Folding and aggregation of DNA chains induced by complexation with lipospermine: formation of a nucleosome-like structure and network assembly

Yuko Yoshikawa[a], Nobuhiko Emi[b], Toshio Kanbe[c], Kenichi Yoshikawa[a,*], Hidehiko Saito[b]

[a] *Graduate School of Human Informatics, Nagoya University, Nagoya 464-01, Japan*
[b] *First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan*
[c] *Laboratory of Medical Mycology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya 466, Japan*

Received 22 July 1996; revised version received 4 September 1996

Abstract Dioctadecylamidoglycylspermine (DOGS) is a cationic lipid vector capable of efficiently introducing DNA into various eukaryotic cells. We investigated the higher-order structure of the DNA/DOGS complex using fluorescence and electron microscopy. Our results show that the DNA/DOGS complex exhibits a nucleosome-like structure in which DNA wraps around an aggregate of DOGS molecules. In addition, DNA/DOGS complexes tend to associate with each other to form network structures. The resulting network assembly may play a role in effective gene transfection.

Key words: Lipospermine; Fluorescence microscopy; Electron microscopy; Higher-order structure of DNA; Gene transfection

1. Introduction

The ability to deliver foreign DNA into eukaryotic cells is a powerful tool not only for studying the mechanism of gene expression but also for human gene therapy. Although viruses are currently thought to be efficient gene-transfer vehicles and the viral genome has been modified to incorporate genes into cells, progress has also been made in developing nonviral synthetic vectors. This approach involves DNA complexes with cationic lipids [1–3], peptides [4] or cationic polymers [5–7] as well as cell surface receptor-targeting ligands [8,9]. However, little is known about the structure of such complexes, especially the higher-order structure. Controlling the higher-order structure of DNA complexes could help in understanding the molecular mechanism of gene delivery, including internalization via cell membrane and nuclear transport.

In general, DNA is tightly packed in native genomes and the manner of this packaging is expected to be involved in the mechanism of gene expression. The in vitro collapse of DNA can be induced by various chemical agents including polyamines [10–12], hexamine cobalt(III) [13,14], neutral polymers and peptides [15,16] in aqueous solution. Although electron microscopy is a powerful tool for observing the morphology of compacted forms of DNA, it is unclear whether such morphological features accurately reflect the structures in solution due to the methods used to prepare samples for electron microscopy. Using fluorescence microscopy, we recently made direct observations of individual long duplex T4 DNA molecules in aqueous solution and found that DNA molecules undergo a transition from an elongated coil into a compacted globule with the addition of various condensing agents such as polyethylene glycol [17], poly-Arg [18], cationic surfactant [19] and even diamines such as diaminopropane and cadaverine [20], which had been thought to have no effect on DNA collapse in aqueous solution [10]. This technique can help us understand the packaging process of individual DNA molecules. In this study, we apply this technique together with electron microscopy to evaluate the conformational changes in DNA complexed with a lipospermine, DOGS (dioctadecylamidoglycylspermine) (Scheme 1). DOGS is a cationic lipid vector developed by Behr and coworkers that has been shown to result in relatively high levels of gene expression [21–23]. It contains a spermine headgroup which strongly interacts with DNA and two hydrophobic chains. It has been assumed that DOGS molecules coat the negatively charged DNA with a cationic lipid bilayer [21,23]. In contrast, the present observations suggest that DNA wraps around a micellar aggregate of DOGS molecules by forming a nucleosome-like compacted complex and that the resulting DNA/DOGS complexes tend to form a multimolecular network.

2. Materials and methods

2.1. DNA and other chemicals

Plasmid pLZRN, which exhibits a β-galactosidase reporter gene, was prepared by standard techniques [24]. T4 phage DNA and pBR322 plasmid DNA were purchased from Nippon Gene (Toyama, Japan). A fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI), and an antioxidant, 2-mercaptoethanol (2-ME), were purchased from Wako Pure Chemical Industries (Osaka, Japan). Spermine·4HCl was obtained from Nacalai Tesque Inc. (Kyoto, Japan). DOGS was obtained from Promega (Madison, WI, USA).

