The structural peculiarities of condensed DNA micro- and nanoparticles formed in PCR

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Studies of DNA condensation have opened new perspectives in biotechnology and medicine. DNA condensation induced by polyamines or trivalent metal ions in vitro at room temperature has been investigated in detail. Our recent studies have demonstrated Mg2+-mediated formation of DNA condensates during the PCR. In this study, we report the unique morphology and fine structure of PCR-generated condensed DNA particles using electron and atomic force microscopy. The principal morphologies of studied DNA condensates are 3D particles of micrometer dimensions, oval microdisks of nanometer thickness, filaments, and compact nano-sized particles. SEM examinations have revealed a new structural type of spherical and elliptical 3D microparticles formed by numerous definitely oriented microdisks and their segments. AFM revealed a granular structure of the microdisk surface and the smallest nano-sized disks and thinnest nanofibrils – that appear to be the primary products of DNA condensation during the PCR. We suggest that the formation of DNA nanofibrils and nanodisks in PCR occurs due to Mg2+–mediated intermolecular (lateral) and intramolecular condensation of ssDNA. Aggregation of elementary nanodisks in the course of thermal PCR cycles, occurring both by magnesium cations and via complementary interactions, give a rise to large nano-sized aggregates and more complex microparticles.

Keywords: inter- and intramolecular DNA condensation; micro- and nanoparticles; polymerase chain reaction; electron microscopy; atomic force microscopy

Introduction

A remarkable feature of DNA is its ability to adopt a compact (condensed) form in vivo and in vitro. In the condensed state, density of DNA is higher by several orders of magnitude than that of DNA in aqueous solutions. The phenomenon of DNA condensation in vitro has been investigated for many years as a model of DNA packing in living systems. Condensation of double-stranded (ds) DNA in vitro at room temperature may be achieved by adding different ligands, such as polyamines (spermine and spermidine) (Chattoraj, Gosule, & Schellman, 1978; Gosule & Schellman, 1976; Vijayanathan, Lyall, Thomas, Shirahata, & Thomas, 2005), cations of trivalent metals (for example, cobalt hexammine) (Arscott, Ma, Wenner, & Bloomfield, 1995; Conwell & Hud, 2004; Plum, Arscott, & Bloomfield, 1990; Widom & Baldwin, 1980; Wilson & Bloomfield, 1979), polylysine (Laemmli, 1975), cationic oligopeptides (Niidome et al., 1997), cationic lipids (Felgner et al., 1987), and other ligands. Condensation of DNA in vitro may be achieved also in the presence of crowding agents such as poly(ethylene oxide) (Laemmli, 1975). The principal morphologies of nano-sized DNA condensates, formed in the result of interactions with ligands, are toroids, rods, and spheroids (Arscott et al., 1995; Chattoraj et al., 1978; Conwell & Hud, 2004; Plum et al., 1990; Vijayanathan et al., 2005; Widom & Baldwin, 1980). The mechanism of ligand-mediated DNA condensation has been unraveled in numerous studies (Bloomfield, 1991; Cherstvy, Kornyshev, & Leikin, 2002; Kornyshev & Leikin, 1998; Teif, & Bohinc, 2011; Todd, Parsegian, Shirahata, Thomas, & Rau, 2008) and condensed DNA particles are promising tools in biotechnology and medicine (Chen et al., 2006; Hart, 2010; Itaka & Kataoka, 2009; Rao, 2010).

Condensed DNA in the form of micro- and nanoparticles (MPs and NPs) may also be produced during polymerase chain reaction (PCR) (Danilevich, Barinova, & Grishin, 2009; Danilevich, Kadykov, & Grishin, 2010; Danilevich, Vasilenko, Pechnikova, & Grishin, 2012; Danilevich, Vasilenko, Pechnikova, Sokolova, & Grishin, 2012). For example, we have demonstrated the formation of DNA MPs during PCR amplification of Saccharomyces cerevisiae and Pichia pastoris ribosomal DNA fragments.

