

[研究論文]

2-DIETHYLAMINOETHYL(DEAE)-DEXTRAN-MMA GRAFT COPOLYMER FOR NON-VIRAL GENE DELIVERY*

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Abstract

For non-viral gene delivery vectors, a stable and soap-less latex of DEAE-dextran-MMA graft copolymer (DDMC) has been developed. Transfection activity was determined with HEK293cell line under the condition with a serum using the X-gal staining method and a higher value of 5 times or more was confirmed for DDMC samples than for the starting DEAE-dextran hydrochloride.

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Introduction

In vivo gene delivery has allowed the study of gene expression via insertion of foreign genes or alteration of existing genes. However, some dangerous adverse effects remain associated with the use of viral vectors. Non-viral gene delivery vectors may be a key technology in circumventing the immunogenicity inherent in viral-mediated gene transfer. DEAE-dextran has been used for a non-viral gene delivery vector^{1,2)}. But these cationic polysaccharides, such as DEAE-dextran, may not be superior to viral vectors with a transfection efficiency. For a safety and a high transfection efficiency, many efforts have been done in the field of non-viral gene delivery vector³⁻⁵⁾. Especially, DEAE-dextran has been investigated increasing a transfection efficiency and found several good conditions for a human macrophage⁶⁾. DEAE-dextran, having strong adsorbing properties with DNA or RNA due to its cationic properties, is found to change its adsorbing power for nucleic acids by

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pH and ionic strength^{7,8)}. It is well-known dextran-MMA graft copolymer having a hydrophilic-hydrophobic micro-separated-domain have a good affinity to a cell membrane⁹⁾. The present paper is related to a novel graft-copolymer having some possibilities for a non-viral gene delivery vector that is composed of a cationic derivative of dextran and an vinyl monomer. DDMC was obtained by graft-polymerizing MMA onto DEAE-dextran, in water using ceric ammonium nitrate(CAN) to obtain a stable latex of DDMC¹⁰⁾, which is very effective for a non-viral gene delivery¹¹⁻¹³⁾.

Experimental

Preparation of DDMC. Samples DDMC1 and DDMC2 in Table 1 were prepared as described below: 2g of 2-diethylaminoethyl(DEAE)-dextran hydrochloride (nitrogen content 3%) derived from dextran having Mw500,000 was dissolved in 100 ml of water, and then 4 ml, and 3 ml of methyl methacrylate (MMA), for samples DDMC1, and DDMC2, was added respectively. With stirring, the air in the reaction vessel was fully replaced with N₂ gas. To the solution was added 0.1 g of CAN and 15 ml. of 0.1N nitric acid, and the mixture was reacted with stirring for 1 hour at 30°C. Then, 3 ml of a 1% aqueous solution of hydroquinone was added to stop the reaction, and the resulting latex of DDMC was purified with a water dialysis using a cellophane tube in order to remove the un-reacted MMA, ceric salts, and nitric acid. The resulting latex of DDMC was stable and soap-less.

Characterization of DDMC. The resulted DDMC precipitated by methanol is insoluble in water and acetone at 25°C. In view of the fact that DEAE-dextran hydrochloride is soluble in water and poly(MMA) is soluble in acetone, it is evident that the DDMC is not a mixture of DEAE-dextran and poly(MMA).

