DNA Diffusion in Mucus: Effect of Size, Topology of DNAs, and Transfection Reagents

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ABSTRACT DNA represents a promising therapeutic and prophylactic macromolecule in treating genetic diseases, infectious diseases and cancers. The therapeutic potential of DNA is directly related to how DNA transports within the targeted tissue. In this study, fluorescence photobleaching recovery was used to examine the diffusion of plasmid DNAs with various size (2.7 ~ 8.3 kb), topology, and in the presence of transfection reagents in mucus. We observed that DNAs diffused slower when size of DNAs increased; supercoiled DNAs diffused faster than linear ones; mucus did not reduce the diffusion of linear DNAs but retarded the diffusion of supercoiled DNAs. Diffusion data were fitted to models of a polymer chain diffusing in gel systems. Diffusion of linear DNAs in mucus were better described by the Zimm model with a scaling factor of −0.8, and supercoiled DNAs showed a reptational behavior with a scaling factor of −1.3. Based on the Zimm model, the pore size of bovine mucus was estimated and agreed well with previous experimental data. In the presence of transfection reagents, e.g., liposomes, the diffusion of DNAs increased by a factor of 2 in mucus. By using bovine mucus as a model system, this work suggests that DNA size, topology, and the presence of transfection reagents may affect the diffusion of DNA in tissues, and thus the therapeutic effects of DNA.

INTRODUCTION

Mucosal surfaces of the gastrointestinal, respiratory, and reproductive tracts have long been used as sites for delivering therapeutics, particularly low molecular weight drugs, but there is increasing evidence that mucosal sites may be appropriate and useful for delivery of vaccines for preventing infectious diseases, e.g., human papilloma virus (HPV)-associated diseases (1–3) and unwanted pregnancy (4,5). However, the mucus gel layer associated with mucosal surfaces may be a significant barrier to the delivery of macromolecules. Mucus can trap and slow the diffusion of macromolecules or particles by several mechanisms: (1), it has a densely packed fibrous structure (for example, the pore size of human cervical mucus is ~100 nm (6)); (2), it can collaborate with secretory antibodies (e.g., sIgA), which can bind to macromolecules or particles (7,8); (3), negatively charged glycosylated regions of mucin fibers can form low-affinity bonds with some molecules; and (4), hydrophobic surfaces in the mucus gel can trap some molecules.

The diffusion of macromolecules (6,9), leukocytes (10), and virus particles (11) through the mucus has been studied using epifluorescence microscope (6,9), direct visualization of movement of cells (10) or particles (12), diffusion chamber (13), and fluorescence recovery after photobleaching (FPR) (6,11). These studies indicate that most proteins, antibodies, and virus-sized particles can diffuse through human cervical mucus almost as rapidly as they diffuse through water, but there are exceptions: IgM, small aggregates of sIgA or HSV diffuse more slowly in mucus than in water. Diffusion appears to depend primarily on the molecular weight, although binding of certain macromolecules (such as sIgA and IgM) to mucin fibers can influence their rates of diffusion significantly (11).

DNA is another potential therapeutic macromolecule. Delivery of plasmid DNAs to mucosal surfaces has been promising as both therapeutic and prophylactic approaches to genetic diseases (e.g., cystic fibrosis (14)) and infectious diseases (e.g., HIV (15)). In this event, diffusion through mucus is the first physical barrier for DNA, which must be crossed to be available to underlying cells. Although DNA is often delivered to the mucosal surface in combination with agents designed to enhance transfection, diffusion of naked plasmid DNA may be important in some applications (see Shen et al. (5), for example).

Plasmid DNA is different in many aspects from other molecules and viruses. First, DNA is a large molecule (several million daltons), which may make it difficult for DNA to penetrate through the densely packed mucin fibers and reach the surface of epithelial cells. Second, a plasmid DNA can exist in different topological configurations such as linear or supercoiled. The supercoiled configuration of DNA shows higher gene expression in cells than a linear one does and, therefore, is most often used in gene therapy or DNA vaccines (16–19). The diffusion of a plasmid DNA through mucus may be affected by its topological configuration. Lastly, a plasmid DNA is usually complexed with a transfection reagent for improving gene transfer. Transfection reagents may change the size, charge, or topology of DNA, and thus affect DNA diffusion through mucus. No previous studies have evaluated the role of DNA size, topology and presence of transfection reagents in DNA diffusion through mucus. The goal of this study is to address these questions

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using FPR techniques and to use existing models to describe diffusion of DNA in bovine cervical mucus.

