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Review

Advances in the separation of bacteriophages and related particles

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Abstract

Nondenaturing gel electrophoresis is used to both characterize multimolecular particles and determine the assembly pathways of these particles. Characterization of bacteriophage-related particles has yielded strategies for characterizing multimolecular particles in general. Previous studies have revealed means for using nondenaturing gel electrophoresis to determine both the effective radius and the average electrical surface charge density of any particle. The response of electrophoretic mobility to increasing the magnitude of the electrical field is used to detect rod-shaped particles. To increase the capacity of nondenaturing gel electrophoresis to characterize comparatively large particles, some current research is directed towards either determining the structure of gels used for electrophoresis or inducing steric trapping of particles in dead-end regions within the fibrous network that forms a gel. A trapping-dependent technique of pulsed-field gel electrophoresis is presented with which a DNA–protein complex can be made to electrophoretically migrate in a direction opposite to the direction of migration of protein-free DNA.

Keywords: Reviews; Bacteriophages; Pulsed-field gel electrophoresis; Gel structure; Large particle fractionation; DNA–protein complex

Contents

1. Introduction

When analyzing events that occur in biological systems, characterization is performed of the components of the system present at each time during the event. To achieve this characterization, the components must first be isolated. Because of its efficiency (in both time and cost), gel electrophoresis is sometimes the technique used for this purpose.

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Although gel electrophoresis is most often used to fractionate monomolecular particles, multimolecular particles can also be fractionated by gel electrophoresis. The multimolecular particles include multienzyme complexes, ribosomes, chromatin, microtubules, protein–polysaccharide complexes used as vaccines, viruses and particles in the assembly pathways of viruses. These applications have been reviewed previously [1–4].

Although multimolecular particles are usually larger than their monomolecular counterparts, the application of nondenaturing gel electrophoresis to a multimolecular particle does not differ from its application to a monomolecular particle, if dissociation of the multimolecular particle does not occur during electrophoresis. In addition to the applications previously reviewed [1–4] for multimolecular particles, in two more recent studies, applications have been found for the gel electrophoretic co-fractionation of both monomolecular proteins and multimolecular protein assemblies, in the analysis of the pathway for the assembly of bacteriophage procapsids. A bacteriophage procapsid is a nucleic acid-free capsid that, in an infected cell, both packages DNA and, after a structural transformation, becomes the capsid of the mature bacteriophage. In a study of bacteriophage T7 [5], nondenaturing agarose gel electrophoresis was used to both separate and quantify both the multimolecular procapsid and the two constituents of the outer shell of the T7 procapsid, a strongly acidic, monomeric scaffolding protein and a weakly acidic, tetrameric protein that is the major outer shell protein of both the procapsid and the mature bacteriophage; the scaffolding protein exits the capsid during DNA packaging. The efficiency of the gel electrophoretic procedure assisted in both exploring conditions for observing assembly and characterizing the assembly process as a function of both time and the ratio of the amount of scaffolding to major outer shell protein. A result was the finding of a partially assembled procapsid that had not been detected previously [5]. In a study of the assembly of the outer shell protein of the procapsid of bacteriophage HK97, both pentameric and hexameric assembly intermediates [6] and a complex of a monomer with the GroEL chaperonin [7] were detected by nondenaturing agarose- and polyacrylamide gel electrophoresis. This analysis of the assembly of HK97 procapsids was assisted by both the efficiency and the sieving-based resolution by size that is achieved with gel electrophoresis.

The above studies of bacteriophage procapsid assembly are examples of the usefulness of nondenaturing gel electrophoresis in the analysis of both the assembly and the function of multimolecular particles. The techniques employed have remained at least as useful today as they were when previously reviewed [1–4]. Presumably, these techniques will be used to solve additional biological problems as time progresses. However, the question remains: To what extent can nondenaturing gel electrophoresis be further developed to yield more information during fractionation of both purified biological particles and biological particles that are in crude cellular extracts? Most particles of interest will be multimolecular; some will be either viruses or virus-related particles. Primarily from the perspective of physics, several recent, theoretical studies have suggested the use of the random (thermal) motion of macromolecules in schemes of fractionation [8–11]. Because these schemes involve an asymmetrical response to a partially symmetrical input, they are sometimes called ratcheting. Beyond its application to separation science, the concept of ratcheting also has potential application to understanding the isothermal transduction of energy in biological systems [9,10]. Primarily from the perspective of molecular biology–virology, the authors will describe here an empirical, ratcheting-based approach to gel electrophoretic fractionation. The context will be both a review and, the authors hope, a preview. The previewlike characteristic was introduced in an attempt to encourage multiresearcher, multidisciplinary participation. Ratcheting-like fractionations will have a basis that is more complex than that of fractionations usually used in molecular biology; participation of physical scientists may not be optional for productive application of these fractionations in molecular biology–virology.

