Biomaterials 35 (2014) 567–577

Contents lists available at ScienceDirect

Biomaterials

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

The effect of serum in culture on RNAi efficacy through modulation of polyplexes size

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ARTICLE INFO

Article history:
Received 28 August 2013
Accepted 25 September 2013
Available online 11 October 2013

Keywords:
RNA interference
Non-viral vectors
Pullulan-spermine
Serum tolerance
Endo-lysosomal escape

ABSTRACT

Serum in the culture medium is one crucial factor that compromises RNAi efficiency of non-viral vectors. However, mechanistic roles of serum in siRNA delivery remain unknown. In this work, we took one cationic polymer, pullulan chemically modified by spermine (termed as pullulan-spermine, Ps), as a siRNA carrier model to investigate the effects of serum on key steps in siRNA delivery including formation of Ps and siRNA polyplexes (Ps-siRNA), cellular uptake, lysosomal escape, and cytotoxicity. We demonstrate that low serum concentration (1.25% and 2.5%) in culture medium results in large particles of Ps-siRNA, while high serum concentration (10%–40%) leads to small particles of Ps-siRNA. The larger particles initiated the internalization of siRNA more effectively in comparison to the smaller ones. The engulfed Ps-siRNA particles mainly locate in lysosomes. The large particles exhibited stronger abilities of destabilizing lysosomes than that of the small particles as large Ps-siRNA particles contain more amines and subsequently elicit a stronger proton sponge effect which results in more effective lysosomal escape of siRNA. Despite the lower RNAi efficiency, the small particle of Ps-siRNA in the high serum medium generates much lower cytotoxicity. These findings explain why serum significantly affects RNAi and also propose a strategy for improving RNAi efficiency and safety by modulating serum concentration and enhancing lysosomal destabilization.

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1. Introduction

RNAi-based therapies hold great promises in fighting against various diseases such as cancers, virus infections and diabetes [1,2]. However, there are still multiple obstacles on the way to their clinical application [3]. One of the major barriers is lack of proper carriers that meet the practical requirements. As virus vectors have high risks of mutagenesis and immunogenicity [4], non-viral delivery carriers for siRNA with low toxicity and high transfection efficiency are urgently expected. Cationic polymers are one favorite candidate in non-viral siRNA carriers, attracting ever-growing attention due to their distinctive properties. However, their efficacy of silencing target genes is not ideal either for laboratory research or for therapeutic applications [5–7], leaving ample space for further study.

Cationic polymers compact siRNA molecules to form polyplexes that is easily taken up by a variety of cells [8,9]. However, the polymers strongly adsorb serum proteins in the culture medium and biological fluids including blood, which renders the transfection ineffective [10–13]. Hence serum-free medium is required in transfection protocols *in vitro* for most of the cationic carriers to ensure transfection efficiency even if the biocompatibility is compromised. However, the serum-free condition is not suitable for all cells, especially primary or stem cells [14]. Furthermore, there are abundant proteins in biological fluids, which make it impossible to create a serum-free environment when applying cationic polymers *in vivo*. Although several studies have suggested that serum plays crucial roles in transfection efficiency when cationic polymers or lipids are used as gene vectors [15–19], the underlying mechanism of the serum effects is unclear.

In this work, we aimed to probe the underlying mechanisms of how serum acts in siRNA delivery when cationic polymers are used as carriers. One typical cationic polymer, spermine-modified pullulan (Ps), is selected as the model. Pullulan is a hydrophilic polysaccharide, which can be cationized by spermine modification for nucleic acids delivery in serum-free conditions [20–22].

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0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.09.102

We hypothesized that serum concentration would play a pivotal role in modulating the size of the polyplexes of Ps and siRNA (Ps-siRNA), which would further determine the cellular uptake and lysosomal escape, and finally affect RNAi efficiency. To address these issues, we measured the size of Ps-siRNA in the culture media with serum concentrations set from 0% to 40%. The uptake, cellular distribution, lysosomal escape and RNAi efficiency of Ps-siRNA were analyzed using flow cytometry and confocal microscopy. Furthermore we examined the RNAi efficiency in a high serum medium for Ps-siRNA preformed in low serum.

