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Monodisperse DNA restriction fragments I. Synthesis and characterization

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Abstract

We present a convenient and low-cost method to prepare milligram amounts of completely monodisperse DNA restriction fragments in a physico-chemical laboratory setting to study (in part II) the effect of limited flexibility on the concentration dependent sedimentation velocity. Four fragments of 200, 400, 800, and 1600 bp were designed to span a range of 1–11 persistence lengths. The fragments were synthesized by cloning fragments of controlled lengths obtained by PCR into bacterial plasmid DNA. The constructs were amplified in large-scale bacterial cultures from which the fragments were obtained by a modified alkaline lysis procedure and subsequent digestion with EcoRV. A method is presented to isolate the DNA from the digestion mixture using horizontal agarose-slab gels and agarose columns in a home-built preparative gel electrophoresis set-up. We show that a combination of optical absorbance readings, ethidium bromide fluorescence, and hyperchromicity measurements allows assessment of both the purity of the DNA solutions and the fraction of double-stranded DNA.

Keywords: DNA restriction fragments; Monodisperse colloidal rods; Preparative gel electrophoresis; DNA quality and quantity

1. Introduction

It is a long-standing challenge to obtain model systems of highly monodisperse colloidal rods for physico-chemical studies; most inorganic and organic synthesis methods yield particles with a certain and often wide size distribution. The problem of a size distribution can be circumvented by using biological compounds like filamentous bacteriophage fd virus, Tobacco Mosaic Virus (TMV), proteins, and DNA, which are essentially monodisperse. DNA occurs naturally in a wide range of sizes, from one to 10^8 base pairs. Depending on the organism, double-stranded (ds) DNA helices may exist in a linear or a circular and/or supercoiled form. DNA is widely used as a model for a wormlike polyelectrolyte due to its well-defined structure, and because much research has been done on the structural aspects of DNA.

Most experimental techniques require large amounts of DNA in comparison to ordinary microbiological research techniques, i.e. milligrams versus micrograms. The main sources for such amounts of DNA have been mononucleosomal DNA from calf thymus [1,2] or chicken erythrocytes [3,4], phage DNA [5,6], and restriction fragments [7–10]. Sizes of the obtained DNA from these sources range from 150 base pairs up to 10^6 base pairs. Except for DNA restriction fragments, the majority of these sources yield DNA fragments with a certain degree of size-polydispersity.

Our aim here is to synthesize sufficiently large amounts, i.e. milligrams, of completely monodisperse restriction fragments specially designed for the analytical ultracentrifuga-

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tion study in the accompanying paper II, where we investigate their sedimentation velocities as a function of aspect ratio and concentration, and measure sedimentation–diffusion equilibrium distributions. We propose a convenient and low-cost synthesis route that can be implemented in a physico-chemical laboratory setting. Linear blunt-ended, double-stranded DNA restriction fragments, consisting of 200, 400, 800, and 1600 bp, were obtained using standard molecular cloning techniques. The contour lengths were chosen to span a range of flexibilities from 1 to 11 times the persistence length (i.e. the length over which the molecule behaves as a rigid rod [11]). The nucleotide sequences, and therefore the lengths as well as the molecular weights of the fragments, could be precisely determined from the template and primers used in the PCR, and from the recognition sequence of EcoRV. We combine here several well-known techniques to assess the quality and purity of DNA fragment solutions by combining optical absorbance readings, ethidium bromide fluorescent agarose-gel electrophoresis, and hyperchromicity measurements.

