Efficient Preparation of Cationized Gelatin for Gene Transduction

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We previously reported gene therapy using cationized gelatin microspheres of ϕ20-32 μm, prepared from pig skin, as a transducing agent, but although the gelatin offered various advantages, its yield was extremely low (only 0.1%). In this study, we markedly improved the yield of ϕ20-32 μm cationized gelatin microspheres and prepared a newly less than ϕ20 μm cationized gelatin. Conventionally, cationized gelatin is prepared by cationization, particulation by agitation, and cross-linking. The yield is determined by the particulation step, for which we had used a three-necked distillation flask of 500 mL and an agitation speed of 420 rpm. The yield was significantly increased from 0.13 ± 0.02% to 8.80 ± 1.90% by using a smaller flask of 300 mL and an agitation speed of 25000 rpm ($p < 0.01$). We could also prepare cationized gelatin of less than ϕ20 μm, which had not been possible previously. We confirmed that efficient gene introduction into peritoneal macrophages could be achieved with the new cationized gelatin.

Key words: gelatine microsphere, macrophage, yield

INTRODUCTION

Efficient gene transduction methods are necessary for gene therapy [1], and currently available methods can be divided into viral vector techniques and non-viral approaches, such as lipofection or electroporation. Viral vectors such as adenovirus or retrovirus offer high transduction efficiency, but there are questions regarding safety [2]. On the other hand, the efficiency of transduction with non-viral vectors is generally poor [3]. In recent years, nucleofection has been developed for highly efficient gene transduction, but it can be applied to only certain cells, and it causes damage in some cases [4].

We showed that intramuscular injection of FGF-4 gene-gelatin complex induced significantly greater angiogenesis than injection of the bare FGF-4 gene [5]. Furthermore, we showed that adrenomedullin (AM) gene-gelatin complex effectively transduced the AM gene into endothelial progenitor cells (EPCs), and the transduced EPCs had a therapeutic effect in pulmonary hypertension [6]. In those studies, we used cationized gelatin microspheres of ϕ20-32 μm, derived from pig skin. However, the yield of gelatin was only about 0.1%. Cascone *et al*. reported that the preparation of nanoparticulate gelatin required an agitation speed of cationized gelatin and olive oil of more than 10,000 rpm [7], so in this study, we examined whether the use of a higher agitation speed during preparation of the gelatin particles would increase the yield and reduce the particle size in our procedure. We also confirmed the efficacy of the gelatin particles thus obtained for gene transduction.

MATERIALS

Gelatin of pig skin origin (PI 9) was purchased from Nitta Gelatin Corp, Japan. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), β-alanine, acetone, glycine, hydrochloric acid, ethylenediamine, olive oil, glutaraldehyde solution (GA), potassium dihydrogenphosphate, disodium hydrogenphosphate and sodium hydrogen carbonate were purchased from Wako Pure Chemical Industry, Japan. Liquid nitrogen was purchased from Tomoe Corporation, Japan.

Rat peritoneal macrophages were collected by intraperitoneal injection of thioglycolate culture medium as previously described [8]. DNA encoding GFP with the cytomegalovirus enhancer-chicken β-actin hybrid promoter was constructed [5].

METHODS

Conventional preparation of gelatin microspheres involves three steps: (1) cationized gelatin production, (2) microsphere production and (3) cross-linking of cationized gelatin microspheres. In order to increase the yield of cationized gelatin microspheres, we aimed to improve the second step, i.e., microsphere production.

Cationized gelatin production

PI 9 (10 g) was completely dissolved in 0.1 M phosphate buffer (PBS, 450 mL) containing potassium dihydrogenphosphate and disodium hydrogenphosphate. Ethylenediamine (31.1 mL) and hydrochloric acid were added, and the pH was adjusted to 5.0. EDC (5.35 g) was added to this solution, which was made up to 500 mL with PBS and left for 18 hours. The solution was

