Synthesis and Characterization of 2-Diethyl-aminoethyl–Dextran–Methyl Methacrylate Graft Copolymer for Nonviral Gene Delivery Vector

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ABSTRACT: A stable and soapless latex of 2-diethyl-aminoethyl (DEAE)–dextran–methyl methacrylate (MMA) graft copolymer (DDMC) was developed for nonviral gene delivery vectors (complex between polycation and nucleic acid). DDMC was newly prepared using MMA and DEAE–dextran. Following a transfection protocol, transfection of HEK 293 cells by DDC1, DDC2, and DDC3 samples was carried out using plasmid DNA. With the transfection efficiency determined using the X-Gal staining method, a higher value of 5 times or more was confirmed for DDMC samples DDC1 and DDC2 (but not for DDC3) than for the starting DEAE–dextran hydrochloride. The absorption spectrum shift at around 3400 cm^{-1} of the complexes between DDMC and DNA may support the formation of more compact structures by a Coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE–dextran, concluding in DNA condensation. The specifically designed molecular structure of DDMC to ensure easy entry of DNA into cells needs not only a positive charge and a hydrophilic–hydrophobic microseparated domain but also more compact structures for transfection steps. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 98: 9–14, 2005

Key words: transfection; DNA; 2-diethyl-aminoethyl–dextran; methyl methacrylate; positive charge; polyion complex; microseparated domain

INTRODUCTION

Recently, *in vivo* gene delivery has allowed the study of gene expression and function in animal models via insertion of foreign genes or alteration of existing genes and/or their expression patterns. The transfection mechanism between transferred DNA or RNA and a cell has been studied and clinical tests for transfection have become easy to carry out using a viral vector. However, some dangerous adverse effects remain associated with the use of viral vectors.

Nonviral gene delivery vectors may be a key technology in circumventing the immunogenicity inherent in viral-mediated gene transfer.

Water-soluble cationic polysaccharides are also of interest for a nonviral gene delivery vector to increase safety by minimizing the incidence of serious diseases resulting from the immunogenicity inherent in viral vectors. 2-Diethyl-aminoethyl (DEAE)–dextran has been used for a nonviral gene delivery vector.[1–3] However, these cationic polysaccharides, such as DEAE–dextran, are not superior to viral vectors with transfection efficiency.

Many efforts have been made for safety and high transfection efficiency in the field of nonviral gene delivery vectors.[4–6] DEAE–dextran has been investigated, and its transfection conditions increase transfection efficiency and several good conditions for a human macrophage have been found.[7]

DEAE–dextran has strong adsorbing properties with nucleic acids, such as DNA and RNA, because of its cationic properties and is able to adsorb specific nucleic acids by changing the pH and ion strength.[8,9]

The interaction between DNA and basic proteins such as histones, known by the appearance of a partially unfolded part on chromatin, plays a key role in the regulation of the gene transfer system.[10] The structural transition of DNA, which is called a coil–globule transition, induces discrete on/off switching on transcriptional activity.[11] This collapse transition in single giant DNA chains has been reviewed as DNA condensation.[12] The *in vitro* collapse of DNA may be induced by various cationic compound vectors such as cationic lipids,[13–15] peptides,[16] or cationic polymers.[17,18] In the case of cationic lipid vectors, the complex of dioctadecylamidoglycylspermine (DOGS)/DNA, which has a nucleosome-like structure in which DNA wraps around a micellar aggregate of DOGS and has an

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association with each other to form a network structure, is very effective for gene transfection.[11]

The complex by cationic polymers/DNA in cytoplasm can be protected from restriction enzymes for the collapse of DNA.[12] In the case of cationic dextran, the complex of DEAE–dextran/DNA in the cytoplasm can be protected from DNase.[5] However, there are problems for the complex to be degraded *in vivo* by dextranase.

DEAE–dextran must have a high facility for endocytosis. However, its transfection efficiency is not so high but the reasons why may not be enough to protect it from degradation of the complex between DEAE–dextran and DNA, especially at the escape from the endocytic vesicle.[3]

Graft polymerization of methyl methacrylate (MMA) onto DEAE–dextran can be very effective for the improvement of the defect of DEAE–dextran with its protective facility from the degradation of the complex in the cytoplasm because of its graft chains of MMA.[19]

These graft copolymers have an amphiphilic domain to form a polymer micelle and should become a stable latex with a hydrophilic–hydrophobic microseparated domain to form a spherical structure.[20] The complex of DEAE–dextran–MMA graft copolymer (DDMC)/DNA to be formed on the spherical structure of the amphiphilic microseparated domain of DDMC should be stable for intracellular surroundings and have a good affinity to the cell membrane because of its hydrophilic–hydrophobic microseparated domain.[21]

The present article is related to a novel graft copolymer having some possibilities as a nonviral gene delivery vector that is composed of a cationic derivative of a water-soluble linear polymer and a vinyl ester monomer.