2. Materials and methods

2.1. Materials

EGFP siRNA, NC siRNA (with no target interior the cells) oligos and fluorescently-labeled siRNA (NC siRNA-FAM) were synthesized by GenePharma (Shanghai, China). The sequences are as follows: EGFP siRNA: 5′-GCAAGCUGACCCUGAAGUUCA-3′ [23], NC siRNA: 5′-UUCUCCGAACGUGUCACGU-3′ [24]. Lipofectamine™ 2000 (Lipo) transfection reagent was purchased from Invitrogen. Deionized water (18.2 $M\Omega cm^{-1}$) produced by Milli-Q Synthesis (Millipore Co., USA) was used in all the experiments. Serum-free medium (opti-MEM) was purchased from Invitrogen. Pullulan and spermine were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma–Aldrich, Inc. (St. Louis, MO.), respectively. All chemicals were used as received.

2.2. Atomic force microscopy (AFM) analysis of Ps-siRNA polyplexes

After the formation of Ps-siRNA polyplexes, 3 μl of the Ps-siRNA was dropped onto the mica sheets and the water was allowed to evaporate at the room temperature. After that, the morphology of Ps-siRNA was visualized using an atomic force microscope (AFM) (Dimension 3100, Veeco, Digital Instruments, Santa Barbara, CA). Imaging was performed in the tapping mode by using oscillating linear silicon tips with a resonance frequency around 350 Hz.

2.3. Dynamic light scattering (DLS) analysis

DLS analysis was performed using a Zetasizer Nano ZS90 (Malvern instruments) and the measurements were carried out at room temperature with a scattering angle at 90°. Briefly, the Ps-siRNA was dispersed in 1 ml water. After 30 min, 24 and 48 h, the size and zeta potential were measured. For the measurements of Ps-siRNA dispersed in serum-containing media, Ps-siRNA with N/P ratio of 40 was dispersed in culture media (1 μg/ml siRNA) with serum content ranged from 0% to 40% for 4 h.

2.4. Cells culture

Human breast cancer cell line MDA-MB-231 was obtained from the Cell Resource Center of Chinese Academy of Medical Sciences (Beijing, China). The MDA-MB-231-GFP cell constantly express EGFP protein was purchased from the Cellcyto Company (Beijing, China). Cells were cultured in Leibovitz's L-15 Medium (Gibco Invitrogen, CA, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin and maintained in a 37 °C humidified incubator with a low-CO_2 environment.

2.5. siRNA transfection procedure

About 1.6×10^5 MDA-MB-231-GFP cells were seeded in the 6-well plates and left overnight to allow cell adherence. Considering the balance between cytotoxicity and transfection efficiency of Ps-siRNA (Supplementary information), N/P ratio of 40 was used in this study. Cells were incubated with Ps-siRNA (1 μg/ml siRNA) in culture media with serum content ranged from 0% to 40% for 4 h. Then the medium was changed and cells were further cultured for 44 h before the flow cytometric analysis. The serum free medium was the opti-MEM (Invitrogen, CA, USA). Cells with no treatment or treated by naked siRNA or Ps-siRNA (NC siRNA) in medium of 10% serum were set as controls and cells treated by Lipofectamine 2000 (Lipo) (Invitrogen) were set as positive control.

2.6. Environmental scanning electron microscopy (ESEM)

About 5×10^4 MDA-MB-231 cells were seeded onto the coverslips in 24-well plate and left overnight to allow adherence. Then the media were replaced with 0.5 ml of fresh media with different serum concentrations containing Ps-siRNA (N/P = 40, 1 μg/ml siRNA) and incubated for 4 h. Cells cultured in normal conditions (10% serum) were set as control. After that, the coverslips were rinsed twice with PBS and fixed with 1% glutaraldehyde of PBS solution. Following gradient dehydration with alcohol, the cell samples were subjected to carbon dioxide critical point drying. The ESEM observation was performed on the environmental scanning electron microscope (ESEM, Quanta 200 FEG, FEI Co.) with different magnification.