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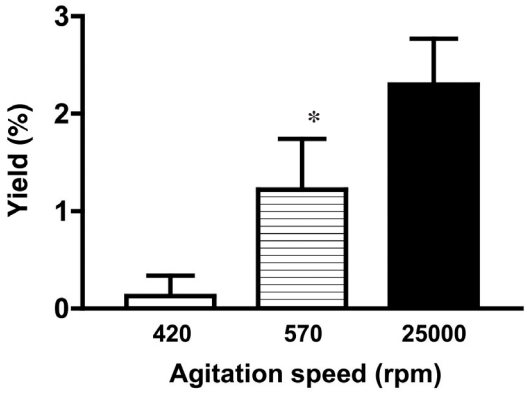

Fig. 1 Yield of ϕ20-32 μm cationized gelatin using a 500 mL three-necked distillation flask.
The open bar shows the control (420 rpm) group, horizontal lined bar, the 570 rpm group and closed bar, the 25,000 rpm group. Data are presented as mean ± SEM. The yield in the 570 rpm group was significantly higher than that in the control group (*p <0.001 vs 420 rpm).

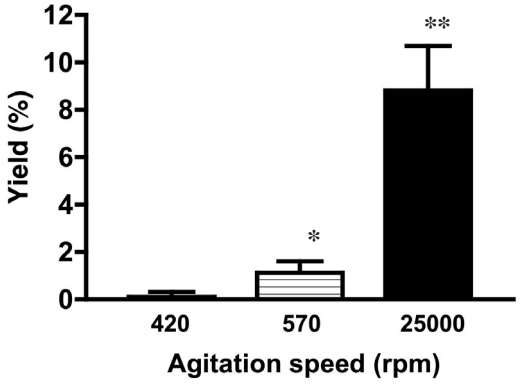

Fig. 2 Yield of ϕ20-32 μm cationized gelatin using a 300 mL three-necked distillation flask.
The open bar shows the 420 rpm group, horizontal lined bar, the 570 rpm group and closed bar, the 25,000 rpm group. Data are presented as mean ± SEM. The yield in the 570 rpm group was significantly higher than that in the 420 rpm group (*p < 0.001 vs 420 rpm). The yield in the 25,000 rpm group was significantly higher than that in the other groups (**p <0.0001 vs other groups).

then dialyzed for 2 days with 16 changes of water. After the dialysis, this solution was freeze-dried for 4 to 7 days to afford cationized gelatin. The conversion rate of carboxyl groups to amino groups was measured by the TNBS method to characterize the product [13].

Microsphere production

In the conventional procedure, cationized gelatin aqueous solution and olive oil were placed in a three-necked distillation flask of 500 mL at 40°C, and centrifuged at 420 rpm for 10 minutes. This solution was stirred for 30 minutes at 0°C. Acetone was added, and the mixture was centrifuged. After centrifugation, the oil layer was removed, and acetone was added. This solution was centrifuged again, homogenized and sieved with ϕ20 μm, ϕ32 μm and ϕ90 μm sieves (Test sieves, #JIS Z 8801, Tokyo Screen Co., Ltd., Japan). The microspheres was dried in a refrigerator overnight, and the yield of each fraction was determined.

In this experiment, we examined the effect of increasing the agitation speed to 570 rpm and 25000 rpm, and the effect of using a smaller three-necked distillation flask (300 mL) to obtain smoother mixing.

Cross-linking of cationized gelatin microspheres

Acetone and hydrochloric acid (7:3) were added to cationized gelatin microspheres, the crosslinking agent GA was added, and the reaction was allowed to proceed for 24 hours. After the reaction, centrifugation was performed and the supernatant was removed. Glycine solution (100 mM) was added to remove GA for one hour. After centrifugation, the supernatant was removed and cross-linked microspheres were cooled with liquid nitrogen, freeze-dried, and weighed.

Gene introduction with the newly developed cationized gelatin microspheres

Gene gelatin complex was prepared by mixing 2 mg of cationized gelatin and 50 μg/100 μl gene (GFP or luciferase). The complex was incubated with rat peritoneal macrophages for 14 days. Effective gene introduction was demonstrated by cellular expression of GFP or luciferase.