MATERIALS AND METHODS

Materials

Plasmids (ϕX RFI (5.4 kb), PUC 19 (2.7 kb), PBR (4.4 kb)) were purchased from New England Biolabs (Beverly, MA). PcDNA3/LDH-C4 (6 kb) and gWiz/*Lac*Z (8.3 kb) were purchased from Aldevron (Fargo, ND). Oestrus bovine cervical mucus was obtained from Humagen Fertility Diagnostics (Charlottesville, VA). The mucus was used directly without further dilution. Two transfection reagents were used, Tfx-20 (Promega, Madison, MI) and Superfect (Qiagen, Valencia, CA).

Linearization of DNA

All the purchased DNAs were in supercoiled form. Supercoiled DNAs were linearized using restriction enzymes. gWiz/*Lac*Z was linearized by either Xho I or BmH I. ϕX RFI was linearized by Xho I. Other DNAs were linearized by *EcoR* I. All enzymes were purchased from New England Biolabs (Beverly, MA). Linearization reactions were carried under conditions as suggested by the manufacturer for each enzyme. The degree of linearization was determined by 1% agarose gel electrophoresis.

Labeling of DNA molecules

Both supercoiled and linearized DNAs were labeled using the Label IT Fluorescein Nucleic Acid labeling kit from Mirus Corporation (Madison, WI) following the procedure described by the manufacturer. The labeled DNAs were purified by ethanol precipitation and resuspended in DNase free TE buffer. Based on the manufacturer's literature, DNAs remain intact after being labeled by Labeling IT reagents.

Diffusion measurement

FPR was used to measure the diffusion of DNA or DNA/transfection reagent complex in mucus or PBS. All the measurements were carried out at room temperature (20°C). Solutions of 5 μg of labeled DNA were prepared in 50 μl of 10 mM PBS or mucus, and then added to a spherical cavity microscope slide (5 mm in diameter, 200 μm in maximum depth). For studies in mucus, a very small amount of DNA solution was used to avoid diluting mucus samples. The well was sealed with a cover slip and equilibrated for 15 min before measurement. FPR was carried out using a confocal laser scanning microscope (LSM 510 META, Zeiss, Thornwood, NY). The 488-nm line of a 30-mW argon ion laser was used for sample bleaching and fluorescence excitation. Emitted light was monitored at 520 nm. Typical settings for bleaching and recovery imaging are 100% and 0.5% of maximum laser power, respectively. From the whole field, a circle ($D = 6.75$ μm) was selected for bleaching. The minimum bleach time was 0.5 ms. For recovery, a time series of images of bleached region were recorded till the recovery approached 100%. The intensity of bleached region was recorded automatically. The diffusion coefficient is calculated by the equation: $D = \gamma R^2/4\, t_{1/2}$, where γ is a parameter depending on the degree of photobleaching, R is the radius of bleaching area, and $t_{1/2}$ is the half-time for diffusive recovery of photobleaching-induced concentration fluctuation (20,21). Both γ and $t_{1/2}$ can be estimated from the recovery curve.

RESULTS

Size and topological structure of plasmid DNAs

We evaluated the size and topological structure of DNA molecules used in our measurements by agarose gel electrophoresis (Fig. 1). By comparison to supercoiled molecular weight markers, all the plasmids maintained supercoiled form with correct size (Fig. 1, *left panel*). Except for plasmid gWiz/*Lac*Z, all the DNA preparations displayed a single band on electrophoresis. In contrast, plasmid gWiz/*Lac*Z had two major bands: the larger band corresponded contaminating DNA of a slightly higher molecular weight. All the linearized DNAs were obtained by digesting the supercoiled DNAs with a single restriction endonuclease for which the plasmid had a single restriction site. All the linearized DNAs migrated as a single band with the anticipated size (Fig. 1, *right panel*) determined by 1-kb molecular weight ladder.