2. Materials and methods

2.1. DNA preparation

DNA fragments of controlled lengths were synthesized using the polymerase chain reaction (PCR). The plasmid pBluescript II SK^+ (2970 bp) with an insert of 2570 bp that is part of the STAT3 gene served as a template (a gift from Ing. B.-J. Wierenga, Groningen University). The universal forward (sense) KS primer with the sequence $^{5'}$CGAGGTCGACGGTATCG$^{3'}$ was used for each fragment. The 200 bp fragment was generated using the reverse (antisense) primer $^{5'}$GGAGATTATGAAACACCA$^{3'}$, and the 400 bp fragment using $^{5'}$GCCCCCTTGCTGGGCCGCA$^{3'}$. For the 800 and 1600 bp fragments the reverse primers $^{5'}$GAT!ATCCAGTTTTCTAGCCGA$^{3'}$ and $^{5'}$GAT!ATCGGAGCTTCTCAGCCA$^{3'}$, respectively, were used, which contain an EcoRV recognition site (GAT!ATC) in contrast to the 200 and 400 bp fragments. We specifically chose EcoRV because it cleaves both strands of ds-DNA at identical sites symmetrically positioned with respect to the twofold symmetry axis, which results in blunt-ended DNA fragments (double-stranded ends). Thus, inter- or intra-molecular interactions between the ends are avoided. The sequence of interest from the template DNA (100 ng) was amplified by 25 cycles of 94 °C (15 s)–55 °C (30 s)–72 °C (90 s) using a DNA Engine (PTC−200 SN EN-005873 MJ Research, Inc.).

The PCR products, which are slightly smaller than 200, 400, 800, and 1600 bp, were each ligated into a plasmid pCR® 2.1-TOPO® (3.931 kb, Invitrogen, linearized plasmid). The ligation is facilitated by the single 3′-adenine-overhangs that are generated by the *Taq*-polymerase in the PCR, the complementary single 3′-thymidine-overhangs of pCR® 2.1-TOPO®, and by the topoisomerase I enzyme (see for mechanism [12]). The additional EcoRV site in the reverse primers for the 800 and 1600 bp fragments ensures that the correct fragment is obtained upon EcoRV digestion of the fragment-plasmid construct, regardless of the orientation of insertion of the PCR product (pre-fragment) into the plasmid.

To replicate the plasmid with the inserted PCR-product, chemically competent *E. coli* bacterial cells (One Shot® TOP10, Invitrogen) were transformed with the constructs, using ampicillin as a selective agent. Single colonies from agar–agar selection plates were inoculated in vials containing Luria Bertani (LB) medium with ampicillin. The cultures were screened for the presence of the construct by lysing the bacteria and purifying the plasmids using a small-scale alkaline lysis procedure [13], digesting the plasmids with EcoRV, and analyzing the digests with agarose-gel electrophoresis. Large bacterial cultures for plasmid harvesting were created by inoculating the small cultures in 800 mL of LB medium with the selective agent ampicillin.

Plasmids were isolated with a large-scale alkaline lysis procedure, which closely follows the protocol in [14]. The bacteria were harvested by centrifuging for 1.5 h at 3800g, and 4 °C in a Beckman centrifuge J6-HC using a JS-4.2 rotor. Subsequently, the cell pellets were resuspended in lysis buffer containing 5 mg mL^{-1} lysozyme (a gift from Dr. R. Tuinier, Forschungszentrum Jülich, Germany), 50 mM glucose, 25 mM Tris-HCl pH 8, and 50 mM EDTA. The cells were lysed by adding 200 mM NaOH/1% SDS. Cellular debris (primarily genomic DNA and proteins) was precipitated by adding saturated ammonium acetate and removed by centrifugation for 20 min at 7710g, and 4 °C (Sorvall centrifuge RC-5B Plus, SS-34 rotor). The plasmid DNA was precipitated from the supernatant by the addition of one volume of isopropanol and isolated after 30 min incubation at −20 °C by centrifugation for 20 min at 12,000g and at 4 °C (Sorvall centrifuge RC-5B Plus, SS-34 rotor). The DNA pellets were resuspended in sterile TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Undissolved material, primarily salts, was pelleted by centrifugation. Residual proteins were removed by phenol extraction using TE-saturated phenol of pH 8.0. Plasmid DNA was precipitated from the aqueous phase by addition of 5 M NaCl and ice-cold ethanol followed by incubation at −20 °C. The DNA pellet was redissolved in TE buffer. RNA was digested by an overnight incubation at 37 °C with ribonuclease A (RNAase, Sigma type I-AS). Plasmid DNA was precipitated, washed with 80% ethanol, and finally dissolved in 10 mM Tris-HCl buffer pH 8.0.