2. Fundamentals of gel electrophoresis for small (<200 nm in radius) particles

Historically, gelled media were initially used for electrophoresis in order to prevent convection during electrophoretic migration. For most of these early

studies, the gel was formed by agar (reviewed in Ref. [12]). Because agar has negatively charged groups added to its disaccharide backbone of β-D-galactose and 3,6-anhydro-α-L-galactose [13,14], the electrophoretic mobility (μ=velocity/electrical potential gradient) was altered by cathode-directed electrical field-induced flow of counterion-associated water (electroendosmosis, or EEO). The gel-associated negatively charged groups also caused some proteins to adhere to the gel (reviewed in Ref. [15]). When most charged groups were removed from agar to form a preparation called agarose, the EEO decreased and separations improved. The most extensively purified agarose preparations yield gels that have an EEO-induced mobility (μ_E) of less than 1.0×10^{-5} $cm^2/V\cdot s$ (calculated from data in Ref. [16] by use of an empirical relationship reviewed in Ref. [15]). For the proteins fractionated in agar gels, the ratio of the size of the protein to the radius of the effective pore of the gel was small enough so that a retarding effect on these proteins was not detectably caused by the fibers that formed an agar gel [12]. The basis of fractionation was the average electrical surface charge density of a particle, proportional to the μ that is observed in the absence of a gel (μ_0) (reviewed in Ref. [15]). The subsequent introduction of the tighter matrices of polyacrylamide gels yielded fractionations of monomolecular proteins on the basis of both μ_0 and the nonadsorptive interaction of particles with the fibrous network of the gel (reviewed in Refs. [17,18]). These nonadsorptive interactions are collectively called sieving.

Even in the case of spheres, understanding of sieving is at a comparatively primitive level. Empirical relationships have been reduced to the following [19]:

$$\mu/\mu_0' = (1 - R/P_E)^2 \quad (1)$$

where μ_0' is μ extrapolated to a gel concentration of 0; R is either the radius of a sphere or the effective radius of a nonspherical particle; P_E is the radius of the effective pore of a gel. A rigorous explanation of why Eq. (1) so accurately represents the data has not yet been developed. Recent efforts have been made to describe the sieving of gels via the fractional volume occupied by a particle in a two-dimensional lattice [20]. For practical purposes, after μ is determined as a function of gel concentration for a sphere of known R, Eq. (1) is used to determine P_E as a function of gel concentration. This P_E value has the following characteristics that indicate its applicability for a variety of particles: (a) The P_E value obtained by quantifying sieving via Eq. (1) is indistinguishable from the R of the largest sphere that enters a gel [19]. (b) This P_E is also indistinguishable from one-half the length of the longest rod that migrates through a gel without exhibiting the effects of end-first migration (called reptation) [21]. (c) This P_E is also proportional to one half the mean interfiber spacing observed in thin sections of the same gels used for the (sieving-based) determination of P_E [22]. The fibers observed by electron microscopy are lateral aggregates (called suprafibers) of more primitive fibers; for the more primitive fibers, a double helical structure has been proposed [23,24]. (d) The use of P_E determined via Eq. (1) yields effective R values for defined-length chromatin that are, quantitatively, in agreement with results obtained by rate zonal centrifugation [25,26]. (e) Even when the sieving per mass of gelled fibers differs by a factor as high as 3.5 for two different gel-forming compounds, equalizing P_E values also equalizes the fractionation of double-stranded DNA shorter than 1000 base pairs, for all DNAs except the longest DNAs resolved. This latter (slight) perturbation may be caused by a difference in either the distribution of pore sizes or the flexibility of the gelled fibers [27].

In this regard, the authors note that several factors in addition to gel concentration determine P_E. At any given concentration of gel (by mass), the value of P_E has been found to decrease as (a) the extent of either hydroxyethylation [19] or carboxymethylation [27] increases, (b) the temperature of gelation decreases [28], (c) the molecular mass of the agarose increases [28] and (d) the ionic strength during gelation decreases [27,29]; in at least some cases, the effect of changing one of these factors depends on the others [27,28]. The reader can, qualitatively and easily, test the effect of hydroxyethylation, temperature of gelation and ionic strength by visually observing the turbidity of the commercially prepared gels cast. Thus far, the authors have always found that the clearer the gel, the smaller the P_E will be, if (light-scattering) precipitates are not present. Although precipitates do form when agarose is gelled in the presence of some polyethylene glycols [30], the formation of precipitates has not yet (to the

authors' knowledge) been observed in the absence of a known precipitating agent, such as a second, nonagarose polymer. In the presence of precipitating agents, formation of microprecipitates is discriminated from the formation of large pores by electron microscopy of thin sections [30].