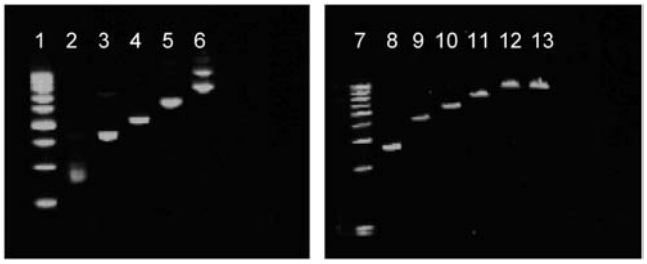

FIGURE 1 Agarose gel picture of supercoiled and linearized DNAs. (*Left panel*) Supercoiled DNAs. (*Lane 1*) supercoiled molecular marker (2–10 kb), (*Lane 2*) PUC 19 (2.7 kb), (*Lane 3*) PBR (4.4 kb), (*Lane 4*) ϕX RFI (5.4 kb), (*Lane 5*) pcDNA3/LDH-C4 (6 kb), and (*Lane 6*) gWiZ/*Lac*Z (8.3 kb). (*right panel*) Linearized DNAs. (*Lane 7*) 1 kb ladder, (*Lane 8*) PUC 19 (2.7 kb), (*Lane 9*) PBR (4.4 kb), (*Lane 10*) ϕX RFI (5.4 kb), (*Lane 11*) pcDNA3/LDH-C4 (6 kb), (*Lane 12*), and *lane 13*) gWiZ/*Lac*Z (8.3 kb). gWiZ/*Lac*Z in lane 12 was digested by Xho I and gWiZ/*Lac*Z in lane 13 was digested by *Bam*HI.

Diffusion of DNA molecules in PBS

The diffusion of both supercoiled and linearized DNAs in PBS varied with DNA size, decreasing as the number of

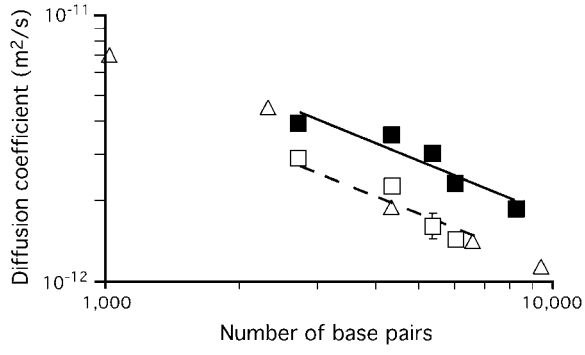

FIGURE 2 Diffusion coefficients of DNA molecules in PBS as a function of their size in basepairs. Open squares indicate linear DNAs and solid squares indicate supercoiled DNAs determined from this work; open triangles indicate the diffusion coefficients of linear DNA from the literatures (27,28). The dashed line (linear DNAs) and the solid line (supercoiled DNAs) represent the prediction by the Zimm model (25). Error bars indicate the standard error of the mean based on 10–20 measurements.

basepair increased (Fig. 2). The diffusion coefficients of linear DNAs in PBS were in agreement with previously published values obtained by various methods (Fig. 2), which validated our FPR method. Compared to linear DNAs of the same size, supercoiled DNAs diffused faster in PBS.

Linear gWiZ/*Lac*Z diffused faster than any other linear DNAs. To test whether this behavior was due to a particular restriction enzyme, we digested supercoiled gWiZ/*Lac*Z with different restriction enzymes. All the enzymes we used only produced a single band with anticipated size on the agarose gel (Fig. 1, *right panel*). The diffusion of all resulting linear gWiZ/*Lac*Z DNAs was faster than expected. This unexpected behavior may be due to the sequence of gWiZ/*Lac*Z DNA, which also affects diffusion of DNA (22,23). Because of its different behavior, we did not include linear gWiZ/*Lac*Z in our analysis.