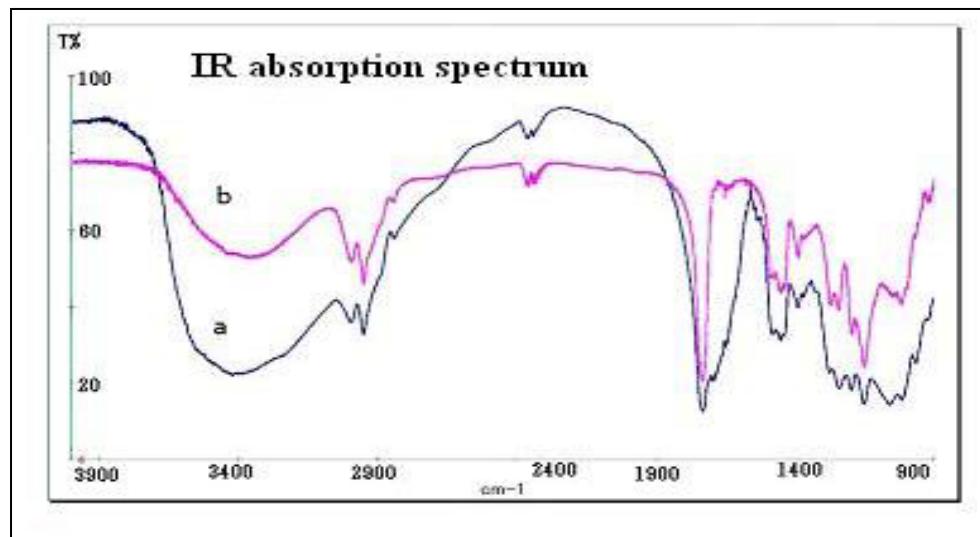


Fig.1. IR absorption spectra of DEAE-dextran-MMA Graft Copolymer and the complexes between DNA and DEAE-dextran-MMA Graft Copolymer: a, complex of DDMC2/DNA; b, DDMC2.

The infrared absorption spectrum of DDMC as shown in Fig.1 has some characteristic absorption bands at 1730 cm⁻¹ and at 1000 to 1150 cm⁻¹, which is attributed to the carbonyl group of poly(MMA) and the pyranose ring of DEAE-dextran, respectively. Thus, the resulting DDMC exhibits different solubility from DEAE-dextran and poly(MMA) and shows the above-described characteristic absorption in infrared absorption spectrum. From this fact, it is judged that the resulting DDMC is a compound graft-polymerized.

Reaction between DDMC and DNA. For reaction between DDMC and DNA, to DNA(EX Salmon Sperm) solution(20mg/ml), solution(10mg/ml) of the resulting latex of DDMC was added drop-wise in order to obtain the complex of DDMC/DNA.

Table 1. Properties of DEAE-dextran-MMA Graft Copolymers

Sample	Weight-increase(%)	Precipitation time(hrs) by DNA
DDMC1	150	2.0
DDMC2	100	1.0
DEAE-dextran	0	96.0

Weight increase (%)=(weight of MMA used/weight of DEAE-dextran hydrochloride used)×100

infrared absorption spectra of the resulting complex between DDMC2 and DNA. The spectrum of the complex has some characteristic absorption bands at 1730cm^{-1} , 1220cm^{-1} , 1000 to 1150cm^{-1} , and at 3450cm^{-1} which is attributed to the carbonyl group of poly(MMA), P-O stretching vibration of DNA, the pyranose ring of DEAE-dextran, and the diethylaminoethyl(DEAE) group of DEAE-dextran, respectively.

Transfection. The HEK293cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 DNA. A pCAGGS/LacZ, which expresses β -galactosidase at eukaryotic cells, was inserted under CAG promoter of a plasmid, pCAGGS. Plasmids were amplified in *Escherichia coli* DH5 α and purified by Qiagn Mega plasmid purification kit(Qiagen). For transfection by DDMC/DNA, HEK293cells (15×10^4 cells) were seeded 35-mm culture dishes and incubated at 37° in a humidified atmosphere of 5% CO₂. In a sterile tube, diluted 10 μg of DNA in 270 μl of 1 × PBS. Added 14 μl of the autoclaved DDMC having a concentration of 10mg/ml to the DNA solution. Then mixed by vortexing briefly. Removed the growth medium(Dulbecco's modified medium with 10% fetal calf serum) from the cells to be transfet. Washed the cells twice with 1 × PBS. Added DDMC/DNA solution to cover the cells. Careened the dish slowly several times to ensure complete cover of the cells, and incubated at 37° for 30 minutes. Slowly careened the dish several times during the incubation. Added 1ml of growth medium, and incubated at 37° for 48-hours. After the incubation, transfection activity was determined using the X-gal staining method. Following transfection protocol, transfection of HEK293 by sample DDMC1 and DDMC2 was carried out using plasmid DNA.

Results

Transfection efficiency.

