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Gelatin nanospheres incorporating siRNA for controlled intracellular release

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ABSTRACT

The objective of this study is to develop a sustained release system of small interfering RNA (siRNA) inside cells aiming at a prolonged time period of gene suppression. Gelatin aqueous solution containing luciferase siRNA was coacelvated by acetone addition, followed by the glutaraldehyde (GA) crosslinking of gelatin to prepare gelatin nanospheres incorporating siRNA. The nanospheres were degraded with time in phosphate-buffered saline solution containing collagenase to release siRNA incorporated. The nanospheres were degraded more slowly as the GA concentration become higher, and consequently the rate of siRNA become lower. siRNA was released from the nanospheres as a result of nanospheres degradation. The nanospheres were internalized into colon 26 cells luciferase stably expressed, irrespective of the GA concentration. The gene expression was suppressed by the nanospheres incorporating siRNA capable for the longer-term release, and subsequently the time period of gene suppression was prolonged. The siRNA release inside the cell was observed, while the release period became longer for the slow-degraded nanospheres. It is possible that the intracellular siRNA release for a longer time period contributes to the prolonged time period of gene suppression.

1. Introduction

RNA interference (RNAi) is a gene silencing process in which mRNA was cleaved in the sequence-specific manner in the cell cytoplasm. The RNAi phenomenon was discovered by Fire et al. in 1998 [1], and demonstrated to occur in mammalian cells with small interfering RNA (siRNA) by Elbashir et al. [2]. siRNA is a synthetic double-stranded RNA (dsRNA) of 21–23 base pairs which can be molecularly designed to suppress the target sequence. It has been applied for biology and medicine to identify and investigate the gene mechanism regulating the biological function of cells as well as genetic engineering technology of cells. There are some problems to be improved, such as the low efficiency of transfection and the short duration of suppression [3,4]. Viral [5–8] and non-viral vectors [9–11] have been investigated to improve the transfection efficiency. However, there are rooms to be developed for the viral vectors, such as the immunogenicity and cytotoxicity. On the other hand, in the case of non-viral vectors, cationic liposomes [12–14], micelles [15,16], and nanospheres [17,18] have been extensively investigated. However, the efficiency is low and the time period of gene expression is short.

As one trial to tackle the short time period of gene suppression by siRNA, it is practically promising to use drug delivery systems (DDS) technologies. Among the technologies, the controlled release of siRNA is one of the possible strategies to resolve the problem. There have been reported on the controlled release of nucleic acids intracellularly [4,19–21] and extracellularly [22–24]. The intracellular controlled release must be promising to regulate the time period of siRNA activity because the active site is in the cytoplasm. Intracellular controlled release of plasmid DNA from gelatin microspheres has been reported to demonstrate the prolonged time period of biological activity [25].

The objective of this study is to evaluate the intracellular release of siRNA from gelatin nanospheres in terms of gene suppression period. Gelatin has been extensively used for food, pharmaceutical, and medical applications, and the bio-safety has been proven through its clinical use as biomaterials and drugs [25]. Another advantage is the availability of samples with various physicochemical natures of gelatin effective in the controlled release of proteins and low-molecular weight drugs [26–29]. Cationic gelatin can readily be prepared by simply introducing amine residues to the carboxyl groups of gelatin. Cationic gelatin carriers can release plasmid DNA extracellularly [30,31] and intracellulary [25].

In this study, gelatin nanospheres incorporating siRNA with different degradabilities were prepared by changing the condition of gelatin crosslinking, and the siRNA release from the nanospheres was evaluated. Gene suppression activity of nanospheres incorporating siRNA was evaluated in terms of siRNA release profiles. We examine the cellular internalization of gelatin nanospheres incorporating siRNA and the subsequent intracellular siRNA release.

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2. Materials and methods

2.1. Materials

Gelatin with an isoelectric point of 9.0 and the weight-avaraged molecular weight of 99,000, prepared by an acidic process of pig skin, were supplied from Nitta Gelatin (Nitta Gelatin Inc., Osaka, Japan). A duplex siRNA targeted at the firefly (GL3) luciferase gene (sense-sequence 50-CUU ACG CUG AGU ACU UCG AdTdT-30, and antisense, 30-dTdT GAA UGC GAC UCA UGA AGC U-50, GL3-siRNA) and that targeted at the green fluorescent protein (GFP) gene (sense-sequence 50-CUA CAA CAG CCA CAA CGU C dTdT-30, and antisense, 30-dTdT GAU GUU GUC GGU GUU GCA G-50, GFP-siRNA) was purchased from B-Bridge International (Sunnyvale, CA). Other chemicals were obtained from Nakarai Tesque (Kyoto, Japan) and used without further purification. Ethylenediamine was purchased from Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC), glutaraldehyde were purchased from Nacalai Tesque. Inc., Kyoto, Japan. Collagenase (Lot. 061M8626V) was purchased from Sigma–Aldrich Co. LLC, Missouri.