The effect of ionic strength on P_E occurs for underivatized agarose preparations that are in frequent use for the analysis of biological macromolecules. For example, in Fig. 1a, the fibers (mostly suprafibers) of a frequently used underivatized agarose gel are revealed by electron microscopy of a thin section of a 1.0% gel cast in a buffer that is sometimes used for the gel electrophoresis of DNA. When this same gel was cast in water, a subtle increase in the number per area of the thinnest fibers causes the mesh of gel-forming fibers (Fig. 1b) to be finer than it was for the gel cast in buffer. This difference, detected by inspection of Fig. 1, was confirmed by measurement of P_E; the P_E of the buffer-cast gel was 1.7× the P_E of the water-cast gel (legend to Fig. 1). Because of the dependence of pore size on several variables in addition to gel concentration, size standards must always be used when the value of P_E is needed. Commercial agarose preparations are usually standardized by EEO, not by the characteristics of sieving. The authors also make the point that, if the (incorrect) assumption is made that the value of P_E depends only on gel concentration, comparing data from different gels may yield inconsistencies that would not have been present if P_E values had been determined by use of spherical standards.

For the ionic strength-dependence of Fig. 1, a possible explanation is ionic strength-induced reduction of electrical charge–charge repulsion by residual negatively charged groups on the agarose (see also Ref. [27]). By this explanation, the reduced electrostatic repulsion promotes formation of both more and wider suprafibers. However, the following observation suggests that knowledge of electrical charge (via EEO) will not be sufficient to predict the response of P_E to ionic strength: The effect of methylation on the strength of an agarose gel correlates primarily with methylation of the 2-OH position of the D-galactose constituent of agarobiose [32]; this hydroxyl is the hydroxyl that has the most inwardly pointing position in the previously proposed (Ref. [24]) agarose double helix [32]. Independent of the extent to which the agarose double helix represents the secondary structure of agarose molecules in an agarose gel, if a

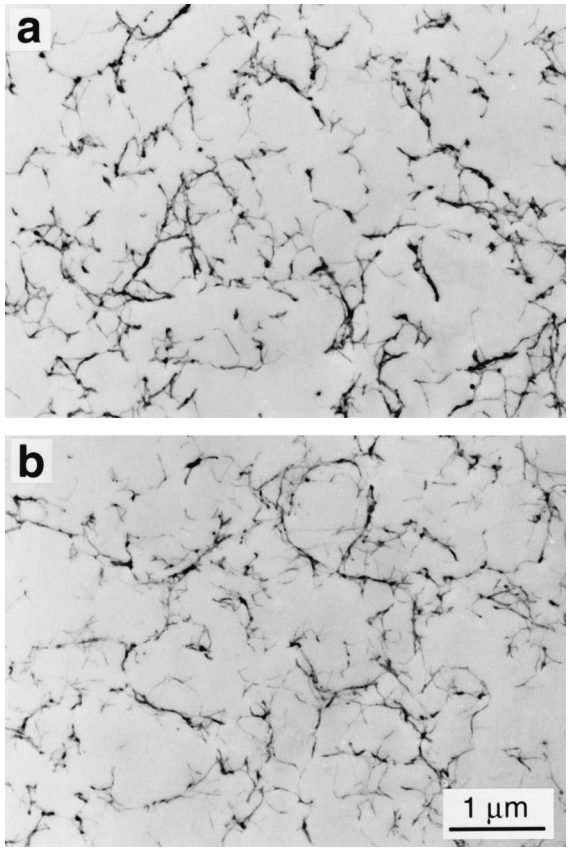

Fig. 1. Electron microscopy of underivatized agarose gels. Agarose gels were prepared for electron microscopy by fixation–dehydration–embedding–thin sectioning–staining, by use of previously described procedures [22] modified in the following way: Potassium ferrocyanide was added at a concentration of 2.5% to the solution of 1.5% osmium tetroxide used as a fixative before dehydration. This addition enhanced staining. The thickness of sections (dark gold) was 120±15 nm; the dimensions of the gel changed by less than 5% during fixation–dehydration. Thin sections were observed in a JEOL 100CX electron microscope at a magnification of 10 000×. Values of P_E for each gel were determined by procedures that were described previously [4]; turbidity at 460 nm was measured at room temperature (22±3°C), in a 1-cm plastic cuvette, using an LKB Ultrospec 4050 spectrophotometer. The following 1.0% gels of Seakem LE agarose (lot #62773; FMC Bioproducts, Rockland, ME, USA) were the samples: (a) gel cast in 0.09 *M* Tris–acetate, pH 8.4, 0.001 *M* EDTA (turbidity = 0.61; P_E = 125±6 nm), (b) gel cast in glass-distilled water (turbidity = 0.30; P_E = 74±4 nm).

derivatizing group is on an hydroxyl that is usually at an intermolecular interface, this derivatizing group will have a comparatively large effect on the properties of the gel. Thus, the working hypothesis is that only a small fraction of derivatizing groups causes the dependence of gel structure on ionic strength. If so, then equality of EEO will not assure equality of response to ionic strength.