The plasmids were digested with EcoRV to excise the fragments. Fragments were separated from the linearized plasmids and residual RNA by preparative agarose-gel electrophoresis in two steps. First, the digestion mixtures were separated by normal horizontal ethidium bromide (powerful mutagen, always wear gloves!) agarose-gel electrophoresis. The fragment bands were excised from the slab-gel and

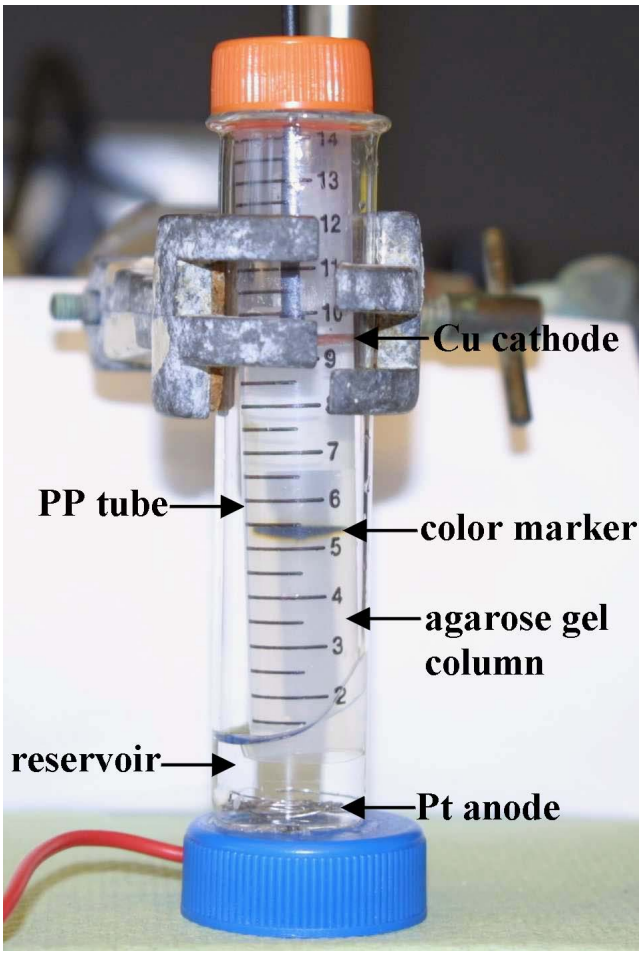

Fig. 1. Home-built preparative gel electrophoresis set-up, used to elute DNA restriction fragments from gel slices of a horizontal agarose slab gel into a reservoir containing TBE-buffer.

eluted from the gel slices by applying an electric field of 5 V cm^{-1} using a home-built electrophoresis set-up (Fig. 1). This electrophoresis set-up can be easily made from a glass tube and a piece of copper and platinum wire. The agarose column is made by pouring microwave melted agarose in a polypropylene tube that serves as a mould. The gel slices, excised from the horizontal slab gel containing the fragments, can be placed on top of the agarose column to elute the DNA fragments. This method is convenient and does not require electrophoresis experience.

After elution was completed, the fragments were precipitated by adding 5 M NaCl and ice-cold absolute ethanol. After overnight incubation at −20 °C, the DNA fragments were collected by centrifugation for 50 min at 15,000 rpm at 4 °C (Sorvall centrifuge RC-5B Plus, SS-34 rotor), and redissolved in a TEN_3-buffer containing 10 mM Tris HCl, 1 mM EDTA, 99 mM NaCl and 7.6 mM NaN_3. The total Na^+ content of the buffer is 107.6 mM. The buffer pH is 7.5 ± 0.1, ensuring that the purine nucleotides are stable (the pH should be above 6). The DNA solutions were stored at −20 °C to prevent degradation.

2.2. DNA characterization

The purity of the DNA solutions was assessed by taking optical density (OD) readings at 260, 270, and 280 nm (Varian Cary 1E UV–visible spectrophotometer). For DNA solutions a 260/280 nm OD ratio of 1.8 indicates the absence of protein [15], while a 260/270 nm OD ratio of 1.2 indicates the absence of phenol [16]. The monodispersity of ds-DNA was further checked by ethidium bromide agarose-gel electrophoresis.

The final yield of DNA restriction fragments was determined from the OD at 260 nm using an extinction coefficient [17] of 20 mL cm^{-1} mg^{-1} and by ethidium bromide fluorescent quantitation of double-stranded DNA using a quantified DNA molecular weight marker (Gene Ruler™ 100 bp DNA ladder, Fermentas).