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using KlenTag polymerase (Danilevich et al., 2009) and fluorescently labeled primers – the derivatives of 5.8SR and LR3 (Vilgalys & Hester, 1990). Single DNA MPs and their aggregates are observed as highly fluorescent particles in PCR products. DNA MPs are detected at the late PCR stages when amplicon DNA accumulates in considerable amounts. Both the size and the morphological features of MPs depend on the amplicon DNA structure, on the template (microbial genomic DNA or plasmid DNA) and type of thermostable DNA polymerase (Tag polymerase) used in PCR (Danilevich et al., 2010; Danilevich, Vasilenko, Pechnikova, & Grishin, 2012). MPs are rather stable in water; however, they dissociate easily to soluble dsDNA of amplicon size in the presence of chelating agents (EDTA, citrate) (Danilevich et al., 2012). A high yield of DNA (Danilevich et al., 2010; Danilevich, Vasilenko, Pechnikova, Sokolova, et al., 2012). The standard PCR regime was the following: denaturing at 94 °C for 30 s, annealing at 63 °C for 1.5 min, and the number of thermal cycles was 35 or 40 (unless otherwise noted). Negative controls were PCR mixtures containing all the components except DNA templates. PCR products were examined by electrophoresis on a 0.8% agarose gel in 1 × TAE buffer (pH 8.3) with ethidium bromide for DNA staining.

As demonstrated in previous TEM studies, PCR-generated MPs are mainly electron-dense spheroids, ellipsoids of different morphological types (smooth, with rugged edges and spikes, star-shaped), and oval disks of tens of nanometers in thickness. Along with MPs, rare filaments with nanometer thickness and compact spherical NPs have been observed, but their total content, as a rule, is less than 1% of bulk condensed DNA (Danilevich et al., 2010; Danilevich, Vasilenko, Pechnikova, & Grishin, 2012). A high yield of filamentous and compact NPs has been observed only in routine PCR amplification of the IST2 sequence (Danilevich, Vasilenko, Pechnikova, Sokolova, et al., 2012). To date, however, both the 3D morphology and details of structural organization of DNA MPs and NPs have remained obscure.

In the present study, we describe the morphology and fine structure of PCR-generated DNA MPs and NPs using SEM, TEM, and atomic force microscopy (AFM). The objects of our study are condensed DNA particles of different type and size, produced upon PCR amplification of insertion elements IST2 and ISAfe1 (Holmes et al., 2001; Kondrat’eva, Danilevich, Ageeva, & Karavaiko, 2005; Yates, Cunningham, & Holmes, 1988). In order to produce condensed DNA structures in quantities sufficient for detailed AFM examinations, we have amplified the IST2 sequence in PCR under the conditions of low background noise (Danilevich, Vasilenko, Pechnikova, Sokolova et al., 2012). The results of the SEM study presented here demonstrate the peculiar 3D structure of DNA MPs and extend our knowledge of the mechanism of their formation. Using SEM, TEM, and AFM, we observed the granular structure of microdisk surface. We suggest that the smallest NPs – nano-sized singular disks of 6–20 nm in size and thinnest nanofibrils of ~10 nm in width, appear to be the primary products of ssDNA condensation in PCR.

Materials and methods

PCR procedure
As templates in PCR we used the plasmids pBS::IST2 and pBS::ISAfe1, the derivatives of pBlueScriptIIsk+ (Promega, USA), with the insertion elements isoIST2 (1400 bp) and isoISAfe1 (1250 bp) from the bacterium Acidithiobacillus ferroxidans (Kondrat’eva et al., 2005). Plasmid DNA was isolated with a Wizard reagent kit (Promega, USA) according to the manufacturer’s protocol. Target sequences were amplified from plasmid DNAs by using primers E2.1f (5′-GAG CTA TAG TCA AAT CTG GTG TAT G-3′) and E2.2r (5′-GCT TCA TTT CAA GTG GGT AG-3′) for IST2 and E1.1f (5′-TCG TCG GAT TGA GTG GGT AG-3′) and E1.2r (5′-TCG TCA TTT CAA GTG GGT AG-3′) for ISAfe1 (Kondrat’eva et al., 2005). PCR experiments were performed with a MJ Research PTC-200 thermocycler (USA) by using 50-μl reaction mixtures containing 2.5 U of Taq-polymerase, each primer (10 pmol), each dNTP (0.2 mM). The template used was 1 to 5 ng of plasmid DNA. PCR buffer contained 67 mM Tris–HCl (pH 8.8), 16.6 mM (NH4)2SO4, 0.01% Tween 20, and 1.5 mM MgCl2.