2.2. β-Galactosidase assay

DOGS stock solution was prepared as described in the protocol from Promega [25]. The adherent cell line 208F (rat fibroblast) was cultured at a density of about 5×10^4 cells per well in 24-well culture dishes in Iscove's modified Dulbecco's medium (IMDM) with 10% (v/v) fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37°C. DOGS or spermine with plasmid DNA was prepared in microcentrifuge tubes by mixing 2 μg pLZRN with serially diluted DOGS (60 μM) or spermine (10 μM to 1 mM) in plain medium in a final volume of 200 μl. In a typical experiment, cells were transfected in PBS with DNA complex at 37°C. After 2 h of incubation, the cells were washed and replaced by medium. Expression of β-galactosidase genes was measured after 24 h of incubation at 37°C in 5% CO_2 with the β-Galactosidase Enzyme Assay System (Promega) according to the manufacturer's instructions. Each assay included 1–5 mU of β-gal as a standard. The activity was normalized.

2.3. Fluorescence microscopic measurements

T4 DNA, 166 kbp with a contour length of 57 μm [26], was dis-

*Corresponding author. Fax: (81) (52) 789-4808.
E-mail: f43943a@nucc.cc.nagoya-u.ac.jp

PII S0014-5793(96)01057-5

solved in pure water, and DAPI and 2-ME were added to the DNA solution. The final concentrations were as follows: 0.6 μM T4 DNA (in nucleotide units), 0.6 μM DAPI, and 4% (v/v) 2-ME. It has been confirmed that the persistent length and the contour length of DNA remain essentially constant at such a low concentration of DAPI [27]. Compaction was induced by the addition of DOGS or spermine to the DNA solution. Fluorescence DNA images were obtained using an Axiovert 135 TV microscope (Carl Zeiss, Germany) equipped with a 100× oil-immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera, which allowed recording of images on video tapes. The video image was analyzed with an Argus 50 image processor (Hamamatsu Photonics, Hamamatsu, Japan). Observation was performed at 20°C.

2.4. Electron microscopic measurements

Samples used for electron microscopy were prepared by the addition of DOGS or spermine to 0.6 μM DNA solutions containing either pBR322, pLZRN or T4 DNA. They were mounted on carbon-coated copper grids (# 200), negative-stained with 1% uranyl acetate, and observed with a JEOL 1200EX transmission electron microscope (Tokyo, Japan) at 100 kV.

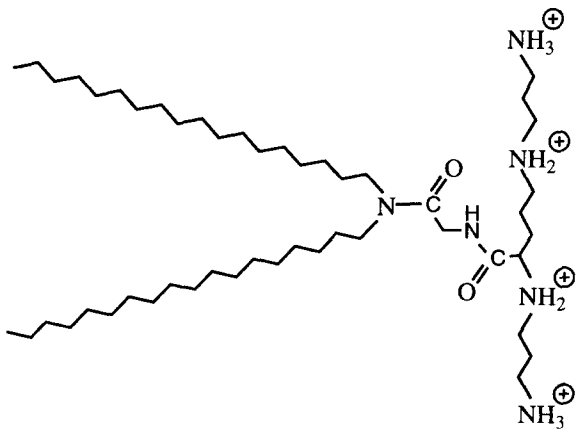
3. Results and discussion

3.1. Transfection of the 208F cell line with DOGS and spermine

Before investigating the higher-order structure of various DNA complexes, the transfection efficiency of DOGS and spermine was compared. Spermine was unable to transfect β-galactosidase activity into cells. On the other hand, DOGS-mediated transfection resulted in a maximal efficiency of 20–30%. This suggests that the hydrophobic chains of DOGS molecules may play a role in effective transfection. These results are consistent with a previous report by Behr et al. [21].

