [b, c], Gabriele Candiani [a, b, *]

[a] Research Unit Milano Politecnico, National Interuniversity Consortium of Materials Science and Technology – INSTM, Via Mancinelli 7, Milan, Italy
[b] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Via Mancinelli 7, Milan, Italy
[c] Università degli Studi e-Campus, Via Isimbardi 10, Como, Italy

ARTICLE INFO

Article history:
Received 13 November 2016
Received in revised form
15 December 2016
Accepted 17 December 2016
Available online 18 December 2016

Keywords:
Gene delivery
Non-viral vectors
PEI
PEG
RGD
Targeting

ABSTRACT

PEGylation is widely used to improve the stability of gene delivery vectors through the decrease of non-specific binding to serum proteins. In order to provide "stealth" vectors with targeting properties, the grafting of specific ligands is mandatory. For instance, the tripeptide arginine-glycine-aspartic acid (RGD) has been shown to confer selectivity towards some integrin-overexpressing tumor cells. Unfortunately, owing to the different RGD grafting degrees, some difference in starting materials and experimental conditions, the literature displays contradictory findings about the efficacy of this strategy.

Starting from branched poly(ethylene imine) (*b*PEI) transfectant and the heterobifunctional linker *N*-hydroxy-succinimide-poly(ethylene glycol)-maleimide, a series of variably substituted PEI-PEGs were synthesized and functionalized with linear and cyclic RGDs. *b*PEI-PEG-RGDs were as effective as *b*PEI in complexing DNA while derivatization did affect the physicochemical properties of polyplexes. A degree of substitution of 1.31% led to enhanced targeting of cognate receptor-expressing cells without impairing the transfection efficiency of *b*PEI, even in the presence of serum. Of note, both RGDs were equally effective to confer selectivity to polyplexes.

These results highlight the key role of the degree of substitution on the effectiveness and selectivity of *b*PEI-PEG-RGDs, suggesting that a systematic approach is needed for the development of more effective targeted transfectants.

1. Introduction

Gene delivery consists in the introduction of exogenous genetic material into cells in order to alter protein expression. The application of such strategies to medicine is called gene therapy and aims at eradicating causes rather than symptoms of diseases. As such, it does represent a promising approach for the treatment of a wide variety of diseases that conventional medicine fails to heal. However, the major challenge hampering effective gene therapy is the development of effective and non-toxic gene delivery vectors that target specific cell types [1,2]. Although viral vectors have demonstrated high transduction efficiencies, major safety concerns remain to be solved [3]. On this ground, the development of non-viral-based approaches to deliver nucleic acids to cells and tissues (i.e. transfection) is a rapidly advancing, interdisciplinary area of research. Among non-viral vectors, cationic polymers display striking advantages over viral vectors in terms of molecular diversity and excellent chemical processability that allows fine tuning of their physicochemical properties. At physiological pH they do spontaneously interact with negatively charged nucleic acids to give rise to complexes named polyplexes [4]. Poly(ethylene imine) (PEI) is considered the gold standard polymeric transfectant [5–7] that exists in a linear (*l*PEI) and branched (*b*PEI) form. Although both PEI forms have been reported effective in transfection regardless of the molecular size, 25 kDa *b*PEI and 22 kDa *l*PEI are by far the most popular reagents reported in literature. Unfortunately, their *in vivo* application is hampered by the still too low transfection efficiency, toxicity and lack of selectivity [8]. In this context, PEGylation does represent a frequently applied strategy to increase the stability, and thus the half-life, of polyplexes in biological fluids by decreasing their binding to serum proteins but also to the cell surface. Indeed, the so called "stealth" behavior of polyethylene

* Corresponding author. Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Via Mancinelli 7, Milan, 20131, Italy.
E-mail address: gabriele.candiani@polimi.it (G. Candiani).

http://dx.doi.org/10.1016/j.jddst.2016.12.005
1773-2247/

glycol (PEG) in PEGylated polyplexes has also proven to impair their cellular uptake [2]. A promising strategy to overcome this major drawback is the grafting of targeting ligands, such as folic acid [9], transferrin [10], certain antibodies and, frequently, the tripeptide sequence arginine-glycine-aspartic acid (RGD) [11–13]. The RGD motif is now known to bind some integrins, such as $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, that play important roles in several pathophysiological conditions [14,15]. Besides, it is worthy of note that the context of the RGD sequence (i.e. flanking residues, 3D presentation, and individual features of the integrin binding pockets) has shown to determine whether productive interactions occur [16]. By means of different chemical grafting strategies and by using distinct spacer arms, some RGD-functionalized PEIs for gene delivery have been proposed thus far [11,17–19]. In this regard, Erbacher et al. synthesized a series of *b*PEI-RGD conjugates by grafting the linear peptide CYGGRGDTP through the heterobifunctional linker succinimidyl 3-(2-pyridyldithio) propionate (SPDP), showing that, at a nitrogen (N) to DNA phosphate (P) ratio (N/P) of 10, the transfection efficiency increased an order of magnitude as compared to unmodified *b*PEI in both serum-free medium and in the presence of 10% fetal bovine serum (FBS) [17]. Conversely, Clements et al. reported that conjugation of *b*PEI with a linear RGD-containing peptide through a short SPDP linker or a longer (2.3 kDa) PEG-based spacer did not improve neither the polyplex binding to bone marrow stromal cells (BMSC) nor the transfection efficiency [20]. Kunath et al. instead reported that SPDP-based coupling of the tetrapeptide RGDC improved transfection efficiency of PEI by about 1–2 orders of magnitude in integrin-expressing MeWo cells transfected in serum-enriched medium, but only at low N/Ps (N/P 3–4), while the use of a 3.4 kDa PEG spacer did impair targeting [11]. On the other hand, *b*PEI-PEG-RGD derivatives with a conjugation ratio of 1 (3.4 kDa PEG/25 kDa *b*PEI, molar ratio) led to a 5-fold increase in transfection with respect to the parent polymer, when used at N/P 3, i.e. far below the optimal N/P of *b*PEI [7,21]. As shown by these and many other examples regarding PEI-*grafted*-RGD derivatives, the heterogeneity of chemistries used and some differences in transfection conditions have led to a number of contradictory reports about their activity and selectivity.