The DDMC graft copolymer was obtained by graft polymerizing a vinyl ester monomer (MMA) onto a cationic derivative of a water-soluble linear polysaccharide (DEAE–dextran) in water using ceric ammonium nitrate to obtain a stable latex of DDMC,[22] which is very effective as a nonviral gene delivery vector.

It is expected that nonviral vectors, such as the DDMC in this article, will increase safety by minimizing the incidence of serious diseases resulting from the immunogenicity inherent in viral vectors.

EXPERIMENTAL

Polymerization procedure of DDMC

Samples DDC1, DDC2, and DDC3 in Table I were prepared by the following procedure: 2 g of DEAE–dextran hydrochloride (3% nitrogen) derived from dextran (weight-average molecular weight = 500,000) was dissolved in 100 mL of water; then, 3, 4, and 6 mL of MMA was added for DDC1, DDC2, and DDC3 samples, respectively. While stirring, the air in the reaction vessel was fully replaced with nitrogen gas. To the solution was added 0.1 g of ceric ammonium nitrate and 15 mL of 0.1*N* nitric acid, and the mixture was reacted with stirring for 1 h 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 by water dialysis using a cellophane tube in order to remove the unreacted MMA, ceric salts, and nitric acid. The resulting latex of DDMC was stable and soapless.

TABLE I
Properties of DEAE–Dextran–MMA Graft Copolymers

Sample	Weight increase (%)	Precipitation time by DNA (h)
DDC1	150	2.0
DDC2	200	1.0
DDC3	300	0.5
DEAE–dextran	0	96.0

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

Synthesis of complex of DDMC/DNA

Two milliliters of a 10 mg/mL solution of the resulting latex of DDMC was added dropwise to 1 mL of a 20 mg/mL DNA (EX salmon sperm) solution to obtain a complex of DDMC/DNA.

Measurement of IR absorption spectra

IR measurements on DDMC samples and DDMC/DNA complexes were carried out by the KBr powder method using Jasco FT/IR-300.

Transfection protocol

The protocol of DDMC for transfection of monolayer cells is a modification of the protocol by Al-Moslih and Dubes.[23]

Cell line and cell culture

The 293 cell line is a permanent line of primary human embryonal kidney (HEK) transformed by sheared human adenovirus type 5 DNA. The cell line was grown at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.1 m*M* nonessential amino acids, 5 m*M* L-glutamine, and antibiotics (100 μg/mL streptomycin, 100 U/mL penicillin).