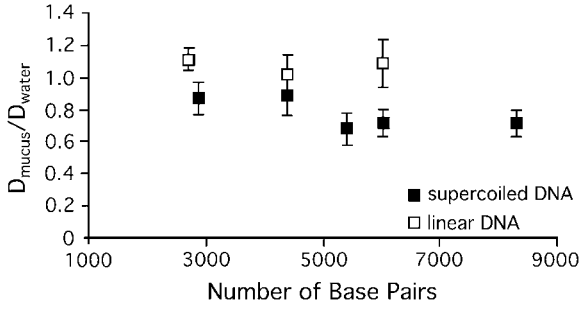

FIGURE 4 Reduced diffusion coefficient (D_{mucus}/D_{water}) of DNAs in mucus as a function of size in basepairs. Open squares indicate linear DNA, solid squares indicate supercoiled DNAs. Error bars indicate standard deviation, which is calculated as $((SD_{PBS}/D_{PBS})^2 + (SD_{mucus} \times D_{mucus}/D_{PBS}^2)^2)^{0.5}$, where *SD* denotes standard deviation and *D* denotes mean of diffusion coefficients from Fig. 2 (D_{PBS}) and Fig. 3 (D_{mucus}).

Diffusion of DNA molecules in mucus

The diffusion coefficients for linear and supercoiled DNA molecules were determined in bovine mucus (Fig. 3). The diffusion coefficients of supercoiled DNAs in mucus were about the same as their linear form. The reduced diffusion coefficient, D_{mucus}/D_{PBS}, was used to demonstrate the effect of mucus on diffusion of DNAs (Fig. 4). The hindrance of mucus to DNA diffusion was dependent on topology. The diffusion of linear DNA was not significantly retarded ($D_{mucus}/D_{PBS} \sim 1$), but the diffusion of supercoiled DNA was significantly retarded ($p < 0.05$), especially when DNA size was larger than 5 kb.

Effect of transfection reagents on DNA diffusion in mucus

Liposomes, such as Tfx 20, and dendrimers, such as Superfect, are commonly used transfection reagents. Both transfection reagents are positively charged, whereas DNA is highly negatively charged, so that mixture of DNA with either reagent leads to the formation of supramolecular complexes; with an increase in the ratio of Tfx 20 or Superfect to DNA, the charge of the complex shifts from more negative to more positive. DNA/Tfx 20 complexes were small and uniformly distributed in mucus (Fig. 5 *a*). With the increase of the ratio of Tfx 20 to DNA, DNA diffused faster in mucus (Fig. 6). Superfect formed large and nonuniform aggregates with DNA (Fig. 5 *b*), therefore, we were not able to use FPR to determine the diffusion coefficient of DNA/Superfect complex because the resulting recover curves was erratic and could not be fit by a single diffusion coefficient.

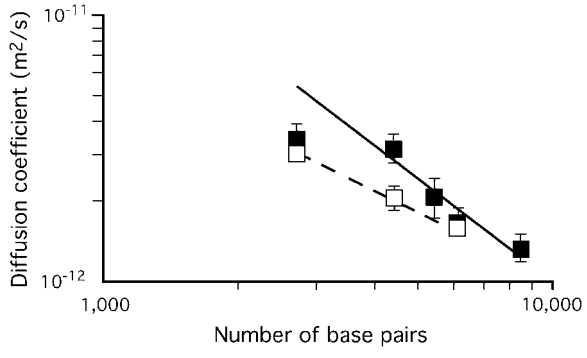

FIGURE 3 Diffusion coefficients of DNAs in mucus as a function of their size in basepairs. Open squares indicate linear DNAs, solid squares indicate supercoiled DNAs determined for this work. The dashed line (linear DNAs) represents the predictions by the Zimm model (25) and the solid line (supercoiled DNAs) represents the predictions by the reptation model (26). Error bars stand for the mean of standard deviation of 10–20 measurements.