3.2. Collapse of single T4 DNA molecules in aqueous solution

With fluorescence microscopy, we observed long duplex T4 DNA, rather than shorter DNA such as pBR322 DNA, to obtain direct information on the change in the higher-order structure in aqueous solution. Fig. 1 shows fluorescent images of individual T4 DNA molecules in aqueous solution. In pure water, as shown in Fig. 1a, individual duplex T4 DNA molecules exist as elongated random coils. T4 DNA transforms into a collapsed globule with the addition of spermine (see Fig. 1b, where the ratio of nucleotide to spermine is 1:5). The complex composed of T4 DNA and DOGS at a ratio of 1:5 also exhibits a compacted structure, and is somewhat swollen compared to the spermine/DNA complex (Fig. 1c). T4 DNA is still in a compacted state even at a DNA/DOGS ratio of 1:3, whereas the DNA chain is unfolded in the coiled state at the same ratio of DNA and spermine. This result indicates that DOGS, which has the same valence as spermine, is a more effective condensing agent than spermine.



Scheme 1.

To evaluate the size of the DNA/DOGS complex in a quantitative manner, we measured the translational diffusion constant D for individual DNA obstacles observed by fluorescence microscopy. Although we tried to minimize convective flow in the aqueous sample, non-negligible convective flow was present during the measurement, possibly due to the thermal effect of the illumination. Since convective motion was almost constant during the period of observation with regard to both the flow rate and flow direction, we could eliminate the effect of convective flow using Eq. 1 [28]

$$\langle (R(t)-R(0))^2 \rangle = 4Dt + At^2, \quad (1)$$

where $R(t) = (R_x, R_y)$ is the position of the center of mass for a DNA, $\langle (R(t)-R(0))^2 \rangle$ is the mean square displacement, and A is a numerical constant related to the convective flow. The effective hydrodynamic radius ξ_H of a single DNA molecule was calculated from the D value based on the Stokes-Einstein equation given in Eq. 2 [29,30]

$$\xi_H = \frac{k_B T}{6\pi\eta_s D}, \quad (2)$$

where k_B is the Boltzmann constant and η_s is the viscosity of the solvent (1.002 mPa·s for pure water at T = 293 K). The ξ_H value of DNA complexed with DOGS at a ratio of 1:5, of which the fluorescent image is shown in Fig. 1c, is calculated to be 0.3 μm. On the other hand, the long-axis length is about 1.2 μm for the fluorescent object (Fig. 1c), indicating that the radius is ca. 0.6 μm. Taking into account a blurring effect on the order of 0.3 μm [18] (see Fig. 1a″,c″), the actual size is estimated to be ca. 0.3 μm, which corresponds well to the hydrodynamic radius ξ_H as estimated from the diffusion constant.

3.3. Nucleosome-like structure of the DNA/DOGS complex

To investigate the morphological details of DNA/DOGS complexes, we observed DNA complexes using electron microscopy (Fig. 2). Consistent with the fluorescence microscopic observations, these photographs indicate that DNA/DOGS complexes form compacted structures (Fig. 2b–e) with a markedly different morphology from the toroidal structures of DNA/spermine complexes (Fig. 2f). Without DNA, DOGS molecules form micelle-like aggregates (Fig. 2a). Thus, these observations indicate that the DNA/DOGS complex is composed of an aggregated core of DOGS wrapped by DNA strands, resulting in the formation of a spool or a nucleosome-like structure. However, it should be mentioned that the size of the DNA/DOGS complex is larger than the actual size of native nucleosome; the diameter is ca. 11 nm [31]. It is also to be noted that the overall morphology is essentially the same for the DNA/DOGS complexes obtained using the three different DNA species: T4 DNA, pBR322 DNA and pLZRN. This means that the shape of the positively charged core plays a critical role in determining the steric structure of the DNA complex, regardless of the length and base composition of the DNA. These results strongly suggest that the DNA-transporting structure is very similar to the nucleosomal structure,