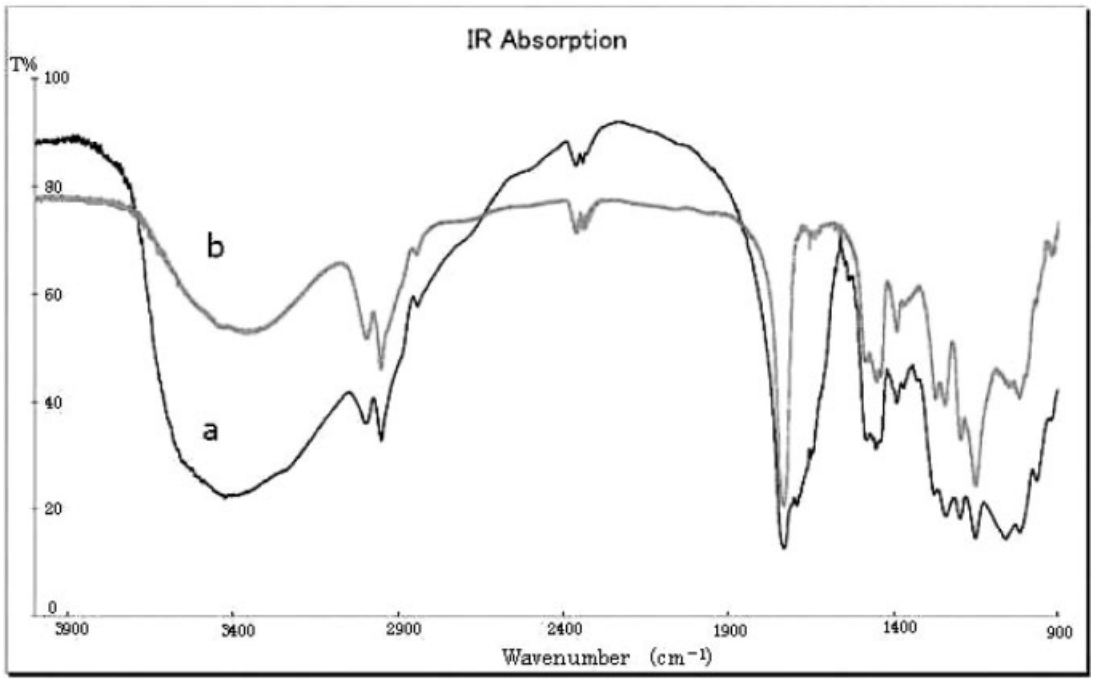

Figure 1 IR absorption spectra of DEAE–dextran–MMA graft copolymer and the complexes between DNA and DEAE–dextran–MMA graft copolymer: DDC2/DNA complex (spectrum a) and DDC2 (spectrum b).

Plasmid DNA and reagents

A pCAGGS/LacZ, which expresses β-galactosidase at eukaryotic cells, was inserted under the CAG promoter of a plasmid (pCAGGS). Plasmids were amplified in *Escherichia coli* DH5α and purified by a Qiagen Mega plasmid purification kit (Qiagen). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution was purchased from Promega. The staining solution for β-galactosidase expression cells was 20 mg/mL X-Gal (stored at −20°C), 50 m*M* potassium ferricyanide, 50 m*M* potassium ferrocyanide, and 1*M* $MgCl_{21}$ in phosphate-buffered saline (PBS). This solution, without X-Gal, can be prepared in advance and stored at room temperature in the dark. X-Gal was added from a stock solution just before use.

Transfection by DDMC/DNA

Cell line 293 cells (15×10^4 cells) were seeded on 35-mm culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2. In a sterile tube, 10 μg of DNA was diluted in 270 μL of 1 × PBS (for ×1 dilute). To the DNA solution was added 14 μL of DDMC (autoclaved) having a concentration of 10 mg/mL. Then, it was mixed by brief vortexing and the growth medium was removed from the cells to be transfected. The cells were washed twice with 1 × PBS, and DDMC/DNA solution was added to cover the cells. The dish was slowly moved side to side several times to ensure complete cover of the cells, and they were incubated at 37°C for 30 min. The dish was slowly moved side to side several times during the incubation. Then, 1 mL of growth medium was added, and it was incubated at 37°C for 48 h. After the incubation, the transfection activity was determined using the X-Gal staining method.

RESULTS AND DISCUSSION

The resulting DEAE–dextran–MMA copolymer 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) (PMMA) is soluble in acetone, it is evident that DDMC is not a mixture of DEAE–dextran and PMMA.

The IR absorption spectrum of the copolymer shown in Figure 1 has some characteristic absorption bands at 1730 and 1000–1150 cm^{-1}, which are attributed to the carbonyl group of PMMA and the pyranose ring of DEAE–dextran, respectively. Thus, the resulting DDMC exhibits different solubility from DEAE–dextran and PMMA and shows the characteristic absorption in the IR absorption spectrum. From this fact, it is judged that the resulting DDMC is a graft-polymerized compound.[22]

Reaction between DDMC and DNA

A solution of the resulting latex of DEAE–dextran–MMA copolymer was added dropwise to the DNA (EX salmon sperm) solution in order to obtain the complex of DDMC/DNA (Fig. 2). The obtained complex was insoluble in water, which is a good solvent