2.2. Preparation of cationized gelatin

The carboxyl groups of gelatin were chemically converted by introducing amino groups to cationize gelatin [32]. Ethylenediamine was added at a molar ratio of 50 to the carboxyl groups of gelatin into 250 ml of 100 mM phosphate-buffered solution (PBS, pH 5.0) containing 5.0 g of gelatin. Immediately after that, the solution pH was adjusted to 5.0 by adding 11 M HCl aqueous solution. Further, EDC was added at a molar ratio of 3 to the carboxyl groups of gelatin. The reaction mixture was agitated at 37 °C for 18 h, and then dialyzed against double-distilled water (DDW) for 48 h at room temperature. The dialyzed solution was freeze-dried to obtain a cationized gelatin. When determined by the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) method [33], the percentage of amino groups introduced into gelatin was 58.8 mol% per the carboxyl groups of gelatin.

2.3. Preparation of gelatin nanospheres incorporating siRNA

Gelatin nanospheres were prepared by a coacelvation method [34] with slight modification. Briefly, 1.25 ml of 5 wt% aqueous gelatin solution containing 174 nmol/ml of siRNA specific to luciferase was heated to 50 °C, and then 3–7 ml of acetone was added to the solution. The resulting nanospheres formed were cross-linked by adding glutaraldehyde (GA) (0.01–0.05 wt%) for 30 min. Next, acetone was removed by evaporation, and the resulting gelatin nanospheres were centrifuged for 5 min at 14,000 rpm at 4 °C, and resuspended in double-distilled water (DDW). The nanospheres were degraded in PBS containing 2 μg/ml of collagenase while the concentration of nanospheres was determined by the BCA Assay kit (Thermo Scientific, Rockford, IL). The nanospheres were placed in 0.1 M glycine solution in PBS to block the aldehyde groups unreacted for 12 h, followed by replacing with PBS with centrifuge at 14,000 rpm, and were stored at 4 °C until to use.

2.4. Iodination of siRNA

siRNA was labeled with ^{125}I [35] with slight modifications. Briefly, 100 μl of siRNA at a concentration of 1750 nmol/ml was incubated at 60 °C for 50 min with 2 μl of 0.3 mM Na_2SO_3, 5 μl of $Na^{125}I$, and 5 μl of 4 mM $TlCl_3$. 100 ml of 0.1 M Na_2SO_3, 900 ml of 0.1 M NaCl and 50 mM Tris, and 1 mM EDTA were added to the solution. After 30 min, free iodide was removed by gel filtration on PD10 column (GE Healthcare Bio-Sciences AB, Uppsalo, Sweden). ^{125}I was determined using a gamma counter (Auto Well Gamma System ARC-380 CL, Aloka Co., Ltd, Tokyo, Japan).

2.5. Dynamic light scattering and electrophoretic light scattering measurements of nanospheres incorporating siRNA

Gelatin nanospheres incorporating siRNA were resuspended in 10 mM PBS. The apparent size and zeta potential of nanospheres were measured by dynamic light scattering (DLS-7000, Otsuka Electronics, Osaka, Japan) and electrophoresis light scattering (ELS-7000 AS, Otsuka Electronics, Osaka, Japan), respectively.

2.6. Measurement of water content of gelatin nanospheres

The water content of gelatin nanospheres was measured by comparing the weight of nanospheres between the wet and dry states. The nanospheres (1 mg/ml) were centrifuged at 14,000 rpm, for 5 min to obtain them in the wet state and measured. Next, the nanospheres were freeze-dried to measure the weight in the dried state. The measurement was done independently 3 times.

2.7. Degradation and release test of siRNA from nanospheres incorporating siRNA

Gelatin nanospheres incorporating siRNA were suspended in 500 μl PBS for initial 6 h, followed by the sampling to collect the supernatant by centrifugation. Then, 500 μl of 2 μg/ml collagenase in PBS solution was added and incubated at 37 °C for various time periods. The solution was centrifuged at 14,000 rpm, 37 °C for 5 min while the supernatant was collected. After that, 500 μl of fresh 2 μg/ml collagenase PBS aqueous solution was added and continued to incubate at 37 °C. To measure the amount of gelatin, the supernatant was determined by the BCA Assay kit. The samples were used for each experiment 3 times independently unless mentioned otherwise.