3. Microscopy of hydrated gels

To observe the fibers that are the source of the sieving of an agarose gel during analyses of particles with a radius between 1 and 200 nm, only two techniques of microscopy have, to the authors' knowledge, been used successfully to observe fibers in the interior of a gel: (a) Electron microscopy of a freeze-fractured gel has been used to observe the agarose fibers emerging from fractured ice [29]. (b) As illustrated by the electron micrograph in Fig. 1, electron microscopy of a stained thin section has been used to obtain a projected image of fibers within a slice of the interior of a gel. Both procedures have a resolution of approximately 2 nm; resolution is limited by the characteristics of the specimen. Either freezing or dehydration–embedding–sectioning has the potential to alter the structure of the gel. However, in the case of sectioning, less than a 5% change in the dimensions of the gel is observed during dehydration–embedding [22,28,33,34]. Thus, the assumption is made that the distribution of fibers in the gel is not detectably altered during preparation for sectioning–electron microscopy. Nonetheless, microscopy of a hydrated gel is a desirable procedure to develop.

For observing the structure of a hydrated gel with a resolution of approximately 500 nm, light microscopy is potentially useful. However, initial attempts to perform light microscopy of agarose gels did not reveal structure [35]. Using a large-pore gel that was known to have micron-sized structure through analysis by electron microscopy, this micron-sized structure has been revealed by phase contrast light microscopy [36]. In agreement with Ref. [35], the technique of phase contrast light microscopy used for the large-pore gel revealed absolutely no structure in a conventional agarose gel, such as the gel in Fig. 1 [36]. To improve the resolution obtained during observation of a hydrated specimen, atomic force microscopy, a new and promising technique, has more recently been used to image the surface (but not the interior) of a conventional agarose gel [37]. Pores were observed in images whose resolution was not stated in Ref. [37]. Examination of the smallest features resolved in Ref. [37] suggests a resolution of approximately 100 nm, an improvement when compared to light microscopy, but still far from the resolution of electron microscopy. Thus, when the pore size distribution of a gel was determined by atomic force microscopy in Ref. [37], no pore smaller than 100 nm could have been resolved; none smaller than 150 nm was observed for a 3% gel in Fig. 2 of Ref. [37]. The presence of smaller, unresolved pores is indicated by the observation that a sphere that is either 115 nm or larger in diameter does not migrate through a 3% conventional agarose gel [38]. Thus, although significant strides have been made, the data from atomic force microscopy do not yet have the resolution needed for understanding gel electrophoretic sieving. To understand sieving, the best technique would image the interior (not the surface) of the gel. To visualize the structure of hydrated gels, a second potentially useful technique is scanning electron microscopy of hydrated specimens. However, although this latter procedure can be used to characterize sponges, it does not yet have the resolution for the characterization of gels at the level of the fibers visualized in Fig. 1 (reviewed in Refs. [39,40]).

4. Fractionation and characterization of spheres and rods

During the gel electrophoresis of either a sphere or a rod, the value of μ depends on both μ_0 and the sieving of the gel, the latter expressed by the right side of Eq. (1). In the authors' experience, these two factors have always been independent of each other. In the case of μ_0, changing the size of the particle is known, for the conditions of electrophoresis usually used, not to change μ_0 (theory [31,41]; practice [42,43]). In the case of R, changing the μ_0 of a particle does not alter the particle's sieving [22]. To observe the sieving of a particle independent of its