The quality of the DNA fragments in terms of double-strandedness and possible degradation was characterized in two ways. The first method compares the apparent DNA concentration from the OD at 260 nm (double-stranded DNA, single-stranded DNA, and single nucleotides all contribute to this OD) and from ethidium bromide fluorescent quantitation (which is much more sensitive to double-stranded DNA than to single-stranded DNA). The second method is based on hyperchromicity (thermal denaturation) measurements. Hyperchromicity measurements were done for each fragment using 3 mL solutions (concentration of approximately 25 µg mL^{-1}) and taking OD readings at 260 nm while the solutions were heated from 25 to 95 °C: at a rate of 2 °C min^{-1} from 25 to 75 °C, and 0.2 °C min^{-1} from 75 to 95 °C. The measurements were performed with the Varian Cary 1E UV–visible spectrophotometer in combination with a temperature controller, and thermal and scan application software using 10 mm 111-QS' Hellma quartz cuvettes with screw caps. Because the 200 bp fragment sample showed only a small increase in absorbance upon heating, it was subjected to an ethanol/NH_4Ac precipitation. NH_4Ac was used to avoid co-precipitation of deoxyribonucleosides [17]. Both supernatant and precipitate were used to record thermal denaturation curves. The melting temperature T_m of each fragment was determined by taking the derivatives of the thermal denaturation curves to position the second moment with respect to the temperature from the maximum of this derivative. The T_m can be calculated from the GC content (computed from the known sequence of the fragments), using that the T_m of DNA from many species increases linearly with GC content from 77 °C for 20% GC pairs to 100 °C for 78% GC pairs [18].

3. Results and discussion

3.1. Purity and yield of DNA

Fig. 2 shows an ethidium bromide-stained agarose gel of the four purified DNA fragment solutions as well as a 100 bp

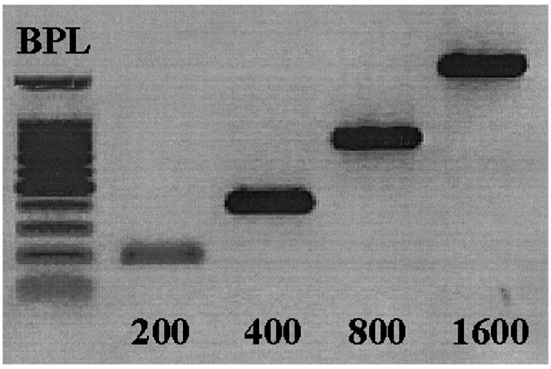

Fig. 2. Scan of an ethidium bromide-stained agarose slab gel, showing from left to right: 100 bp DNA Ladder (Promega), 200, 400, 800, and 1600 bp fragments.

Table 1
Raw yield (after digestion of plasmids and before purification) and pure yield (after gel electrophoresis purification) of DNA restriction fragments

Fragment (bp)	Raw yield[a]		Pure yield	
	# Cultures of 1.6 L LB	mg[b]	# Cultures of 1.6 L LB	µg[b]
200	10	7.07	7	900
400	6	8.01	7	704
800	5	13.14	3	297
1600	3	12.55	7	827

[a] Estimated from the weight ratio of plasmid and fragment in combination with optical absorbance readings at 260 nm, assuming that each plasmid has a single insert and that the samples contain only plasmid DNA.

[b] Calculated from the total volume of solution and the concentration of the solution obtained from the OD at 260 nm using an extinction coefficient of 20 mg mL^{-1} cm^{-1}.

DNA Ladder (further denoted as BPL). The gel confirms the presence of the desired fragments and shows no traces of any undesired DNA or RNA molecules. The ratio of the optical densities (OD) at 260 and 280 nm of the DNA solutions ranged from 1.75 to 1.85, indicating that the DNA solutions are free of RNA or protein contamination [17]. The absence of RNA is confirmed by the ethidium bromide-stained agarose gel (Fig. 2). In addition, there are no proteins present as determined by SDS-PAGE using coomassie blue staining (results not shown). Ethidium bromide contamination is ruled out by the absence of any significant absorption at 302 and 366 nm.

The efficiency of isolation of DNA fragments from the original plasmid digest, shown in Table 1, appears low when the raw and pure yields are compared, but it should be noted that the raw yield is overestimated since it is computed from the weight ratio of fragment to plasmid. In addition, the raw yield contains RNA that contributes to the OD.