DISCUSSION

We investigated the diffusion of DNAs with various size and topology (linear or supercoiled configuration). Within the size range of DNA we examined, mucus did not retard

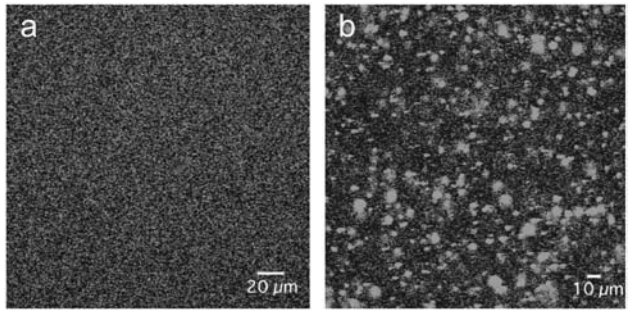

FIGURE 5 Confocal microscopy pictures of supercoiled gWiZ/*Lac*Z/transfection reagent complex in mucus. (*a*) Supercoiled gWiZ/*Lac*Z/Tfx-20 complex, which was formed by mixing 5 μl 5 μg/μl of DNA solution with 2.5 μl Tfx 20 following the protocol suggested by manufacturer. (*b*) Supercoiled gWiZ/*Lac*Z/Superfect complex in mucus, which was formed by mixing 5 μl 5 μg/μl of DNA solution with 2.5 μl Superfect reagent following the protocol suggested by manufacturer. Gray dots represent the complex of DNA with transfection reagents. Scale bars represent 20 μm.

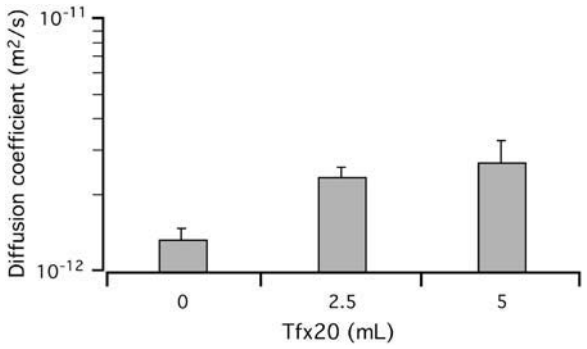

FIGURE 6 Effect of Tfx 20 on the diffusion coefficient of supercoiled DNA in mucus: 5 μg supercoiled gWiZ/*Lac*Z was mixed with various amount of Tfx 20 reagents following the protocol suggested by manufacturer. Data show mean ± SE (n = 10–13).

the diffusion of linear DNAs, but significantly reduced the diffusion of supercoiled DNAs compared to diffusion in water (Fig. 4). This result demonstrates that mucus is a potential barrier to supercoiled DNAs, especially to larger DNAs (with over 5,000 basepairs). Two DNA delivery vectors (liposomes and dendrimers) were used for examining if delivery vectors would affect the diffusion of DNA in mucus. Liposomes (Tfx 20) increased the diffusion of supercoiled DNA (Fig. 6), whereas Superfect formed large aggregates with DNA which we expect to hinder DNA diffusion in mucus (Fig. 5 *b*).

Several mechanisms may hinder the diffusion of DNA molecules through a mucus gel. DNAs can either bind to mucin fibers or they can be trapped by the size of mesh spacing between the mucin fibers. Because of the geometric complexity of fibrous gels, it is difficult to predict the rate of diffusion of a macromolecule through a fibrous gel even in the absence of binding interactions. Several models have been proposed to describe the diffusion of macromolecules in gels or fibrous media (reviewed in Pluen et al. (24)). These models are applicable for small spherical macromolecules, but not appropriate for flexible polymer chain molecules as DNAs.