2.7. MTS assay

MTS assay kit (CellTiter 96 @ AQueous Non-Radioactive Cell Proliferation Assay, Promega) was chosen to evaluate the viability of MDA-MB-231 cells following the manufacturer's protocol. The cells were seeded in the 96-well plate at a density of about 8000 cells per well and cultured overnight to allow cells adherence. After that, cells were incubated in 100 μl culture media containing different concentrations of serum with Ps-siRNA (N/P = 40, 1 μg/ml siRNA) for 0.5, 1, 2, 3, 4 h. Cells cultured in media containing different concentrations of serum without Ps-siRNA were taken as the corresponding controls. Next, the cells were washed twice with PBS and then incubated with 100 μl fresh medium combined with 20 μl MTS at 37 °C for 90 min. The absorbance at 490 nm of wavelength was recorded with BioTek Synergy™ 4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, USA). Measurements were conducted in quadruplicate and the relative viability of cells after the treatment with Ps-siRNA was expressed as follow:

$$\text{Relative cell viability} = (\text{Absorbance cultured with Ps} - \text{siRNA})/(\text{Absorbance cultured without Ps} - \text{siRNA})$$

2.8. Measurement of Ps-siRNA sedimented on the plate

About 8000 cells/well were seeded onto the 96-well plates (Corning). The plate well with cells was negatively charged and the bare plate well was positively charged. The involved siRNA was siRNA-FAM. 200 μl of medium with various concentrations of serum-containing Ps-siRNA was added in the plate wells with or without cells seeded in. After 4-h incubation at 37 °C, the fluorescence of supernatant (FLs) was measured. The plate was washed twice with 100 μl PBS and the fluorescence from the 200 μl washing PBS (FLw) was measured. Finally, the fluorescent intensity on the plate well (FLp) was measured. The corresponding fluorescent intensity from medium, PBS and control cells without Ps-siRNA was deducted as backgrounds. The fluorescent intensity was determined on a BioTek Synergy™ 4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, USA) with the excitation and emission wavelengths set at 488 and 520 nm, respectively. Experiments were conducted in triplicate and the relative amount of Ps-siRNA on plate was expressed as follow:

$$\text{Relative amount of Ps} - \text{siRNA on plate} = \text{FLp}/(\text{FLp} + \text{FLw} + \text{FLs})$$

2.9. Flow cytometry

The internalization of siRNA was evaluated using a C6 AccuriR flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) with siRNA labeled by FAM (siRNA-FAM). After the incubation with Ps-siRNA (N/P = 40, 1 μg/ml siRNA) for 4 h, cells were rinsed thrice in PBS, detached by trypsination and collected by centrifugation at 1000 rpm for 5 min. The fluorescent intensity of the siRNA-FAM in cells was determined in FL1-A channel and the experiment was carried out in duplicate. The acquired data was analyzed using CFlow™ software (Accuri Cytometers).

2.10. Confocal microscopy

Cells were seeded on the coverslips in 24-well plate at a density of 5×10^4 cells per well. After adhesion overnight, cells were treated in culture media containing Ps-siRNA (N/P = 40, 1 μg/ml siRNA-FAM) with different serum concentrations for 4 h. For membrane staining, cells were washed twice with PBS and stained by 5 μM DiI (V22885, Invitrogen) for 10 min at room temperature. Then cells were rinsed twice with PBS and fixed in 1% paraformaldehyde/PBS for 15 min. After washing for 3 times with PBS, the coverslips were mounted with an aqueous mounting medium containing DAPI (Zhongshan Goldenbridge biotechnology Co, Beijing, China). For lysosomal staining, cells were treated by

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