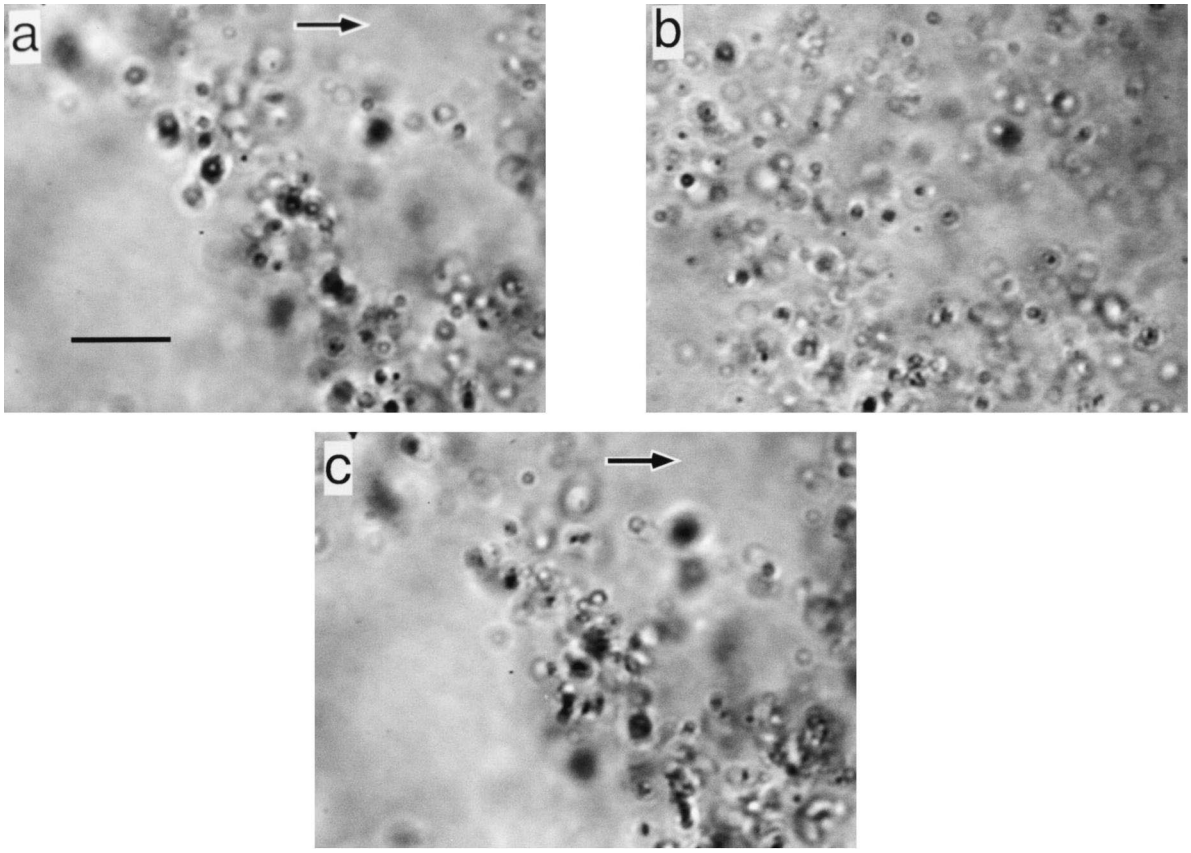

Fig. 2. Decoration of an agarose gel by elevated field-induced arrest of latex spheres. To produce a gel with a comparatively nonuniform distribution of pore sizes, the following agarose was used to cast a 0.4% gel, in 0.01 *M* sodium phosphate, pH 7.4, 0.001 *M* EDTA, at room temperature: glycerylated agarose that had been degraded by exposure to gamma radiation at 750 krad. This agarose is described in Ref. [28]. After casting the gel in a miniature electrophoresis apparatus that fit on the specimen stage of an Olympus BH phase-contrast light microscope, latex spheres (radius = 0.24 μm) were soaked into the gel. After covering the gel with a glass coverslip, phase contrast light images were videotaped. The following sequence of images is shown here for a spatial field that is fixed: (a) latex spheres trapped in the gel by exposure to an electrical field of 4 V/cm (the direction of the field is indicated by the arrow), (b) latex spheres in the same spatial field, after subsequent reduction to zero of the magnitude of the electrical field, (c) latex spheres in the same spatial field, after a second trapping induced by restoring the electrical field to 4 V/cm (the direction of the field is indicated by the arrow). For (a) and (c), 20 frames, equally spaced in time, were averaged for a period of 6 s. For (b), only a single frame was used. The length of the bar is 10 μm. In (b), most spheres are undergoing Brownian motion; in (a) and (c), most spheres are not undergoing Brownian motion.

μ_0 and vice-versa, either μ must be determined at more than one gel concentration or the value of μ_0 must be made identical for all particles. The latter approach is used when denatured proteins are fractionated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The sodium dodecyl sulfate coats all proteins and, therefore, causes the proteins to have the same μ_0 [44,45]. For either native proteins or native multimolecular protein complexes, the former approach is used by either electrophoresis in several gels, each of different concentration, or two-dimensional electrophoresis in which the first dimension gel is more dilute than the second dimension gel (these latter procedures are reviewed in Refs. [1–4]). Whatever procedure is used for sieving-based fractionation, Eq. (1) implies that separation by an inflexible sphere's R should progressively increase as P_E decreases, as long as P_E

does not become so small that the sphere does not enter the gel. The limit in this type of analysis is set by a nonfundamental aspect of the experimentation: The product of the time of electrophoresis and the electrical field must be progressively increased as P_E approaches R. As the time and (or) field increase, either the particles fractionated will be taxed to the point of changing properties or the experimenter's patience will be taxed to the point of finding something else to do.

The above discussion is for inflexible spheres. The question of what spheres are flexible under conditions of gel electrophoresis is not, to the authors' knowledge, yet answered. A key indication of flexibility is the entry of a sphere into a gel at higher, but not lower, electrical field strengths. In the case of latex spheres, electrical fields as high as 30 V/cm do not cause entry into a gel when the sphere would not enter at a lower electrical field strength. In reality, the opposite is the case: As the electrical field increases, eventually, the magnitude of μ decreases progressively to zero. This effect is discussed in the next section. The authors are not aware of any spherical particle that can be made to enter a gel by raising the electrical field strength. In contrast, linear double-stranded DNA, a random coil, does undergo electrical field-induced deformation that increases as the magnitude of the field increases. This deformation causes end-first migration of the deformed DNA, a form of reptation [46–49].

As mentioned above, reptation is also experienced by flexible rods when the P_E of the gel is smaller than one-half the length of the rod. When they are reptating, rod-shaped particles can be detected by their response to the electrical field. Empirically, as the magnitude of the electrical field increases, the magnitude of the μ of a rod (but not a sphere) increases [4,21]. If the persistence length is greater than P_E, then this increase is predicted theoretically [50]. In contrast to the reptation of a multipersistence length random coil, such as 0.1–10.0 megabase pair double-stranded DNA [persistence length = 50 nm (= 147 base pairs) [51]], a flexible rod is not expected to herniate laterally [52,53] during migration through the fibrous network of a gel. Thus, analysis of the sieving of flexible rods should, in the future, provide comparatively uncomplicated tests of the assumptions on which the theory is built.