Interestingly, concentrated DNA solutions stored at −20 °C contained flocs after thawing on ice. For DNA concentrations below circa 550 µg mL^{-1} all visible flocs disappeared when the solutions were repeatedly incubated at 65 °C for 1 h. At higher concentrations, flocs remained. This reversible aggregation is not uncommon for duplex DNA in aqueous buffer solutions. Wissenburg et al. [2], for instance, demonstrated that the critical DNA concentration above which aggregation occurs increases with added salt. Considering the balance between double-layer repulsions between charged DNA rods [19] and dispersion forces [20], no such aggregation is predicted. The aggregation has been explained instead in terms of the long-range attractions predicted to occur between like-charged polyelectrolytes [21–23].

Table 2
Comparison of DNA concentration determined by spectrophotometry (OD 260 nm) and by ethidium bromide-stained agarose-gel electrophoresis

Fragment (bp)	OD (µg mL^{-1})	Gel (µg mL^{-1})	OD/gel
200	34	5	6.8
400	29	10	2.9
800	25	15	1.7
1600	30	25	1.2

3.2. Characterization of DNA fragment quality

The OD measurements at 260 and 280 nm and the agarose gel in Fig. 2 show that the DNA solutions are free from contaminants. However, from these measurements the conclusion cannot be drawn that the DNA fragments are fully double-stranded, or that there is no degradation of DNA. To assess the quality of the DNA fragments, we first compare quantitation by OD readings and ethidium bromide gel electrophoresis, and second, perform hyperchromicity measurements.

OD measurements are sensitive to all components in solution that absorb radiation of 260 nm, i.e. ds-DNA, single-stranded DNA, and single nucleotides, and may therefore overestimate the actual concentration of double-stranded DNA. In contrast, ethidium bromide agarose-gel electrophoresis allows the determination of double-stranded DNA, because the fluorescence of ethidium bromide is greatly enhanced when the dye is intercalated between the stacked base pairs of ds-DNA. In addition, the separation with respect to size, which is determined from the molecular weight marker (BPL), enables the quantitation of the desired fragments. Quantitation by densitometry of the gels is less accurate, however, than the OD measurements. Table 2 compares the DNA concentrations measured with gel electrophoresis and spectrophotometry. Allowing for either a twofold over- or under-estimation of the actual DNA amount with densitometry, the concentrations determined by these two methods for the 800 and 1600 bp are similar, which was confirmed by the results of the thermal denaturation curves reported in the next paragaraph. For the two shorter DNA fragments of 200 and 400 bp, a larger and probably significant difference between the two concentration determinations is seen. The concentrations obtained with gel elecrophoresis are lower, suggesting a significant fraction of single-stranded DNA and/or single nucleotides (deoxyribonucleoside monophosphates) originating from degradation of the fragments. In the case of the 200 bp fragment,

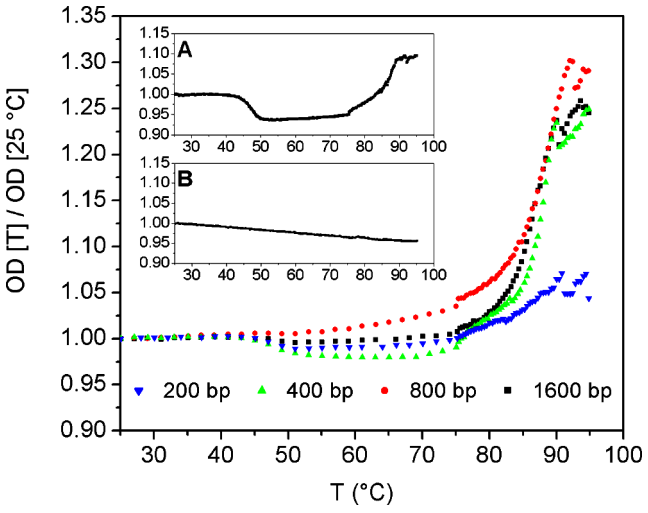

Fig. 3. Thermal denaturation curves for dilute purified solutions of 200, 400, 800, and 1600 bp DNA restriction fragments. The OD at 260 nm relative to that at 25 °C is plotted as a function of temperature, T. The denaturation curve for the redissolved precipitated 200 bp is shown in inset (A) and the curve of the supernatant in (B).