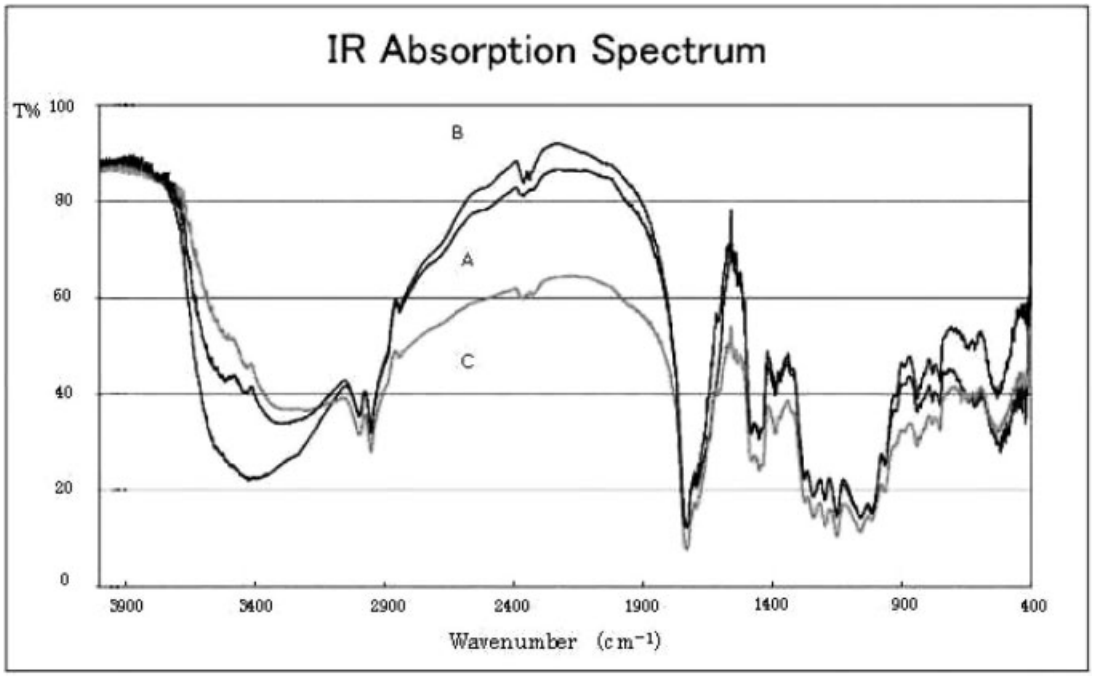

Figure 2 IR absorption spectra of complexes between DNA and DEAE–dextran–MMA graft copolymer: DDC1/DNA complex (spectrum A), DDC2/DNA complex (spectrum B), and DDC3/DNA complex (spectrum C).

for nucleic acids. These results show that the complex between DDMC and DNA must form a polyion complex. In the case of sample DDC2, a complex between DDC2 and DNA having a 200% weight increase needed 1 h to precipitate.

The complex between DDMCs (DDC3 and DDC1) having 300 and 150% weight increases and DNA needed 0.5 and 2 h to precipitate, respectively. However, a complex between DNA and DEAE–dextran hydrochloride needed 96 h to precipitate under this condition.

Figure 1 also shows the IR absorption spectra of the resulting complex between DDC2 and DNA. The spectrum of the complex has some characteristic absorption bands at 1730, 1220, and 1000–1150 cm^{-1}, which are attributed to the carbonyl group of PMMA, P—O stretching vibration of DNA, and the pyranose ring of DEAE–dextran, respectively.

As shown in Table I, the complex between DDC1 having a 150% weight increase and DNA was formed in 2 h. The complex between DDMC (DDC2 and DDC3) with 200 and 300% weight increases and DNA were formed in 1 and 0.5 h, respectively. However, a complex between DNA and DEAE–dextran hydrochloride was formed in 96 h.

Transfection by DDMC

Following the transfection protocol, transfection of HEK 293 by the DDC1, DDC2, and DDC3 samples was carried out using plasmid DNA. As shown in Figure 3, with the transfection efficiency, the transfection activity was determined using the X-Gal staining method and a value 5 times higher or more than for the starting DEAE–dextran hydrochloride was confirmed for DDMC samples DDC1 and DDC2 (but not for DDC3).

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.

Figure 4 shows the change of the transfection efficiency when using 2 times as much as the protocol quantity of both DNA and DDMC, for example, 20 mg of DNA. As shown in Figure 4, transfection of HEK 293 by DDC1 and DDC2, carried out using 2 times as much as the protocol quantity of both DNA and DDMC shows 2 times higher efficiency than the original by the transfection activity determined using the X-Gal staining method. From the results, its cytotoxicity for the transfection should be confirmed to decrease and improve when using DDMC hydrochloride instead of DEAE–dextran hydrochloride.

DDMC transfection of cells was carried out using the following steps:

1. formation of a complex between DNA and DDMC,
2. uptake,
3. endocytosis (endosome),
4. escape from the endocytic vesicle,
5. DNA release in cytosol,
6. nuclear entry, and
7. DNA release and transcription in the nucleus.