RESULTS

Effect of agitation conditions on yield of cationized gelatin

In the conventional method (500 mL/420 rpm), the yield of the ϕ20-32 μm cationized gelatin was 0.13 ± 0.02%, but when the agitation speed was increased to 570 rpm, the yield rose to 1.22 ± 0.52% (*p < 0.001 vs 420 rpm). The yield was further increased to 2.30 ± 0.47% by increasing the agitation speed to 25000 rpm from 570 rpm, but this further increase was not statistically significant (p=0.136)(Fig. 1). Next we used a smaller (300 mL) three-necked distillation flask with agitation at 420 rpm, 570 rpm and 25,000 rpm. When the agitation speed was increased to 570 rpm, the yield rose to 1.12 ± 0.49% from 0.11 ± 0.01% (*p <0.001 vs 420 rpm). The yield was markedly increased to 8.80 ± 1.90% by increasing the agitation speed to 25,000 rpm from 570 rpm (**p <0.0001 vs 420 or 570 rpm) (Fig. 2).

The yields of different-sized microsphere fractions are summarized in Table 1. The use of the highest agitation speed and the smaller flask allowed us to obtain cationized gelatin microspheres of less than ϕ20 μm, which we had not been able to prepare with the conventional method, in addition to increasing the total yield of the cationized gelatin microspheres.

Gene introduction with the new cationized gelatin

We examined the efficiency of the new, smaller-sized cationized microspheres for gene introduction into rat peritoneal macrophages. As shown in Figure 3A, after coincubation of the macrophages and GFP

Table 1 Yields of different-sized cationized gelatines (%).

Flask size (mL) / Agitation speed (rpm)	$>\phi$20 μm	ϕ20-32 μm	ϕ32-90 μm	ϕ90 μm $>$
500/420	0	0.13 ± 0.02	15.1 ± 3.21	14.0 ± 5.30
500/570	0	1.22 ± 0.52*	13.2 ± 3.52	14.1 ± 6.13
500/25000	0	2.30 ± 0.47	17.2 ± 4.10	7.23 ± 1.60
300/420	0	0.11 ± 0.01	19.1 ± 5.32	17.4 ± 7.12
300/570	0	1.12 ± 0.49**	22.1 ± 7.62	11.3 ± 2.52
300/25000	2.12 ± 0.21	8.80 ± 1.90***	32.2 ± 11.0	9.10 ± 2.10

Data are presented as mean ± SEM. *$p < 0.001$ vs 500/420, **$p < 0.001$ vs 300/420
***$p < 0.0001$ vs 300/420 or 300/570