Table 3
Fraction of GC bases and melting temperatures

Fragment (bp)	Actual %GC[a]	%GC from T_m	Calculated T_m (°C)[b]	Experimental T_m (°C)
200	51.5	52	89.5	89.8
400	52.5	49	89.9	88.5
800	53.0	51	90.0	89.4
1600	52.1	47	89.7	87.8

[a] Calculated from the DNA sequence.
[b] Calculated from the %GC.

a certain extent of degradation is not unlikely, given that for complete removal of RNA, repeated separation by fractionated elution from agarose columns was needed.

Hyperchromicity measurements were performed to get independent information concerning the fraction of double-stranded DNA in the purified solutions. Fig. 3 presents melting curves obtained for dilute solutions of the four DNA fragments, showing the temperature dependence of the OD at 260 nm, normalized by the OD at room temperature. The absorbance above 90 °C is noisy, probably due to bubbles that arise from solvent evaporation in the sample that arises upon heating an aqueous solution close to the boiling point. The melting temperatures following from the denaturation curves agree well with the theoretical prediction based on the known GC content of the fragments (see Table 3). For a solution containing double-stranded DNA only, a rise of the normalized OD to 1.4 is expected, assuming that the DNA strands are completely melted. For the 400, 800, and 1600 base pair fragment solutions, a rise to 1.25–1.3 is observed, suggesting that the solutions contain almost exclusively double-stranded DNA. This result confirms the close agreement between the concentrations determined from ethidium bromide-stained agarose gels and spectrophotometry, as discussed in the previous paragraph (Table 2). The relative absorbance for the 200 bp DNA solution upon heating rises to a much lower value of 1.07. Assuming that any double-stranded DNA is melted completely, the hyperchromicity measurements suggest that the solution contains ca. 20% ds-DNA fragments and 80% material that absorbs at 260 nm but is not double-stranded DNA. The contamination may consist of single-stranded DNA and/or low-molecular weight species absorbing light of a wavelength of 260 nm. The most likely low-molecular weight contaminant is single nucleotides originating from partial degradation of the restriction fragments. The discrepancy between the concentration determinations of the 200 bp solutions with gel electrophoresis and spectrophotometry (Table 2) likewise suggests contamination by single-stranded DNA and/or nucleotides.

In an effort to characterize the contaminant, the 200 bp fragment was precipitated and wavelength spectra and denaturation curves were recorded from the supernatant as well as from the dissolved precipitate. For the redissolved precipitate we find an increase of the relative absorbance upon melting to 1.12, inset A in Fig. 3, which is higher than before precipitation but still much lower than 1.4 as expected for fully double-stranded DNA (perhaps due to co-precipitation of the contaminant). The supernatant contained no double-stranded DNA, since the relative absorbance did not change with increasing temperature, inset B in Fig. 3.

4. Summary and conclusions

We prepared sub-milligram amounts of monodisperse, blunt-end, double-stranded DNA restriction fragments of 200, 400, 800, and 1600 bp using a strategy that offers full control of the sequence and molecular weight of the DNA fragments. Our strategy is a low-cost method to obtain completely monodisperse wormlike cylinders that can be carried out in a physico-chemical laboratory setting. We show that a combination of spectrophotometry, ethidium bromide-stained agarose-gel electrophoresis, and hyperchromicity measurements enables a characterization of both the purity of the DNA preparation and the quality of the DNA restriction fragments. We obtained 400, 800, and 1600 bp DNA fragment solutions containing almost exclusively double-stranded, non-degraded DNA. In contrast, the 200 bp fragment solutions contain a large (ca. 80%) fraction of single-stranded DNA and/or low-molecular weight components. Additional evidence for low-molecular weight contaminants is given in Part II of this work, where sedimentation equilibrium measurements are presented.

Interestingly, we observed reversible aggregation of DNA in concentrated solutions. Flocs in solutions that were stored at −20 °C disappeared upon heating the solutions. The visual observation of DNA aggregation in highly concentrated solutions, which were turbid and much more viscous compared to diluted DNA solutions, is further addressed in the sedimentation study in Part II, where the effect of aspect ratio on the hydrodynamic friction is also investigated.

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