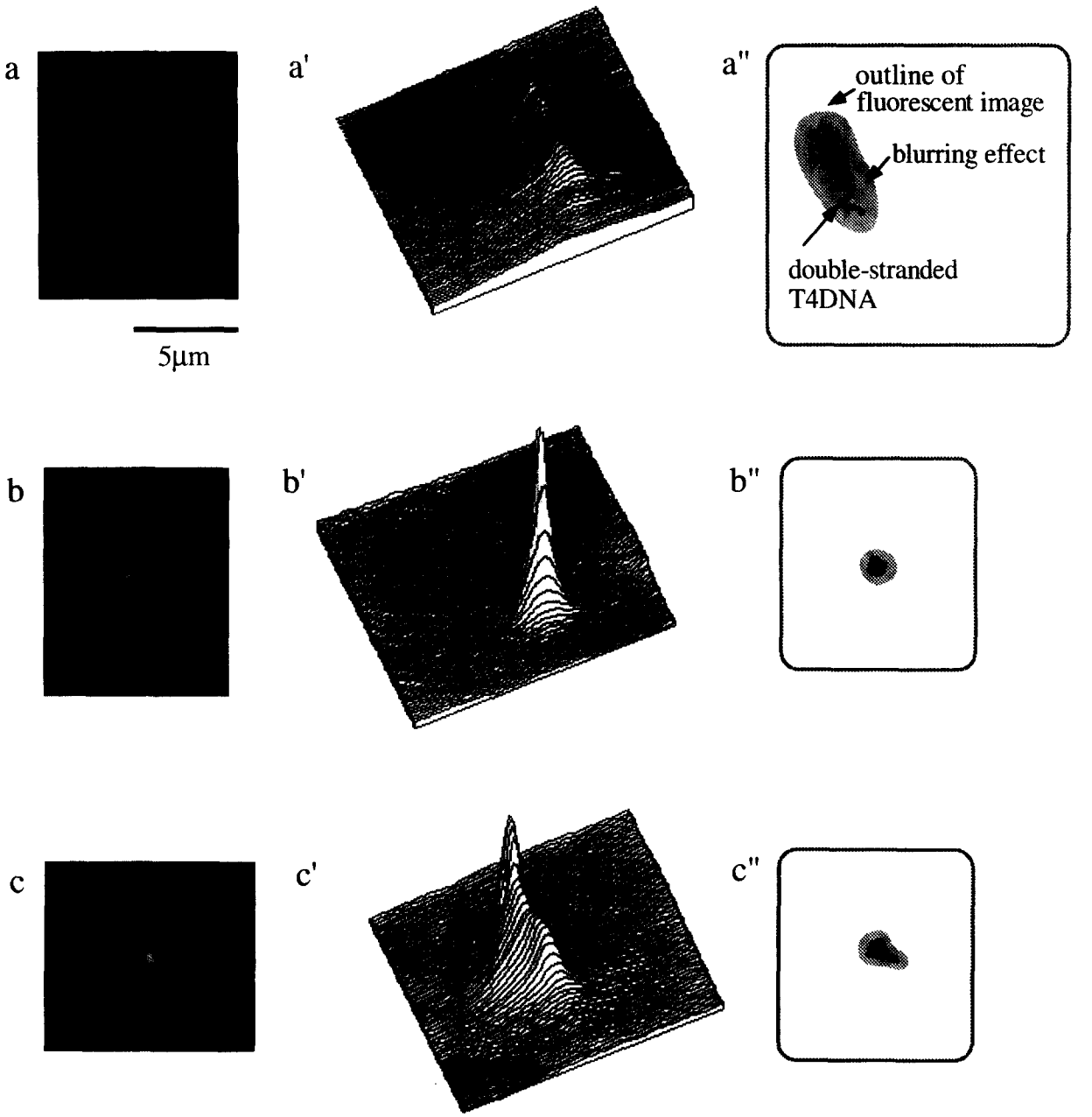


Fig. 1. Fluorescent images of individual T4 DNAs moving freely in aqueous solution (left panels) and the corresponding light-intensity distributions (middle panels) and schematic illustrations (right panels); (a) in pure water, (b) in the presence of spermine at a nucleotide:spermine ratio of 1:5 and (c) in the presence of DOGS at a nucleotide:DOGS ratio of 1:5.