The standard PCR regime was the following: denaturing at 94 °C for 30 s, annealing at 63 °C for 30 s, synthesis at 72 °C for 1.5 min, and the number of thermal cycles was 35 or 40 (unless otherwise noted). Negative controls were PCR mixtures containing all the components except DNA templates. PCR products were examined by electrophoresis on a 0.8% agarose gel in 1 × TAE buffer (pH 8.3) with ethidium bromide for DNA staining.

Isolation and purification of condensed DNA particles
After thermal cycling, the reaction mixtures (50 μl) were diluted with MilliQ water to 400 μl volumes and then centrifuged at 8,000 g (12,000 rpm) for 2 min (Eppendorf MiniSpeen centrifuge, Germany). Supernatants with water-soluble DNA were carefully transferred to clean tubes for subsequent electrophoretic analysis. Pellets of condensed DNA were washed with 400 μl of water and then suspended in 40 μl of water or 1 mM MgCl2 solution. Suspensions of DNA particles were analyzed immediately or after storage at 4 °C for a few days. The content of DNA was measured by fluorometry (the Quibit® 2.0 Fluorometer with fluorescent DNA-binding dye (Invitrogen/Life technology)).
**Treatment of DNA condensates with nuclease S1**

In experiments, we used nuclease S1 (100 U/μl) and the appropriate reaction buffer (Fermentas, Lithuania). Freshly prepared suspensions of DNA particles (20 μl) were treated with nuclease S1 (2U per 40 μl of the reaction mixture). The controls were samples of DNA microparticles in the nuclease S1 buffer without the supplemented enzyme. The samples were incubated under static conditions (without stirring) with periodic vortexing every 10 min. After incubation at 37 °C for 1–2 h, the reaction was stopped by addition of 10 volumes of water and mixtures were centrifuged as above. Supernatants were discarded, and pellets were washed with 100 μl of water and then resuspended in 20 μl of 1 mM MgCl₂. Suspensions of nuclease-treated DNA particles and control samples were analyzed by light and electron microscopy. Counterpart aliquots (10 μl) of nuclease-treated mixtures (prior to centrifugation) were analyzed by electrophoresis in 0.8% agarose gel.

**Bright-field and fluorescence microscopy**

Suspensions of DNA condensates were incubated with propidium iodide (60 μM), acridine orange (3 μM), or DAPI (6 μM) at 37 °C in the dark for 15 min, placed with 5 μl aliquots onto slides, and air-dried. The slides were examined under an Olympus CK40 epifluorescence microscope (Germany) equipped with 41,004 Chroma Texas Red filters (excitation band, 532–588 nm; emission band, 645–675 nm), and an Olympus DP50 digital camera. The number of particles per 1 μl was calculated by counting in 10 fields.

**TEM and SEM of DNA MPs and NPs**

The suspensions (5 μl) were dropped on ploioform-coated copper grids (Pelco, Sweden) and brought to dryness at room temperature. Negative contrasting with aqueous uranyl acetate (1% w/v) (Kleinschmidt, 1968) was used in some experiments only. TEM images were produced using a Tecnai G² spirit twin microscope (FEI Company, Netherlands) with a Block Mega View III digital camera. SEM was performed using a JSM-7401F microscope (Jeol, Japan) equipped with a special grid holder. We used the mode of secondary electron imaging at accelerating voltage 1 kV to obtain more detailed information about the surface topology of DNA MPs. Their average size was calculated by measuring the dimensions of 100 particles.

The prepared specimens were also analyzed by SEM-EDS (FEI Quanta 200 3D scanning microscope with EDAX Genesis system).

**AFM imaging**

The suspensions (5 μl) were deposited onto a freshly cleaved mica surface and air-dried. Samples were examined using the tapping mode of AFM (Hansma, Cleveland, et al., 1994; Zhong, Inniss, Kjoller, & Elings, 1993) in air under a P47-SPM-MDT scanning probe microscope (NT MDT, Russia). We used silicon TL02 and H'RES NSC15 cantilevers with different nominal tip curvature radius available from Mikromasch (Estonia). The preparation and examination of specimens were carried out in a Trackpore Room’02 under controlled humidity and temperature. All images were treated using the Images Analyses program (NT MND, Russia).