5. Large (>200 nm in radius) particle gel electrophoresis

The use of μ to describe migration during electrophoresis is, to some extent, based on the observation that μ is usually independent of the magnitude of the electrical field. In support, the spheres usually fractionated by gel electrophoresis (see Refs. [1–4]) are 'small enough' so that increasing the electrical field strength does not alter sieving. 'Small enough' means that, for the electrical fields normally used during gel electrophoresis (1–10 V/cm), Brownian motion is large enough to prevent electrophoretic trapping of spheres within dead-ends in the fibrous network that forms a gel. However, Brownian motion decreases as the radius of a sphere increases [54]. When negatively charged latex spheres have a radius of either 200 nm or more, they are no longer 'small enough'. As the electrical field increases in the range 1–10 V/cm, the magnitude of μ decreases [55,56], eventually to zero. Light microscopy of the spheres during gel electrophoresis reveals field-induced trapping of the spheres, presumably in the smaller pores of the gel; both Brownian and electrophoretic motion are lost during trapping [55]. When the magnitude of the field is reduced to zero after trapping, Brownian motion is regained [55].

To determine whether the trapping of latex spheres occurs at preferred, rather than random sites, the following experiment was performed: By application of a field of 4 V/cm, latex spheres were trapped in a gel of degraded glycerylated agarose, which is known ([28]) to have a comparatively broad distribution of pore sizes; large pores are surrounded by a network of comparatively small pores. By averaging 20 frames equally spaced over 6 s, images of only the trapped spheres were obtained (Fig. 2a); the images of moving spheres were too weak to see. After the trapping shown in Fig. 2a, detrapping, followed by diffusion of the spheres, was accomplished by reducing to zero the magnitude of the electric field (Fig. 2b; averaging was not used). Finally, the spheres were retrapped (Fig. 2c; averaging was used), as they had been for Fig. 2a; Fig. 2a–c all show the same spatial field. After retrapping (Fig. 2c), the position of the trapped spheres was not exactly the same as it had been in Fig. 1a, but, at low resolution, the spheres were concentrated in the same

zones. The zones preferred for trapping presumably have comparatively small pores. Similar zones of small pores were also observed when the fibrous network of a 0.4% agarose gel was observed by electron microscopy of a thin section [28]. Thus, the trapped spheres have decorated the gel, thereby marking the zones of comparatively small pores. As even an underivatized agarose gel becomes more dilute, it increasingly partitions to form zones of comparatively small pores that surround comparatively large pores [22].

By lowering the magnitude of the electrical field, progressively larger particles can be subjected to gel electrophoresis [55]. By raising the magnitude of the electrical field, progressively smaller spheres should undergo trapping. However, trapping depends on both (a) the extent of the sphere's Brownian motion and, therefore, its R and (b) the electrostatic force on the sphere and, therefore, its μ_0. Presumably, trapping also depends on both the density, the distribution and the shape of the traps. Because of both the complexity of the analysis and the anticipated need for comparatively high fields when trapping is applied to spheres that are 10–100 nm in radius, including most virus particles, exploration of practical uses of trapping has thus far been limited to spheres with a radius of either 95 nm or more [57]. In this latter study, both the direction and the magnitude of the electrical field were discontinuously varied (pulsed) such that the time integral of the field was zero (zero-integrated pulsed-field gel electrophoresis; see Ref. [58]). This use of zero-integrated fields has at least two major advantages: (a) Resolution can be improved to the point that the direction of electrophoresis can be changed by small changes in R. (b) Because pH gradients are comparatively small (zero when the number of pulses is even), recirculation of buffer is not necessary during electrophoresis. Recirculation of buffer is the most difficult, though essential, part of analysis by nondenaturing gel electrophoresis, when the field is not zero-integrated. Thus, if a compact, battery-driven power supply were developed, in situ analysis of both viruses and other large particles is a potential use for trapping-based, zero-integrated pulsed-field gel electrophoresis. The most frequent current use of pulsed-field gel electrophoresis, including the zero-integrated mode, is the fractionation of comparatively long (greater than 50 kilobase pair) double-stranded DNA (reviewed in Refs. [59–63]).