Although at present it is not clear whether the DNA that is wrapped around the DOGS aggregate is naked or coated with lipid. The size of DNA/DOGS complexes in electron micrographs is about 50–300 nm, which is smaller than that estimated from the diffusion constant of individual obstacles in fluorescence microscopy. Considering that the structure may have changed during preparation of the specimen for electron microscopy, it is reasonable to suggest that the original structure in aqueous solution is not very rigid and has conformational flexibility.

3.4. *Time course of the network assembly of DNA/DOGS complexes*

DNA/DOGS complexes, as in Fig. 1c, tend to aggregate with each other. Fig. 3 shows the result of fluorescent microscopic observation of this aggregation. These time-course observations indicate that a DNA/DOGS complexes initially bind to one another (Fig. 3a–c), resulting in the formation of large clusters of compacted vesicles (Fig. 3d–f). This assembly process is characterized by the formation of a network structure between micelles of DOGS molecules, in which the micelle is stabilized in the presence of DNAs. On the other hand, we could not find any network structure in electron microscopic observations, indicating that the association between DNA/DOGS complexes is rather weak. Similar tendencies have been observed in vitro with poly-Lys- or poly-Arg-based transfection (Emi, Kidoaki, Yoshikawa and Saito, submitted for publication). Reimer et al. suggested that aggre-