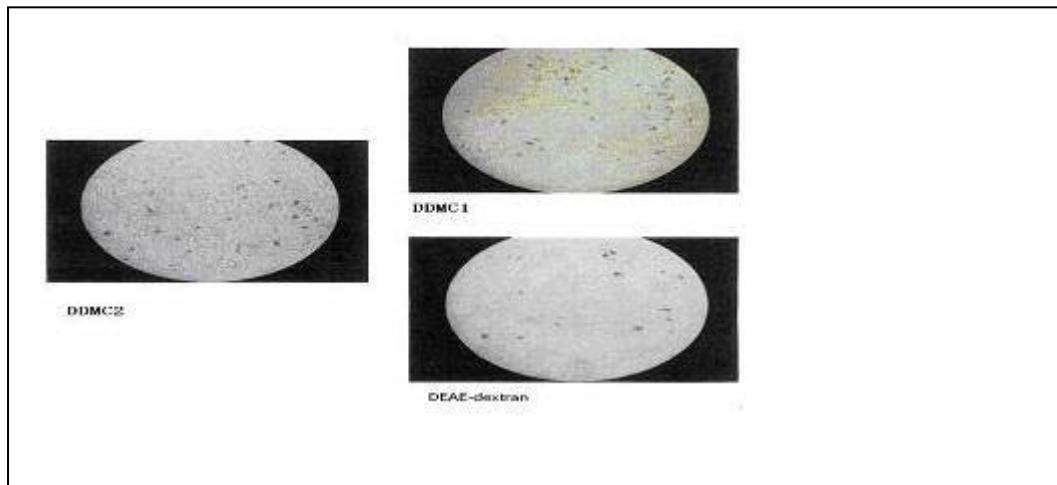


Fig. 2. Transfection of a monolayer of HEK 293 cells by DEAE-dextran-MMA Graft Copolymer

The obtained complex was insoluble in water, which is a good solvent for nucleic acids. These results show that the complex between DNA and DDMC must form a polyion complex(PIC). In the case of sample DDMC2, a complex between DNA and DDMC2 having a 100% of weight increases needed 1 hour to precipitate.

The complex between DNAand DMC1 having 150% weight increases needed 2 hours to precipitate, respectively. However,a complex between DNA and DEAE-dextran hyhydrochloride needed 96hours to precipitate at this condition. Figure 1 also shows the

As shown in Fig.2, with the transfection efficiency, transfection activity was determined using the X-gal staining method and a higher value of 5 times or more was confirmed for samples of DDMC1 and DDMC2 than for the starting DEAE-dextran hydrochloride. From the results, the transfection efficiency and the reaction rate of formation of the complex should increase when using DDMC hydrochloride instead of DEAE-dextran hydrochloride.

Cytotoxicity for the transfection. Figure 3 shows the change of transfection efficiency when using 2 times quantity of DDMC, for example 20 μ g DNA, of both DNA and DDMC as much as the protocol. Transfection of HEK293 by sample DDMC1 and DDMC2, carried out using 2 times quantity of both DNA and DDMC as much as the protocol, has shown 2 times higher efficiency than original by a transfection activity determined using the X-gal staining method. From these results to be impossible for DEAE-dextran, cytotoxicity for the transfection should be confirmed to decrease and improve when using DDMC hydrochloride instead of DEAE-dextran hydrochloride.

Discussion

DDMC transfection of cells has been carried out using the steps below: (a) Formation of a complex between DNA and DDMC. (b) Uptake. (c) Endosytosis (endosome). (d) Escape from endosomal vesicle. (e) DNA release in cytosol. (f) Nuclear entry. (g) DNA release and transcription in nucleus.

For transfection efficiency, it is very important to examine factors such as Uptake in step (b), Resistance of nuclease in step (c), Escape from endosomal vesicle in step (d), Nuclear targeting in step (f), and DNA release in step (g). The positively charged DEAE-dextran copolymer interacts with the negatively charged phosphate backbone of DNA. The resulting

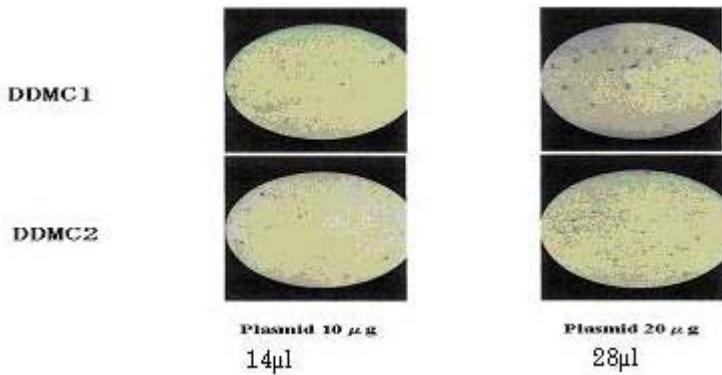


Fig. 3. Effect of cytotoxicity by DEAE-dextran-MMA Graft Copolymer on transfection of a monolayer of HEK 293 cells.

complex in step (a) is absorbed into cells by endocytosis. The specifically designed molecular structure of DDMC having a positive charge and a hydrophilic-hydrophobic micro-separated-domain ensures easy entry of DNA into cells for steps (b), (c), (d), (f), and (g). The complex by DDMC/DNA in cytoplasm also can be stable in steps (b), (c), (d), (f), and (g) to be protected from both DNase and Dextranase.