For transfection efficiency, it is very important to examine factors such as the uptake in step 2, resistance

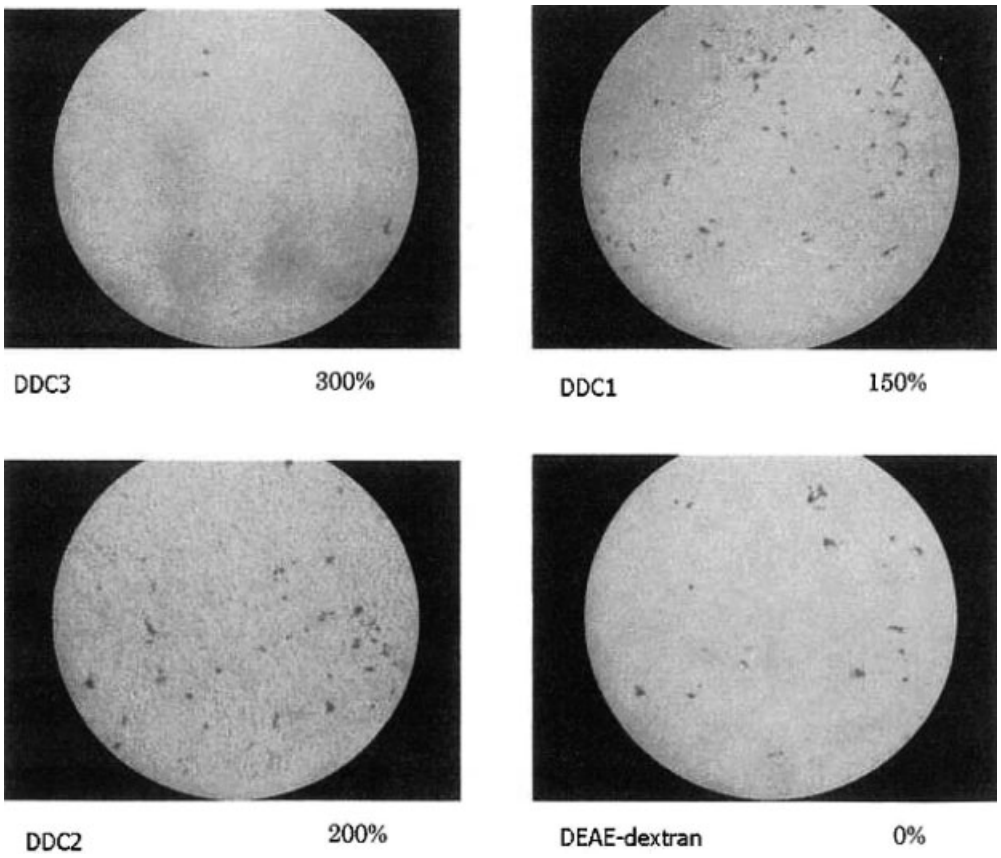

Figure 3 The transfection of a monolayer of HEK 293 cells by the DEAE–dextran–MMA graft copolymer.

of nuclease in step 3, escape from the endocytic vesicle in step 4, nuclear targeting in step 6, and DNA release in step 7. The positively charged DEAE–dextran copolymer interacts with the negatively charged phosphate backbone of DNA. The resulting complex in step 2 is absorbed into cells by endocytosis.

The specifically designed molecular structure of DDMC having a positive charge and a hydrophilic–hydrophobic microseparated domain ensures easy entry of DNA into cells for steps 2, 3, 4, 6, and 7.

Formation of a complex between nucleic acids (DNA or RNA) and cationic graft copolymers, such as DDMC, is accomplished by a Coulomb force between the phosphoric acid of nucleic acids and the DEAE group of DEAE–dextran.

Figure 1 shows the IR absorption spectra of the resulting complex between DDMC (sample DDC2) and DNA. The spectrum of the complex has some characteristic absorption bands at 1730, 1220, 1000–1150, and 3450 cm^{-1}, which are attributed to the carbonyl group of PMMA, the P—O stretching vibration of DNA, the pyranose ring of DEAE–dextran, and the DEAE group of DEAE–dextran, respectively.

Figure 2 also shows the IR absorption spectra of the resulting complexes (samples DDC1, DDC2, and DDC3) between DDMC and DNA. The spectrum of the complexes has some characteristic absorption bands at around 3400 cm^{-1}, which is attributed to the N—H stretching vibration of the DEAE group of DEAE–dextran, following the absorption shift in the order DDC2 > DDC1 > DDC3 (to high energy). The absorption spectrum shift at around 3400 cm^{-1} of the complexes may support formation of more compact structures by a Coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE–dextran, to conclude DNA condensation.