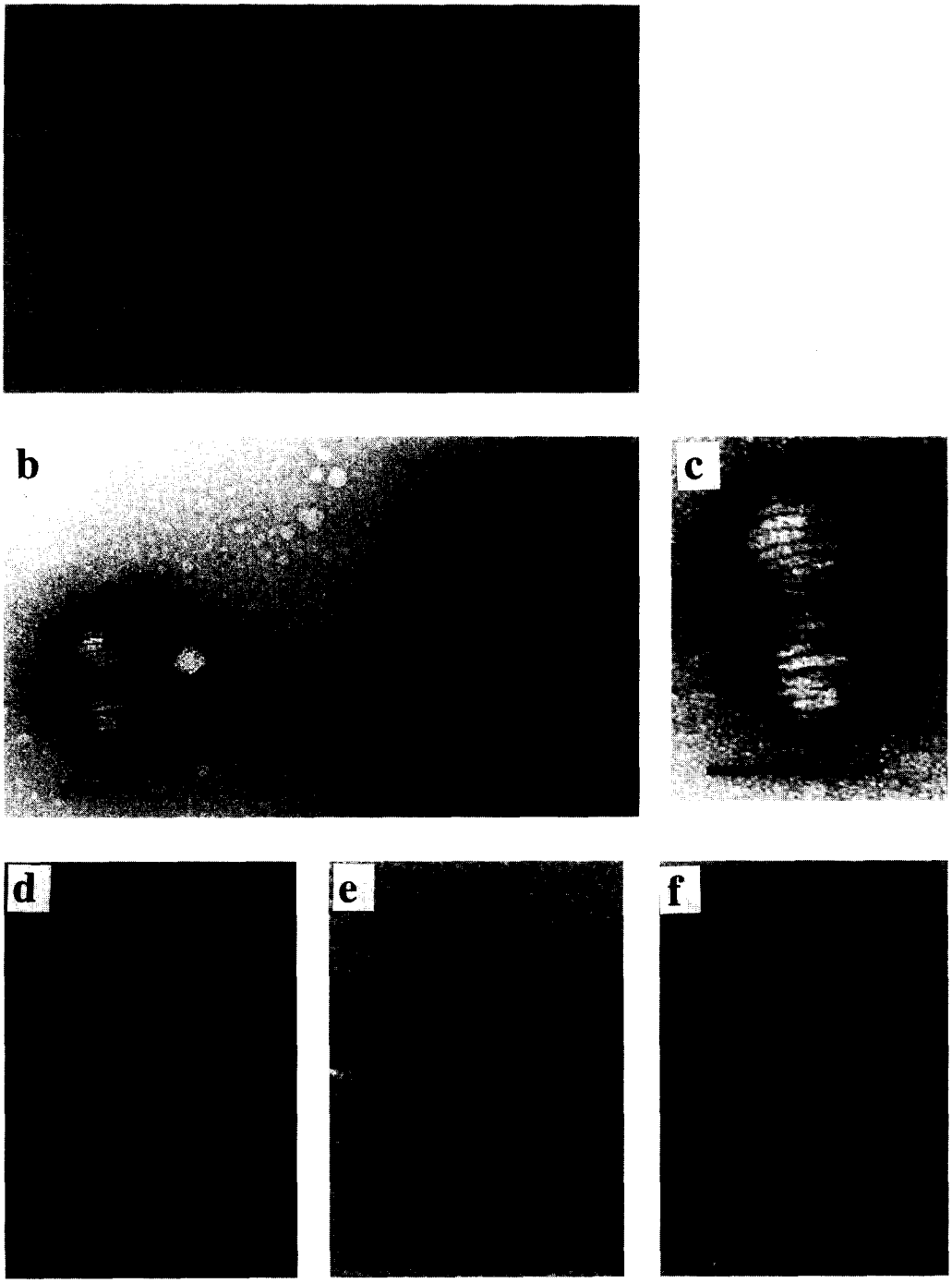


Fig. 2. Electron microscopic image of DOGS aggregates (a), DNA/DOGS complexes (b–e) and DNA/spermine complex (f). (b) and (c) pBR322 DNA, (d) and (f) T4 DNA, (e) pLZRN. (c) is the magnification of (b). Scale bar is 100 nm.

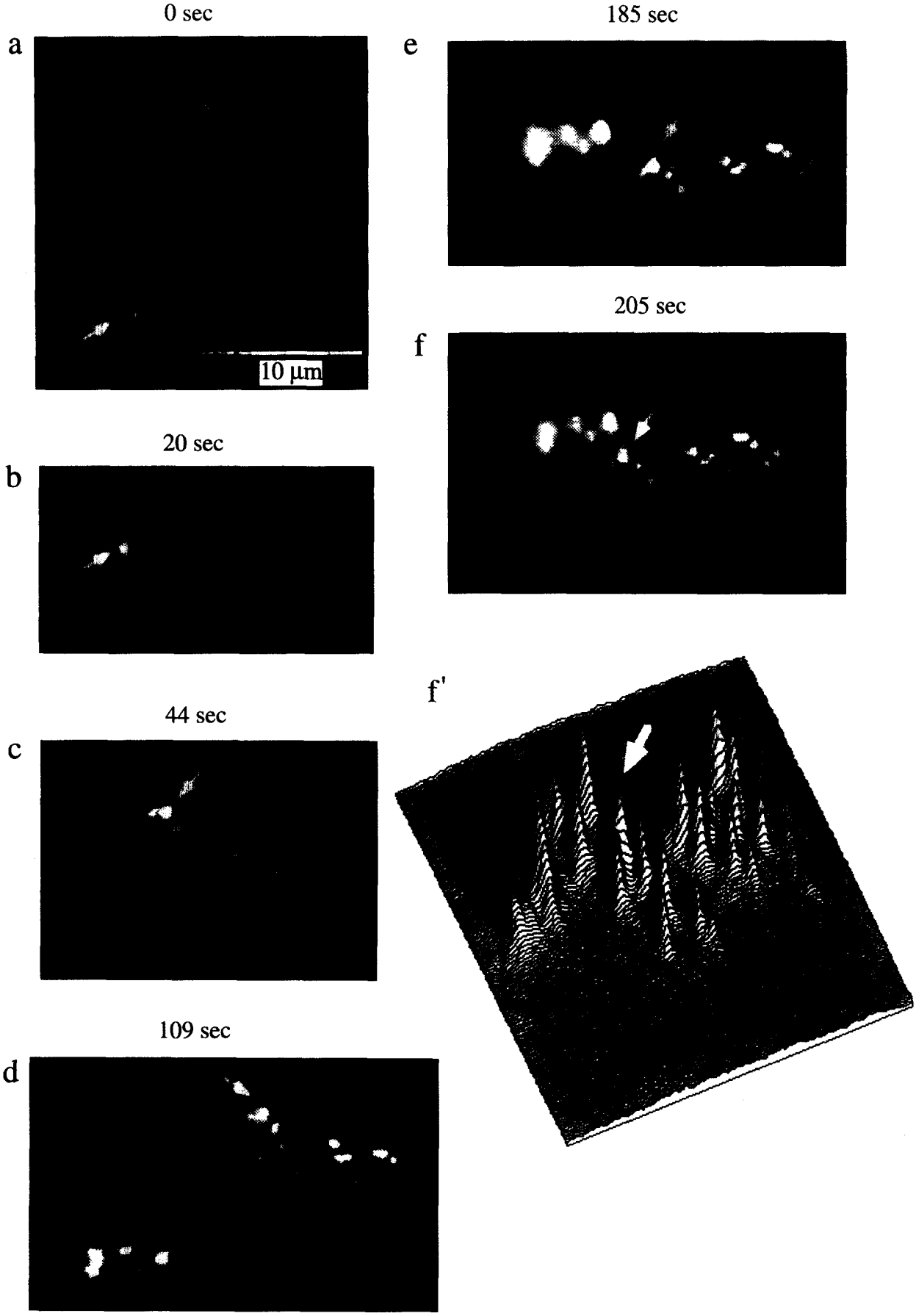
gates may play a role in effective gene transfection in cationic liposome-based transfection in vitro [32]. Thus, we assume that the resulting loose aggregation of DNA/DOGS complexes stabilizes the DNA-transporting structure and is associated with the effective delivery of DNA. The present technique allows us to observe the formation of this loose network.