Formation of a complex between nucleic acids (DNA or RNA) and DDMC is accomplished by a Coulomb force between the phosphoric acid of nucleic acids and the diethylaminoethyl(DEAE) group of DEAE-dextran to ensure easy entry of DNA for steps (b) and endosome buffering for osmotic destruction (proton sponge properties) for steps (d). Figure 1 also shows the infrared absorption spectra of the resulting complex between DDMC(sample DDMC2) and DNA. The spectrum of the complex has some characteristic absorption bands at 1730cm^{-1} , 1220cm^{-1} , 1000 to 1150cm^{-1} , and at 3450cm^{-1} which is attributed to the carbonyl group of poly(MMA), P-O stretching vibration of DNA, the pyranose ring of DEAE-dextran, and the diethylaminoethyl(DEAE) group of DEAE-dextran, respectively. The absorption spectrum shift at around 3450cm^{-1}

of the complexes may mean to form more compact structures by a Coulomb force between the phosphoric acid of DNA and the diethylaminoethyl(DEAE) group of DEAE-dextran to conclude to DNA condensation¹⁴⁾. This phenomenon may be interested, because DNA is usually tightly packed in native genomes and the manner of this packaging should dominate the mechanism of gene expression. As shown in Fig.4, the resulted DDMC having amphiphilic domain, to form a polymer micelle, should become a stable latex with a hydrophilic-hydrophobic micro-separated-domain¹⁵⁾. The complex by DDMC and plasmid may be formed on the spherical structure of the amphiphilic micro-separated-domain of DDMC and have a good affinity to cell membrane. The complex by cationic polymers/DNA in cytoplasm can be protected from restriction enzyme for the collapse of DNA. In the case of DEAE-dextran, the complex by DEAE-dextran/DNA in cytoplasm can be protected from DNase, but degraded by Dextranase. However the complex by DDMC/DNA in cytoplasm can be protected from both DNase and Dextranase. The high efficiency of transfection of DDMC must be depend on its stable property in cytoplasm and its good affinity to biomembrane ,such as cell membrane, owing to its amphiphilic domain.

The high efficiency of this graft-copolymer autoclaved can make it a valuable tool for gene delivery in vivo.

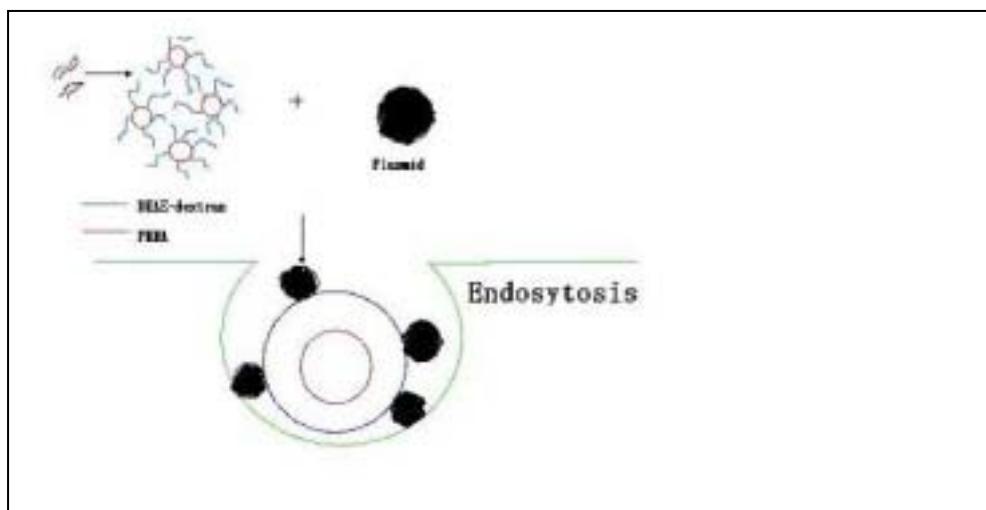


Fig.4. Schematic representation of endocytosis by a complex between DDMC and Plasmid.

Conclusions

Specification of DDMC Vector with DEAE-Dextran MMA Copolymer were as below:

- 1 Fast and easy procedure
- 2 Stable for autoclaving Sterilization
- 3 Applicable in high-throughput-screening(HTS)
- 4 No serum inhibition
- 5 High efficiency by use of low DNA amounts
- 6 Excellent reproducibility
- 7 Low toxicity in comparison with DEAE-dextran

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