To shed light on these issues, herein we synthesized, characterized and assessed in *in vitro* gene delivery experiments a series of PEI-PEG copolymers with different PEG substitution degrees, further functionalized with two different RGD peptide sequences. In this study, we sought to identify the optimal substitution degree and the best *in vitro* transfection conditions allowing for high transfection activity and selectivity towards integrin expressing cells. Furthermore, we compared a cyclic RGD peptide (hereafter referred to as *c*RGD) and a linear consensus sequence (*l*RGD) in terms of ability to target cancer-derived cells expressing a number of cognate receptors.

2. Materials and methods

2.1. Materials

The plasmid DNA (pDNA) pGL3-Control Vector (5.2 kb), encoding for the modified firefly luciferase and the Luciferase Assay System were purchased from Promega (Milan, Italy). Bicinchoninic acid (BCA) protein assay kit was from Pierce Chemical (Rockford, IL, USA). *N*-hydroxy-succinimide-poly(ethylene glycol)-maleimide (NHS-PEG-MAL; 3.4 kDa) was from Nanocs Inc. (New York, NY, USA) while cyclo-RGDfC (*c*RGD) and CYGGRGDTP (*l*RGD) peptides were purchased from ProteoGenix SAS (Schiltigheim, France). 25 kDa *b*PEI ($M_w \approx 25$ kDa; $M_n \approx 10$ kDa) and all other chemicals and biologicals were from Sigma-Aldrich (Milan, Italy) if not differently specified.

HeLa (human epithelial ovarian carcinoma cells, CCL-2.2) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

^{1}H NMR spectra were run on Bruker spectrometer 400 MHz. Chemical shifts are expressed in ppm (δ), using tetramethylsilane (TMS) as internal standard ($\delta_H = 0.00$). Fluorimetric and spectrophotometric analyses were performed using GENios Plus reader (Tecan, Segrate, Italy).

2.2. Synthesis and characterization of bPEI-PEG copolymers

The determination of the amino groups of 25 kDa *b*PEI was obtained from the ^{13}C NMR spectrum of *b*PEI in D_2O ($\approx$ 150 mg/mL, 2048 scans, probe temperature 30 °C) using inverse gated decoupling sequences to avoid NOE effects, as reported in literature by von Harpe and colleagues [22]. Similarly to their results, the relative ratio of different amino functions in 25 kDa *b*PEI was 0.32 primary, 0.38 secondary, 0.30 tertiary amines. 25 kDa *b*PEI was dissolved in phosphate buffered saline (PBS) to a concentration of 1.7 mg/mL (pH 7.0) and 8.0×10^{-2}, 4.0×10^{-2}, 2.0×10^{-2}, 1.0×10^{-2} and 5.0×10^{-3} equivalents of NHS-PEG-MAL (calculated with respect to *b*PEI primary amines ([NH_2])) dissolved in DMSO were added dropwise under stirring to 8 mL of *b*PEI solution. The solution was incubated at 30 °C for 3 h (Fig. 1a). The products were next purified by repeated ultrafiltration through a Vivaspin 6 column MWCO 10,000 (GE Healthcare Europe, Milan, Italy) and finally freeze-dried.

The copolymers were next solubilized in D_2O and ^{1}H NMR analysis was performed to quantify the amount of PEG grafted on *b*PEI. The degree of substitution of the *b*PEI-PEG copolymers was calculated from the ^{1}H NMR spectra by using the following formula:

$$DS = 100 \times \frac{area\ CH_2CH_2O/n}{area\ CH_2CH_2N \times \frac{\%NH_2}{100}}$$

where *area* CH_2CH_2O and *area* CH_2CH_2N are the area of the signals at δ: 3.7 ppm (associated to PEG) and at δ: 2.6–3.2 ppm (associated to the *b*PEI) respectively. The parameter n is the average number of -CH_2CH_2O- units of NHS-PEG-MAL. A value of $n = 70$ can be assumed on the basis of the 3.4 kDa average molecular weight of NHS-PEG-MAL. Finally, $\%NH_2$ represents the percentage of NH_2 of 25 kDa *b*PEI as determined by the ^{13}C NMR analysis using an inverse gated decoupling sequences to avoid NOE effects (32%, see Ref. [23]).

2.3. Synthesis of bPEI-PEG-RGD derivatives

After ^{1}H NMR characterization, copolymers were freeze-dried again and then solubilized in PBS (pH 7.0) to a final concentration of *b*PEI-PEG of 0.35 mg/mL. The amount of copolymers recovered was evaluated by 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay, as previously reported [6]. Copolymers were next added with L-cysteine (*L*-cys) or with the *L*-cys-containing *c*RGD and *l*RGD peptides, in excess (1.5 equivalents) with respect to maleimide moieties of PEG-MAL. Each maleimide reacts with the sulfhydryl of *L*-cys forming a roughly stable thioether bond. Solutions were incubated under stirring for 2 h at room temperature (r.t.) (Fig. 1b) and then purified by repeated ultrafiltration by means of a Vivaspin 6 column MWCO 10,000. Unreacted thiols present in the filtrate were quantified by Ellman's test to evaluate the coupling efficiency. Briefly, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was dissolved in 100 mM potassium phosphate (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) to a final