6. Electrophoretic ratcheting of DNA–protein complexes

Although trapping-based pulsed-field gel electrophoresis is not yet practical for the spheres most often fractionated, trapping-based pulsed-field gel electrophoresis appears to have a more immediate application in the fractionation of DNA–protein complexes. Like the comparatively large spheres, DNA–protein complexes can be trapped by elevating the electrical field. The apparent reason for the trapping is migration of the DNA through a pore that is too small to allow passage of the DNA–attached protein. As the strength of the electrical field increases, the balance between trapping and reverse Brownian motion of the DNA shifts towards trapping. This type of trapping has been demonstrated for both 500–2000 base pair single-stranded DNA bound to streptavidin [64] and 40 kilobase pair double-stranded bacteriophage T7 DNA bound to a T7 capsid that was 30 nm in radius [65]. The T7 DNA–capsid complex was obtained by elevated temperature-induced expulsion of DNA from the T7 capsid; expulsion yields both DNA–capsid complexes and capsid-free DNA. When zero-integrated pulsed-field gel electrophoresis (high, trapping field = 5 V/cm; low, non-trapping field = 0.5 V/cm) was applied to this mixture of DNA and DNA–capsid complex, the capsid-free DNA was found to migrate in the high-field direction (band marked DNA in lane 1 of Fig. 3a). In confirmation of the identity of the DNA that forms this band, the distance from the electrophoretic origin is equal to the distance from the origin of the band formed by T7 DNA that had been purified by extraction with phenol and is, therefore, capsid-free (the latter DNA is fractionated in lane 2 of Fig. 3a). Even though a zero-integrated pulsed-field was used, net migration in the high field direction occurs for capsid-free DNA in Fig. 3a because of an electrical field-induced increase in the magnitude of the μ of double-stranded DNA; this increase is a result of reptation.

In contrast to the protein-free DNA in lane 1 of Fig. 3a, the DNA–capsid complexes underwent net

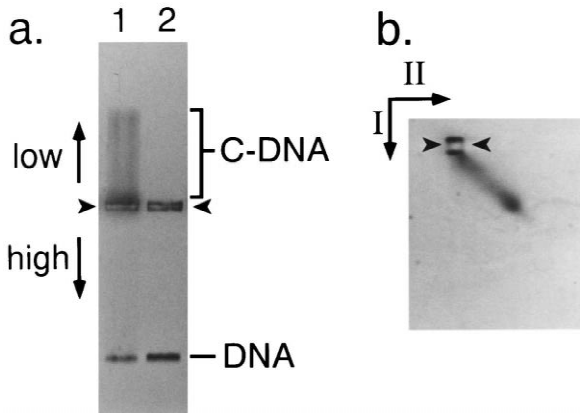

Fig. 3. Ratcheting-based fractionation of T7 DNA–capsid complexes. DNA was expelled from the capsid of bacteriophage T7 by incubation at 51°C; DNA was also extracted from T7 using phenol [65]. To minimize adherence to the gel, the T7 bacteriophage had a hybrid tail fiber as described previously (the A1 hybrid of Ref. [75]). (a) One-dimensional electrophoresis was performed of (1) DNA expelled at elevated temperature and (2) phenol-extracted DNA, by layering these samples in the sample wells of a 0.4% Seakem LE agarose gel cast in 0.01 *M* sodium phosphate, pH 7.4, 0.001 *M* EDTA. Electrophoresis at room temperature was performed by cyclically repeating (25 times) the following sequence of two pulses: first, 5.0 V/cm for 5 min in the high field direction; second, 0.5 V/cm for 50 min in the low field direction. (b) Using a 0.5% agarose gel, the following two-dimensional electrophoresis was performed of the DNA expelled at elevated temperature: Both the first and the second dimension were performed by executing 26 cycles that were the same as those of the one-dimensional electrophoresis used for (a). After electrophoresis, both gels were stained with 1 μg/ml ethidium bromide, destained and photographed through a Tiffen 23A filter. The arrowheads indicate the origins of electrophoresis. The arrows indicate the direction of electrophoresis: (a) low, direction of the 0.5 V/cm pulses; high, direction of the 5.0 V/cm pulses, (b) I, first dimension; II, second dimension. In (b), only the low field quadrant is shown.

migration in the low-field direction (zone marked DNA-C in lane 1 of Fig. 3a). This low field-directed net migration is the expected consequence of trapping during the high field pulses of zero-integrated pulsed-field gel electrophoresis. In contrast to the capsid-free DNA, the DNA–capsid complexes formed a comparatively broad zone that included a comparatively broad band at its leading edge. The comparatively broad distribution of DNA–capsid complexes could be the result of either μ-dependent resolution of particles of variable μ or non-μ-dependent dispersion of particles (or both). Non-μ-dependent dispersion could occur by either adhesion to the gel or nonuniformity of sieving. To determine whether the μ of the DNA–capsid complexes was variable or not, a two-dimensional fractionation was performed for which both the first and the second dimensional fractionation was similar to the fractionation of Fig. 3a. After this two-dimensional fractionation, particles were found near a diagonal line (Fig. 3b). That is, the DNA–capsid complexes do have a variable μ.