This phenomenon is very interesting, because DNA is usually tightly packed in native genomes and the manner of this packaging should be expected to dominate the mechanism of gene expression.

The specifically designed molecular structure of DDMC to ensure easy entry of DNA into cells needs not only a positive charge and a hydrophilic–hydrophobic microseparated domain but also more compact structures for steps 2, 3, 4, 6, and 7. This might be the reason why the transfection efficiency of sample DDC3 with a 300% weight increase was inferior to the starting DEAE–dextran.

CONCLUSIONS

It was recently discovered that the resulting latex of a cationic graft copolymer is superior to other high ef-

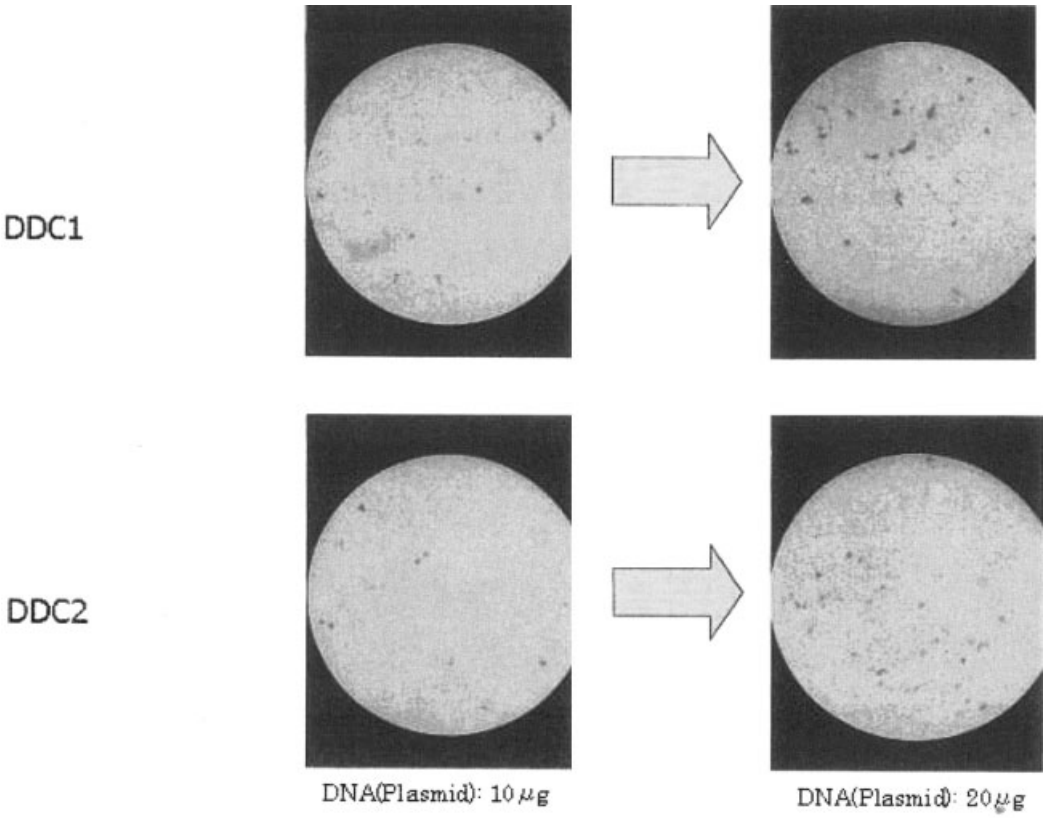

Figure 4 The effect of the cytotoxicity of the DEAE–dextran–MMA graft copolymer on transfection of a monolayer of HEK 293 cells.

ficiency transfection reagent vectors for cells, particularly for mammalian cells.

This report is on a new class of polycationic transfection reagents based on reacting the cationic derivative of the water-soluble linear polysaccharide having hydroxyl groups with a polymerizable vinyl ester monomer in the presence of a redox initiator. The specifically designed molecular structure of the cationic graft copolymer having a hydrophilic–hydrophobic microseparated domain[24,25] ensures easy entry of DNA or RNA into cells (i.e., transfection) by condensing DNA to compact structures (graft copolymer/DNA complex or transfection complex) and endosome buffering. The high efficiency of the graft copolymer makes it a valuable tool for gene delivery or gene transfer experiments.

It is very important that these gene delivery systems consist of a first elementary step of the formation of the complex between the cationic graft copolymer thus obtained and DNA.

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