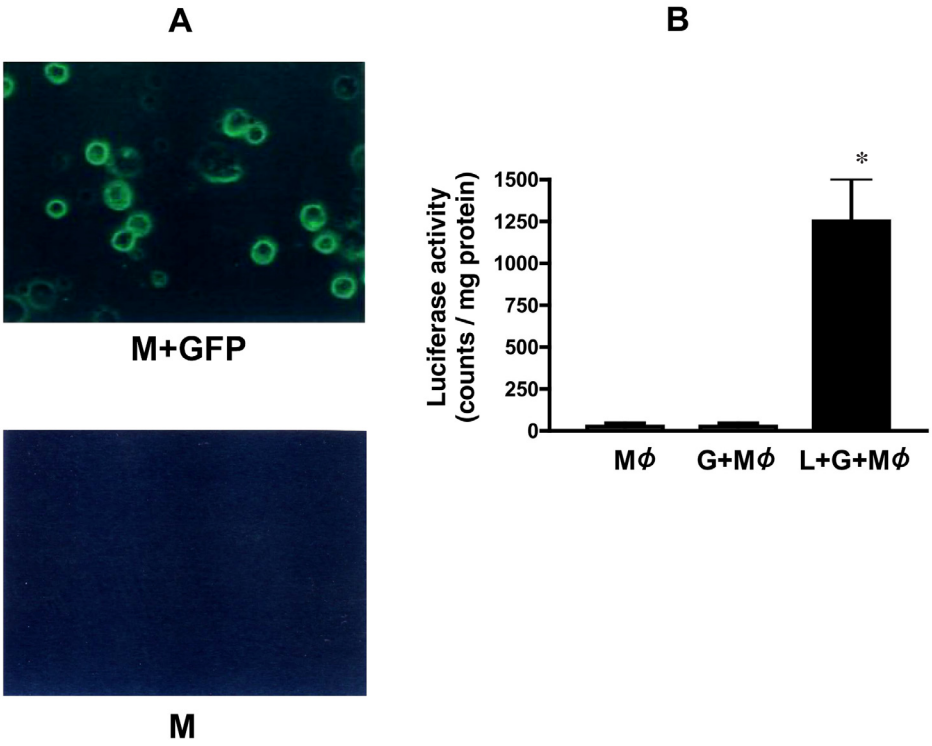

Fig. 3 Gene introduction into rat peritoneal macrophages with cationized gelatin.
A. Upper column: Peritoneal macrophages were coincubated with GFP gene-cationized gelatin complex (M + GFP) for 14 days. GFP was expressed in the cytoplasm of the macrophages. Lower column: Peritoneal macrophages were coincubated with cationized gelatin complex (M) for 14 days. GFP was not expressed in the macrophages.
B. Luciferase activity. Peritoneal macrophages were coincubated with luciferase gene-cationized gelatin complex (L + G + Mϕ, closed bar) or with cationized gelatin (G + Mϕ, horizontal lined bar) or with no additive (Mϕ, open bar) for 14 days. Data are presented as mean ± SEM. The luciferase activity of L+G+Mϕ was significantly higher than that of Mϕ or G+Mϕ (*$p < 0.01$).

gene-cationized gelatin complex, the cells expressed GFP. Coincubation of the macrophages and cationized gelatin alone did not result in expression of GFP. As shown in Figure 3B, after coincubation of the macrophages and luciferase gene-cationized gelatin complex, the cells expressed luciferase activity of 1251 ± 257 (counts/mg protein) on day 14, while the activity in the control group, in which macrophages were cultured alone, was only 5 ± 2 (counts/mg protein) (*$p < 0.01$). The luciferase activity was 8 ± 2 (count/mg protein) in the macrophage + cationized gelatin group.

DISCUSSION

In this study, we showed that the yield of cationized gelatin microspheres of ϕ20-32 μm increased with increasing agitation speed and with the use of a smaller three-necked flask for the agitation of cationized gelatin with olive oil. In addition, cationized gelatin microspheres smaller than ϕ20 μm could be prepared for the first time with the highest agitation speed and the smaller flask.

In the conventional method, 30% of cationized

gelatin finally formed microspheres, but almost all were larger than ϕ32 μm [9]. Gene introduction with cationized gelatin microspheres involves cellular phagocytic activity, which is inefficient for particles as large as ϕ30 μm [6]. By using a speed as high as 25,000 rpm and a smaller flask (300 mL), we were able to increase the yield of cationized gelatin microspheres of ϕ20-32 μm to 8.80%. Furthermore, we could manufacture cationized gelatin microspheres smaller than ϕ20 μm, which could not be obtained by the conventional method, in a yield of 2.12%.

Gedanken *et al.* succeeded in the production of nanoparticles from various chemicals by using ultrasonic irradiation [10]. However, when we used an ultrasonic homogenizer for agitating cationized gelatin and olive oil, the yield decreased to 1.30% .

We previously showed that our gelatin microsphere-gene complexes were introduced into cells by phagocytosis. Since the efficiency of cellular phagocytotic activity is greater for smaller particles, the development of smaller-sized cationized gelatin microspheres is expected to increase the efficiency of gene introduction via phagocytosis. Further, Kaul *et al.* showed that gene introduction into fibroblasts, which exhibit endocytosis but not phagocytosis, was possible by using nanoparticles of polyethylene glycol [11]. Therefore, if our method can be extended to obtain cationized gelatin microspheres in the nano size range, the variety of cells to which they would be applicable may be considerably extended. It is still the case that a gene introduction method with adequate safety and efficiency for clinical application is not yet available [12]. The ingredient, gelatin, used in this study is already in clinical use, and is considered to be safe. Cationized gelatin microspheres cannot be used to introduce genes into all types of cells, and the efficiency of gene introduction is lower than that of viral vectors. However, the use of cationized gelatin microspheres to introduce a gene into endothelial progenitor cells did have an apparent and prolonged therapeutic effect [5, 6]. The smaller cationized gelatin microspheres developed in this study may provide increased efficiency of gene introduction into various cells.

In conclusion, we have improved the preparation of cationized gelatin microspheres for gene transduction, obtaining a greater yield, as well as smaller microspheres, which may have clinical potential.

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