4. Conclusion

The present results provide new information on the higher-order structure of the DNA/DOGS complex and also on the dynamics of multimolecular assembly. The addition of DOGS to a DNA solution allows the formation of nucleosome-like structures that then interact with one another. These com-

→

Fig. 3. Time-course of the process of network assembly as observed by fluorescence microscopy. The arrows indicate the same DNA/ DOGS complex. The complex moved freely in (a) and (b). It then attached to a small cluster in (c), and the cluster grew by forming a larger network through fusion with another cluster in (d), (e) and (f). (f') is the light-intensity distribution for (f).


0 sec
a
10 μm
20 sec
b
44 sec
c
109 sec
d
185 sec
e
205 sec
f
f'

pacted globular structures may be stabilized by their interaction with other DNA/DOGS complexes.

From a physicochemical point of view, the effect of DOGS on the condensation of DNA is interesting. Condensing agents such as spermidine, spermine and poly-Lys collapse DNA into a toroidal or rod-like structure [12]. In contrast, DOGS condenses DNA in a way that resembles the nucleosomal structure of cellular chromatin. This finding may be of value for studying the higher-order structure of compacted DNA.

Since the nucleotide:DOGS ratios of 1:3 and 1:5 used here are optimal for transfection [25], these structures should reflect the DNA-transporting structure. In addition, as suggested by Loeffler and Behr, the excess positive charge ratio may also promote association between DNA/DOGS complexes and cells since the cell membrane possesses an overall negative charge [23]. Although these findings cannot explain all of their behavior in vivo, the nucleosome-like structure may increase the overall efficiency of gene transfection and the formation of network structures may effectively mediate DNA delivery. We thus propose that controlling the higher-order structure of the DNA-transporting structure is important for obtaining efficient gene transfection.