Two models have been proposed for the diffusion of a polymer chain in gels based on the chain length relative to pore size. First, when the gyration radius, R_g, is smaller than half of the pore size and the polymer chain migrates in an ellipsoidal conformation, the diffusion of DNA in gel is described by the Zimm model (25):

$$D_g = 0.196 \times k_B T/6^{1/2}\eta R_g \sim N_0^{-1/2} \quad (1)$$

where D_g is the diffusion coefficient in gel, k_B is Boltzmann's constant, T is temperature, η is the solvent viscosity, and N_0 is the number of basepairs. This model was first developed for the movement of polymer chains in dilute solution, but it has also been used for diffusion of polymer chains in a porous gel system, where the movement of polymer chains is not constrained or does not interact with the gel network. Second, when R_g is greater than the half of pore size, the reptation model, proposed by de Gennes (26), describes the movement of an unattached chain by Brownian motion in a gel system as

$$D_g = a^2 k_B T/(3N_K^2 \delta_K b^2), \quad (2)$$

where a is the pore size, N_k is the number of Kuhn segments, δ_k is the friction coefficient of a Kuhn segment, and b is the Kuhn length ($b = 2p$, p is the persistent length of the chain).

When diffusion coefficients of DNAs in PBS are plotted as the function of the number of basepairs, N_0, on a logarithmic scale, the slope represents the scaling exponent of D as a function of N_0. Our results for linear DNA diffusion in PBS yield a scaling exponent of −0.63, which is in good agreement with Zimm's prediction of −0.6 for a polymer chain diffusing in a good solvent, as well as other experimental observations for DNA diffusion (−0.611 ± 0.016 (27), −0.68 (28), and −0.5 (24)). For supercoiled DNAs, D varied as a power of N_0, giving a scaling factor of −0.68 ± 0.01, which is also close to Zimm's predictions for a good solvent. Therefore, the diffusion of both linear and supercoiled DNAs in PBS can be described reasonably well by the Zimm model (Eq. 1). Using this model, we can estimate the radii of gyration of DNAs in PBS (Table 1). The radii of linear DNAs agree with the predictions by Smith et al. (27). The radius of supercoiled DNA was smaller than that of their linear form.

DNA diffusion in mucus can also be compared to the Zimm model (Eq. 1); the diffusion coefficients of linear DNAs in mucus yield a scaling factor of −0.81 as a power of N_0, and the diffusion coefficients of supercoiled DNAs in mucus yield a scaling factor of −1.3 (Fig. 3). The diffusion of DNAs in mucus revealed a steeper dependence on size of DNA (i.e., a more negative value of the scaling factor) than in PBS. Although no previous studies have reported DNA diffusion coefficients in mucus, Pluen et al. reported that the diffusion of small linear DNAs ($N_0 < 6000$ kb) in 2% agarose gel followed the Zimm model with a scaling exponent of −0.52 (24). This difference from the scaling coefficient obtained in mucus suggests that diffusion of DNA in other gel systems (e.g., agarose gel, collagen) can be very different from mucus; similar observations have been made in the study of protein diffusion in various gels (6).

TABLE 1 Radius of gyration of DNA in PBS

	Basepairs	$R_{g,\,L}$(nm)	$R_{g,\,s}$ (nm)
PUC 19	2686	115	86
PBR	4361	149	95
λ *Hin*dIII F4*	4361	180	
ϕ xRFi	5386	212	112
pcDNA3/LDH-C4	6000	235	146
λ *Hin*dIII F3*	6557	240	
gWiZ/*Lac*Z	8278		181
λ *Hin*dIII F2*	9416	310	

*Data from Smith et al. (27).

The diffusion of supercoiled DNA in mucus with a scaling factor of −1.3 is more consistent with that described by the reptational model, with the exception of the supercoiled PUC plasmid (see Fig. 3). PUC is 2.7 kb—the smallest of the plasmids tested—and, as a result, more rigid than other larger plasmids. We did not thoroughly investigate the diffusion of small supercoiled DNAs (<2.7 kb), but we suspect that smaller supercoiled DNAs may follow the Zimm model, as other studies suggested for diffusion of small size of linear DNAs in agarose gel (24). Though the theoretical prediction of the scaling factor for a purely reptation model is −2.0 (26), other experimental observations and simulations suggest that the actual scaling factor can ranging from −1.0 to −2.0, depending on the type of polymer chains and gels used (29,30).