**Results**

**DNA microparticles produced by IST2 and ISAfe1 amplicons**

As already demonstrated in our previous studies, spherical or elliptical microparticles were major products among condensed DNA generated from the IST2 or ISAfe1 sequence; flat microdisks occurred very rarely (Danilevich, Vasilenko, Pechnikova, & Grishin, 2012; Danilevich, Vasilenko, Pechnikova, Sokolova, et al., 2012). In this work, we used the protocol of low background PCR amplification of the IST2 or ISAfe1 sequences with Taq-polymerase to generate condensed DNA microdisks and filaments in sufficient amounts (Danilevich, Vasilenko, Pechnikova, Sokolova, et al., 2012). To produce them with a high yield, it was optimal to use freshly prepared solutions of the primers and dNTPs and to perform 35 or 40 cycles of PCR amplification under the standard conditions. DNA condensates in the form of pellets (after centrifugation) were detected at late PCR stages. After 25 cycles of PCR, water-soluble DNA amplicons were produced at sufficient amounts but precipitates were absent.

In all the PCR mixtures, we revealed the bands corresponding to dsDNA amplicons of IST2 (1400 bp) and ISAfe1 (1250 bp) and insignificant amounts of nonspecific products visible as background smears on electrophoregrams (Figure S1). The bands corresponding to target DNA amplicons were absent in the negative control samples. After washing of the precipitated PCR products with de-ionized water, almost all soluble DNA, primers, and salts were removed, and no salt crystals were present in specimens analyzed by TEM.

To prove the presence of DNA amplicons in the suspensions of condensates, we used the previously reported procedure (Danilevich et al., 2009). Briefly, 10-μl aliquots of microparticle suspensions (total volume, 40 μl) were mixed with loading buffer (1.5 μl) containing dyes and 5 mM EDTA (Fermentas, Lithuania) and placed into
Obviously, the studied condensates contained amplicons (with size of 1400 bp) were detected in all the wells designated for the supernatants (50 μl) produced upon centrifugation of PCR mixtures produced by amplification of the IST2 sequence with Taq polymerase. Lanes 1, 3, and 5: DNA in supernatants; lanes 2, 4, and 6: DNA in precipitates; lane 7: negative control (without template DNA); lane 8: length marker for DNA fragments (1 kb DNA ladder). The number of thermal cycles: 28 (1 and 2); 34 (3 and 4) and 40 (5 and 6). Aliquots of the supernatants (2 μl, 1/25 of the sample) and the suspensions of DNA condensates (10 μl, 1/4) were applied into wells.

wells of 0.8% agarose gel. In parallel, 2 μl-aliquots of the supernatants (50 μl) produced upon centrifugation of PCR mixtures were added to the corresponding wells. After electrophoretic separation, the bands of ds DNA (with size of 1400 bp) were detected in all the wells designated for the supernatants and condensates (Figure 1). Obviously, the studied condensates contained amplified DNA and dissociated in the presence of EDTA. The estimated DNA amount in the pellets (condensates) produced in PCR with Taq polymerase achieved 50–250 ng per 50 μl of reaction mixture, that is, about 1–5% of the total amplified DNA (~5 μg) as assayed using UV spectrophotometry.

In addition, abundant DNA condensates in the form of microparticles (MPs) possessed intense fluorescence after staining with propidium iodide, acridine orange, or DAPI as revealed by epifluorescence microscopy examinations (images not shown).

In special experiments, we used SEM-EDS to analyze the morphology and composition of DNA microparticles. Carbon, nitrogen, oxygen, and phosphorus (elements of DNA) and magnesium as condensing agent were detected in particles (Figure S2). Finally, the presence of DNA was supported additionally by sensitivity of the studied condensates to nuclease as reported below.