Gelatin nanospheres incorporating radioiodinated siRNA were resuspended in PBS similarly and the similar experiment was performed. The radioactivity of supernatant was determined by the radioactivity measurement described above.

2.8. Cytotoxicity test

Mouse colon 26 tumor cells were seeded on each well of 24 well multi-dish culture plate (Corning Inc., Corning, NY) at a density of 1×10^4 cells/cm^2 and cultured in 500 μl of DMEM (Invitrogen Corporation, Ltd., Carlsbad, CA) with 10 vol% fetal calf serum (FCS) for 24 h. Then, the medium was exchanged to OPTI MEM (Invitrogen Corporation, Ltd., Carlsbad, CA), and the nanospheres incorporating siRNA were added to each well. The transfection culture was performed for 3 h, and thereafter the number of cells proliferated was evaluated with a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) -2H-tetrazolium (WST-8) assay kit (Nacalai Tesque. Inc., Kyoto, Japan). Each experiment was performed 3 times independently.

2.9. Microscopic observation of nanospheres internalized into cells

Gelatin nanospheres were fluorescently labeled with Rhodamine B according to the method reported [36]. Colon 26 were seeded on each well of 6 well multi-dish culture plate (Corning Inc., Corning, NY) at a density of 1×10^4 cells/cm^2 and cultured in 1 ml of DMEM medium with 10 vol% FCS for 24 h. Then, the medium was exchanged to OPTI MEM, and the nanospheres incorporating siRNA (0.68 mole/mole gelatin) were added to each well. The transfection culture was performed for 3 h, and thereafter the cells were incubated in the DMEM containing FCS further for 3 days. At different time intervals, cells were washed with PBS and fixed with 4 vol% paraformaldehyde. The nuclei of cells were stained with Hoechst 33258 (Sigma–Aldrich Co., St. Louis, MO). Imaging data of cells were collected on a Nikon ECLIPSE 90i confocal laser scanning microscope (Nikon Corp., Tokyo, Japan).

2.10. Quantification of cellular internalization of nanospheres

Colon 26 were seeded on 100 mm-cell culture dish (Corning Inc., Corning, NY) at a density of 1×10^4 cells/cm^2 and cultured in 10 ml of DMEM medium with 10 vol% FCS for 24 h. Then, the medium was exchanged to OPTI MEM, and the gelatin nanospheres incorporating radioiodinated siRNA were added to the dish. The transfection culture was performed for 3 h, and thereafter the cells were incubated in DMEM with 10 vol% FCS further for 3 days. At different time intervals, the cells were collected by the trypsinization and centrifuge at 1000 rpm. The amount of siRNA was measured similarly by the radioactivitiy. Experiments were performed independently 3 times for each group.

2.11. Evaluation of gene suppression by nanospheres incorporating siRNA

Gelatin nanospheres incorporating siRNA specific to luciferase or green fluorescent protein (GFP) were prepared similarly. Colon 26 cells stably luciferase expression were seeded on each well of 12 well multi-dish culture plate (Corning Inc., Corning, NY) at a density of 1×10^4 cells/cm^2 and cultured in 1 ml of DMEM medium with 10 vol % FCS for 24 h. Then, the medium was exchanged to OPTI MEM, and the nanospheres incorporating siRNA (10 μg/ml) were added to each well. The transfection culture was performed for 3 h, and thereafter the cells were incubated in DMEM with 10 vol%FCS further for 3 days. At different time intervals, luciferase gene expression was quantified using a commercial kit (Luciferase Assay System, Promega). The light unit was determined using a luminometer (Micro Luminat Plus LB 96V, Berthold). The total protein of each well was determined using the BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL) to normalize the luciferase activity in terms of cell number.

2.12. Statistical analysis

Data were expressed as the means ± standard deviations. They were analyzed using Tukey–Kramer paired comparison test, while the significance was accepted at $p < 0.05$.

3. Result

3.1. Characterization of gelatin nanospheres incorporating siRNA

Fig. 1 shows the effect of acetone amount on the amount of siRNA incorporated in the nanospheres. The amount of siRNA incorporated saturated at 4 ml of acetone. The following experiments were performed at 5 ml of acetone added. Fig. 2 shows the