The length of the high field pulse used for Fig. 3a was 5 min; the length of the low field pulse was 50 min. If the length of the high field pulse during zero-integrated pulsing is much greater than the mean time before trapping of a DNA–capsid complex, then the following response is expected to increasing pulse times: No change in the electrophoretic pattern will be observed, except for changes that are indirectly related to pulse time, for example, changes in electrophoretic pattern caused by changes in either temperature or, if buffer is not recirculated, pH during the high field pulse. No direct effect of the pulse time should occur. In the absence of indirect effects, a pulse sequence of one high field pulse, followed by one low field pulse, would be sufficient to achieve fractionations like the fractionation in Fig. 3a. When the pulse time was progressively increased during zero-integrated pulsing (high field = 5 V/cm; low field = 0.5 V/cm), the fractionation of DNA–capsid complexes did become progressively less sensitive to pulse time as the pulse time increased; the results indicate that the mean time before trapping of a DNA–capsid complex was between 0.2 and 0.5 min (data not shown).

For fractionating DNA–protein complexes in general, either zero-integrated pulsing or zero-integrated pulsing superimposed on a biased field can produce a fractionation that is dramatically improved for all DNA–protein complexes that can be trapped. The following are among the anticipated advantages of these fractionations: (a) DNA–capsid complexes can be made to migrate in a direction opposite to the direction in which the capsid-free DNA migrates. Thus, a background formed by other DNAs is less of a limitation when the DNA–capsid complex is a minor component of a mixture of different DNAs. (b) Because changes in trapping can cause a change in the direction of electrophoretic migration, as well as a change in the average speed of migration,

improved resolution by both size and shape appears to be possible. (c) As discussed above for spheres, the use of a zero-integrated field for the fractionation of DNA–capsid complexes makes possible the elimination of buffer circulation during electrophoresis. This fractionation of DNA–protein complexes will assist studies of both viral DNA packaging, a principal interest of the authors, as well as studies in several other areas, including control of gene expression via interaction of DNA with regulatory proteins [66–70]. In addition, some forms of protein-free DNA undergo field-induced trapping during agarose gel electrophoresis; open circular DNA is an example [71,72]. The open circular 48.5 kilobase pair double-stranded DNA of bacteriophage λ can, like T7 DNA–capsid complexes, be made to move in a direction opposite to that of linear λ DNA during zero-integrated pulsed-field gel electrophoresis (G.A. Griess and P. Serwer, unpublished observation). Thus, the use of a zero-integrated field is also of interest for improving fractionation of the conformers of both DNA and other negatively charged polymers. As the DNA or DNA–protein complex decreases in size, the increased Brownian motion raises the magnitude of the threshold electrical field at which trapping begins [64].

The use of a zero-integrated electrical field to achieve trapping-based fractionations is a specific example of the use of the following more general gel electrophoretic fractionation: zero-integrated pulsed-field gel electrophoresis of a particle that has a μ that is a function of the magnitude of the electrical field. Previously, the use of zero-integrated fields was directed towards improving the fractionation by length of long double-stranded DNA. Pulse times were chosen to capitalize on changes in μ that occur immediately after reversing the field (relaxation effects) [58,73]. However, fractionation also occurs when pulse times are long enough so that relaxation effects do not have a significant effect on the fractionation achieved. For example, reptation of rod-shaped (as well as randomly coiled) particles causes μ to increase in magnitude as the electrical field increases in magnitude [4,21]. Thus, even though comparatively short (0.1–1.0 kilobase pair) double-stranded DNA is not randomly coiled, the use of a zero-integrated field should (a) yield a net μ whose magnitude is greater than zero and (b) produce a fractionation that is significantly different from fractionations obtained using a constant electrical field. These predictions are correct: The use of a zero integrated pulsed-field has been made to achieve a fractionation in which the magnitude of (the net) μ increases as the length of a double-stranded DNA molecule increases, for DNA lengths of between 0.1 and 1.0 kilobase pairs (G.A. Griess and P. Serwer, in preparation). This relationship is (a) the opposite of the relationship that exists after constant field gel electrophoresis and (b) predicted by the observation that the reptation-induced increase in the magnitude of μ increases as the length of a rod increases [4,21]. Sufficient studies have not yet been performed to determine whether resolution can be improved by use of this type of procedure for either double-stranded DNA or, possibly, single-stranded DNA.

7. A perspective

During the future use of variable-magnitude pulsed-fields to improve the gel electrophoretic fractionation of particles with a field-dependent μ, the authors anticipate the need for a database that is more complex than the calibrations currently used to determine characteristics, such as DNA length, as a function of μ. For example, when trapping is the basis of fractionation, the net μ will probably be a function of the structure of the gel, as well as the structure of the particle, the magnitude of the electrical field and the μ_0. The structure of the gel is, as described in Section 2, a complex function of both the composition of the gel and the condition of gelation. Thus, to simplify both the obtaining and the using of a database for predicting characteristics of fractionations, the need for computer-based models is anticipated. In a recent study, computer-modeling of long pulse time–ratcheting-based separation of DNA–protein complexes has been performed; prediction has been made of both the width and the asymmetry of bands [74]. Extension of this type of modeling should greatly assist the finding of conditions for optimal fractionations.

8. Abbreviations

μ	electrophoretic mobility
EEO	electroendosmosis

μ_E EEO-induced mobility
μ_0 μ in the absence of a gel
μ_0' μ extrapolated to a gel concentration of zero
R radius of a sphere
P_E radius of the effective pore of a gel

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