The main morphological types of MPs (as revealed by TEM) were semi-transparent oval microdisks with the smooth surface and electron-dense microparticles (Figure 2), similar to those described in (Danilevich, Vasilenko, Pechnikova, Sokolova, et al., 2012). MPs of the two types were present in all the suspensions of condensed DNA generated in PCR under the selected optimal conditions (35 or 40 thermal cycles, fresh stock PCR solutions).

Using TEM we observed some variations in the number and dimensions of MPs. Thus, oval microdisks with a mean long axis of 1.5 μm and a short axis of 1 μm represented ~80% of the total microparticles (2 × 10^5 μl^{-1} or 10^7 per the total 50 μl reaction mixture) produced upon IST2 amplification in the initial experiments (Figure 2(A) and (B)). Then, the experiments were repeated using the stored stock solutions of primers and dNTP to produce DNA condensates. However, the yield of DNA MPs decreased to 2 × 10^5 μl^{-1}, and oval microdisks had the greater lateral dimensions (4 μm × 3 μm) and constituted ~50% of the total MPs (Figure 2(C) and (D)).

Along with MPs, singular and assembled filamentous structures and compact NPs (>30 nm in size) were found in DNA condensates with the use of TEM (Figure 2). Similar results were obtained in the case of the ISAfe1 amplicon, although the yield of DNA filaments and microdisks was less.

**Unique structure of DNA MPs as viewed by SEM**

SEM examinations proved the presence of oval microdisks of several micrometers in dimensions and ten nanometers thick in the suspensions of DNA particles formed upon PCR amplification of IST2 and ISAfe1 sequences (Figures 3 and 4). Besides these disks, we found spherical and elliptical 3D MPs with an unusual structure formed by definitely oriented micrometer disks and their segments are 30–40 nm thick. As established from the analysis of many SEM images, 3D MPs were similar in their shape but not identical in the structural details. In other words, numerous geometrical structures of 3D MPs of the same type were formed in PCR. Indeed, MPs possessed a simple or sophisticated organization, from singular disks with sail-like leaflets to structures containing numerous smooth intersecting disks (Figures 3 and 4). Evidently, microdisks can be considered as basic structural elements of 3D MPs. Similar 3D structures were found upon SEM examinations of DNA condensate samples produced during PCR amplification of IST2 and ISAfe1 sequences with KlenTaq polymerase (data not shown).

To a certain extent, the structural characteristics and the size of DNA MPs depended on the amplicon structure. Thus, all the disks in 3D structures formed by IST2 amplicon were similar in thickness (25–40 nm) and dimensions (Figure 4(A–C)). In the case...
Figure 2. TEM images of DNA MPs formed during low background amplification of the IST2 element in PCR with Taq polymerase. (A, B) Clusters of small electron-semitransparent microdisks, electron-dense MPs with lateral size of 1.5 μm × 1 μm, and filamentous structures near microdisks formed after 35 thermal cycles of PCR. (C) Clusters of large microdisks (4 μm × 3 μm) and filaments after 40 cycles; (D) Numerous compact NPs visualized as electron-dense dots near large microdisks.

Figure 3. SEM images of DNA MPs formed by IST2 amplicon. (A) A cluster of small microdisks and 3D MPs (lateral size, 1.5 μm × 1 μm), 35 PCR cycles. Note the unique structure of 3D condensed DNA particles consisting of arranged disks and their segments. (B) The honeycomb structure of 3D MPs as viewed at higher magnification, cell thickness is about 25 nm. (C, D) Large microdisks (4 μm × 3 μm) and structurally diverse 3D MPs found in a repeated experiment after 40 thermal cycles.
of ISAfe1 amplicon, 3D MPs had, as a rule, the similar size and structure, but in some experiments they were heterogeneous in the mean diameters (20 and 3 μm), and some disks in large microparticles were thicker (>100 nm) than others. Among these condensates, we found singular microdisks (with the mean diameter 3 μm) constituting a minor part (1–5%) of total MPs (Figure 4(D)–(F)). Also, filamentous structures and compact NPs (d > 30 nm) were present in DNA condensate suspensions (Figure 4(B) and (C)). Their fine structure is described below.