Assuming that the diffusion of supercoiled DNA in mucus obeys the reptational model, we can estimate the effective pore size of bovine cervical mucus using Eq. 2 (Table 2). The estimated pore size is 12.5 ± 1.4 μm, which is in agreement with confocal microscopic (31) and electron microscopic evidence (pore size ranges from 1 to 20 μm) (32) for bovine cervical mucus. Previous theoretical calculations and electron microscopic evidence showed that interspacing of mucin fibers of human mucus is between 100 and ~380 nm (6), we expect that the diffusion of DNAs in human mucus could be different from what we observed in bovine cervical mucus.

Mucus hinders the diffusion of supercoiled DNAs in mucus, especially when the size of the DNA molecule is >5 kb (Fig. 6). The liposomal reagent, e.g., Tfx 20, increased the diffusion of supercoiled DNA in mucus. One feature of Tfx 20 is its ability to condense DNA. The size and structure of the reagent/DNA complex depend on the procedure used for mixing the components, the technique used to observe them, as well as the liposome composition. The size of the complex ranges 50 ~ 300 nm (33). Previous studies suggest that there are mainly three types of structure formed on complexation: one where DNA is packed between a short-range lamellar structure composed of flat lipid bilayers; another where the DNA is encapsulated inside a lipid bilayer, forming cylindrical complexes that are closely packed on a hexagonal network; and finally the so-called ''bean on a string'' complexes, where DNA attaches to the outer surface of the positively charged liposomes (33). In all three of the structures, DNA is no longer a flexible chain, but a more rigid particle, which is not properly described by either the Zimm model or the reptation model.

Many empirical models have been developed to analyze diffusion of rigid particles in polymer gels. One model that fits a range of experimental data is the obstruction-scaling model (34), in which the ratio of diffusion in a gel to the diffusion in water is given by:

$$D_g/D_0 = \exp\{(-\pi/4)[(R_H + R_f)/(a + R_f)]^2\}, \quad (3)$$

where D_0 is the diffusion coeffient in water, R_H is the hydrodynamic radius, a is pore size, and R_f is the radius of gel fiber. For mucin fibers, R_f ranges 5 ~ 30 nm (6,11). This model was developed for chemically cross-linked hydrogels and treats the case in which the gel pore size is greater than the hydrodynamic radius of the diffusion particle. Though mucin fibers are not chemically cross-linked, they do form a physically entangled gel, which is stabilized by low-affinity bonds between hydrophobic domains of mucin fibers (7). As discussed above, based on the diffusion of supercoiled DNAs in mucus, the pore size of bovine mucus (~15 μm) is much larger than the hydrodynamic radius of the DNA/Tfx 20 complex (50 ~ 300 nm), consistent with the model assumptions. We can estimate D_g/D_0 based on this model. In contrast to D_g/D_0 of free DNA (~0.7 for gWiZ/*Lac*Z, as shown in Fig. 4), D_g/D_0 of DNA/Tfx 20 is close to one, which indicates that Tfx 20 enhances the diffusion of DNA in mucus.

In summary, the mucus gel diffusion barrier has been evaluated for delivery of DNA to the mucosal surface. Our data suggest, within the size range commonly used in gene therapy or DNA vaccines, that linear DNA can diffuse through mucus readily whereas supercoiled DNA is mildly hindered. Though transfection reagents can facilitate gene transfer into cells, they can either hinder or facilitate DNA diffusion through mucus. Therefore, it is crucial to carefully evaluate the effect of transfection reagents on DNA diffusion through mucus when choosing transfection reagents for delivering DNA to the mucosal surface.

TABLE 2 Estimation of pore size of bovine mucus from the experimental diffusion coefficients of supercoiled DNA in mucus

	Basepairs	No. of Kuhn segments, N_k	Pore size, a (μm)
PBR	4361	148	11.8
ϕ xRFi	5386	183	11.8
pcDNA3/LDH-C4	6000	204	11.8
gWiZ/*Lac*Z	8278	281	14.6

Note: to solve Eq. 2, the following parameters were used: the Kuhn friction coefficient $\delta_k = 2.69 \times 10^{-10}$ Pa × m (24), the Kuhn length = $2p$ =100 nm (35), the chain diameter d = 2.4 nm (24), and the temperature T = 293 K.

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