Acknowledgements: This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987) Proc. Natl. Acad. Sci. USA 84, 7413–7417.
[2] Gao, X. and Huang, L. (1991) Biochem. Biophys. Res. Commun. 179, 280–285.
[3] Kojima, H., Ohishi, N., Takamori, M. and Yagi, K. (1995) Biochem. Biophys. Res. Commun. 207, 8–12.
[4] Farber, F.E., Melnick, J.L. and Butel, J.S. (1975) Biochim. Biophys. Acta 390, 298–311.
[5] Holter, W., Fordis, C.M. and Howard, B.H. (1989) Exp. Cell Res. 184, 546–551.
[6] Haensler, J. and Szoka, F.C. (1993) Bioconjugate Chem. 4, 372–379.
[7] Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D. Scherman, D., Demeneix, B. and Behr, J.P. (1995) Proc. Natl. Acad. Sci. USA 92, 7297–7301.
[8] Wu, G.Y. and Wu, C.H. (1987) J. Biol. Chem. 262, 4429–4432.
[9] Wagner, E., Cotten, M., Foisner, R. and Birnstiel, M.L. (1991) Proc. Natl. Acad. Sci. USA 88, 4255–4259.
[10] Gosule, L.C. and Schellman, J.A. (1978) J. Mol. Biol. 121, 311–326.
[11] Schellman, J.A. and Parthasarathy, N. (1984) J. Mol. Biol. 175, 313–329.
[12] Bloomfield, V.A. (1991) Biopolymers 31, 1471–1481.
[13] Widom, J. and Baldwin, R. (1980) J. Mol. Biol. 144, 431–453.
[14] Arscott, P.G., Ma, C., Wenner, J.R. and Bloomfield, V.A. (1995) Biopolymers 36, 345–364.
[15] Lerman, L.S. (1971) Proc. Natl. Acad. Sci. USA 68, 1886–1890.
[16] Laemmli, U.K. (1975) Proc. Natl. Acad. Sci. USA 72, 4288–4292.
[17] Minagawa, K., Matsuzawa, Y., Yoshikawa, K., Khokhlov, A.R. and Doi, M. (1994) Biopolymers 34, 555–558.
[18] Minagawa, K., Matsuzawa, Y., Yoshikawa, K., Matsumoto, M. and Doi, M. (1991) FEBS Lett. 295, 65–67.
[19] Melnikov, S.M., Sergeyev, V.G. and Yoshikawa, K. (1995) J. Am. Chem. Soc. 117, 2401–2408.
[20] Yoshikawa, Y. and Yoshikawa, K. (1995) FEBS Lett. 361, 277–281.
[21] Behr, J.P., Demeneix, B., Loeffler, J.P. and Perez-Mutul, J. (1989) Proc. Natl. Acad. Sci. USA 86, 6982–6986.
[22] Loeffler, J.P., Barthel, F., Feltz, P., Behr, J.P., Sassone-Corsi, P. and Feltz, A. (1990) J. Neurochem. 54, 1812–1815.
[23] Loeffler, J.P. and Behr, J.P. (1993) Methods Enzymol. 217, 599–618.
[24] Li, X., Yee, J.K., Wolff, J.A. and Friedmann, T. (1989) Virology 171, 331–341.
[25] Promaga Technical Bulletin (1994) 116.
[26] Yoshikawa, K., Matsuzawa, Y., Minagawa, K., Doi, M. and Matsumoto, M. (1992) Biochem. Biophys. Res. Commun. 188, 1274–1279.
[27] Matsuzawa, Y. and Yoshikawa, K. (1994) Nucleosides Nucleotides 13, 1415–1423.
[28] Matsumoto, M., Sakaguchi, T., Kimura, H., Doi, M., Minagawa, K., Matsuzawa, Y. and Yoshikawa, K. (1992) J. Polym. Sci. Part B Polym. Phys. 30, 779–783.
[29] Doi, M. and Edwards, S.F. (1986) The Theory of Polymer Dynamics, Clarendon, Oxford.
[30] Oono, Y. and Kohmoto, M.J. (1983) J. Chem. Phys. 78, 520–528.
[31] van Holde, K.E. (1989) Chromatin, Springer Verlag, New York.
[32] Reimer, D.L., Zhang, Y., Kong, S., Wheeler, J.J., Graham, R.W. and Bally, M.B. (1995) Biochemistry 39, 12877–12883.