**Fine structure of microdisks and NPs resolved by AFM and TEM**

**Microdisks.** High-resolution AFM demonstrated the specific morphology and topography of PCR-generated microdisks. Condensed DNA microdisks with the long and short axes of 4 μm × 3 μm differed in their maximum thickness (25–117 nm) and were not absolutely flat (Figure 5(A)), at least some of them possessed a lens-shaped profile. Using high-resolution scanning allowed us to visualize a finely granular texture of the micro-
disk’s surface. It consisted from linear or branched cords from dozens of adhered and flattened spherical NPs (Figure 5(C) and (D)). The diameter of NPs, constituting the cords, varied from 10 to 15 nm, and their thickness were of 6–7 nm. Hence, results of our AFM study suggest that microdisks are large aggregates of NPs.

**Filaments.** Besides microdisks, we observed by ASM long filamentous structures in the form of tapes consisting of thinnest nanofibrils (Figure 6). They were not absolutely flat and had bulges of ~5 nm in height, which were located at a different distance from each other (Figure 6). It is likely that these bulges are formed by sticking of compact NPs to nanofibrils. As visible at higher resolution, the majority of nanofibrils had the thickness of 2.2 nm and the width of 14–15 nm, and some of them were 1.1 nm thick (Figure 7). Since real height and width of NPs may not be evaluated exactly upon AFM examination, we also determined these parameters using TEM with uranyl acetate contrasting of specimens. As shown in Figure 8, nanofibrils had the shape of smooth threads with the width of ~11 nm but the details of their fine structure were still unresolved by high-resolution TEM.

**Nanodisks.** Thorough AFM examinations of areas around single microdisks showed a grainy textured background formed by numerous NPs (up to several hundreds per 1 μm²) with different size. Large NPs were agglomerates from smaller NPs (Figure 9). A population of the smallest singular NPs (referred to as nanodisks) was heterogeneous in their geometry: 14% represented 6 nm long and 3 nm wide disks, 44% had the mean length of 12 nm and width of 8 nm, and the others were more than 12 nm long. The thickness of one-third of total nanodiscs was slightly more than 1 nm; the other nanodisks were twice higher.

The presence of compact nanodisks (10–15 nm in diameter) among DNA condensates was proved using TEM with uranyl acetate contrasting. Singular nanodisks had similar diameter as NPs constituting microdisks and 3D MPs, but differed in the thickness (Figures 5 and 9). This difference can be explained partially by the errors in measurements of the

Figure 5. AFM images of DNA condensates. (A) A singular microdisk (4 μm × 3 μm). (B) Its sectional profile along the long axis; the thickness is about 25 nm. (C, D) Fragments of the microdisk surface at higher resolution. Note that the surface layer consists of NPs arranged in linear and branched cords. Images were taken using a P47-SPM-MDT scanning probe microscope (tapping mode) and H'Res NSC15 cantilevers.
thickness by AFM. Nonetheless, we assume that nanodisks are structural units of microdisks and 3D MPs.

Sensitivity of condensed DNA particles to nuclease S1

Freshly prepared suspensions of DNA condensates, which were obtained in PCR amplification of IST2 sequence, were then treated with S1 nuclease at the concentration of 0.05 U/μl. As proved in tests with native and denatured pBS:IST2 plasmid DNA, this concentration was optimal for selective degradation of ssDNA, but not dsDNA. Before the treatment, microdisks with the lateral size of 1.5 μm × 1 μm were predominant over 3D MPs and constituted ~80% of the total DNA microparticles (Figure 2(A) and (B)). In control samples (without nuclease S1), the concentration and morphology of MPs remained unchanged during incubation at 37 °C for 1.5 h. In contrast, the treatment with S1 nuclease caused a 90% reduction in the total number of MPs as judged from the data of microscopy counting. SEM examinations of nuclease-treated specimens confirmed the drastically decreased occurrence of both MPs and NPs and revealed the signs of prominent destruction of remaining disks and filaments. Figure 10 illustrates the rough and thinned surface with holes in some places in MPs, undergoing destruction under the effect of nuclease, in contrast to untreated particles (Figure 3(A)). After the treatment with nuclease S1, we observed the defragmentation of filaments to short threads (<1 μm) as compared to their length of several microns in the control (Figure 2) and the decrease in the number of compact NPs. The presence of incompletely destructed microdisks and filaments can be explained by inaccessibility of some DNA particles to nuclease due to their precipitation under static conditions of incubation.

Complexity of DNA MPs and NPs

Notwithstanding to differences in the structural details and size for each type of DNA MPs and NPs, we tried to estimate the number of DNA molecules for some selected structures (Table 1). Based on the determined yield of total DNA in PCR (5 μg per 50 μl) and the average MW...
of nucleotide (340), the number of nucleotides in synthesized DNA was $9 \times 10^{15}$. Having the IST2 DNA length of 1400 bp, the total number of ssDNA molecules in 50 μl of PCR mixture was estimated to be $6.5 \times 10^{12}$. Since the ratio of condensed DNA accounted for 5% of total amplified DNA; therefore, all DNA condensates contained $1.3 \times 10^{11}$ ssDNA molecules. The number of all microparticles amounted to $1 \times 10^7$ (in 50-μl PCR mixture): $8 \times 10^6$ microdisks and $2 \times 10^6$ 3D MPs. With an assumption that one 3D MPs consisted of five microdisks, the number of ssDNA molecules might be of $2 \times 10^4$ or $1 \times 10^5$ in a single microdisk or a 3D MP, respectively. Another approach to estimate the number of ssDNA in DNA microparticles relied on our supposition that one elementary nanodisk (Figure 9) contained one or two ssDNA molecules. Based on the volume ratios for microparticles and elementary discs with corrections for packing density ($F = 3$, as suggested from Figure 5(D)) and threefold underestimated thickness of elementary nanodisks by AFM, we could derive similar orders of the number of DNA molecules in them.

Indeed, it is impossible so far to determine the exact number of DNA molecules in MPs and the above rough estimates illustrate the complexity of each type of the particles.

Discussion

Taken together, the present and our previous studies have demonstrated that PCR-generated MPs and NPs consist of DNA. Previous studies have shown that these condensed products of the PCR are sensitive to micrococcal nuclease, stainable with DNA-binding dyes, detectable in PCR with fluorescent-labeled primers (Danilevich et al., 2009), and their UV-spectra are consistent to nucleic acids (Danilevich et al., 2010). Here, we also report sensitivity of the MPs (microdisks) and NPs (filaments) to nuclease S1 (Figure 10).

We have already shown that Mg$^{2+}$ ions are crucial for the formation and stability of condensed DNA particles produced in PCR. Indeed, binding of Mg$^{2+}$ with chelating agents causes destruction (dissociation) of
DNA condensates (Danilevich et al., 2009). As it is known well, Mg\(^{2+}\) and other divalent metal ions (Connell & Hud, 2004; Ma & Bloomfield, 1994), except for Mn\(^{2+}\) [40], do not cause dsDNA condensation in aqueous solutions at room temperature. Furthermore, Mg\(^{2+}\) and alkaline earth metal ions (Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\)) are not aggregating agents for dsDNA even at heating up to 95 °C (Duguid & Bloomfield, 1995; Hud, 2009). On contrary, transition metal ions Mn\(^{2+}\), Cd\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), Pd\(^{2+}\), Cu\(^{2+}\) aggregate dsDNA at heating (Duguid & Bloomfield, 1995; Hud, 2009). Interestingly, natural DNA condensates may have the structure of stacked plates, as already demonstrated for isolated chromatin (Castro-Hartmann, Milla, & Daban, 2010). The other fascinating example are recently described sponge-like microparticles from siRNA (Lee, Hong, Bonner, Poon, & Hammond, 2012) which resemble, to a certain degree, 3D DNA microparticles obtained in our work.

Fine structure of both MPs and NPs from PCR-generated DNA condensates has been resolved by AFM. Previous AFM studies have been conducted with specimens of water-soluble ssDNA and dsDNA mounted on cleaved mica surface and scanned under the specially chosen conditions (Hansma, Sinsheimer, Li, & Hansma, 1992; Lyubchenko et al., 1992; Lyubchenko, Shlyakhtenko, Harrington, Oden, & Lindsey, 1993). Also, AFM has found application in studies of nano- and microparticles, containing nucleic acids (Chen et al., 2006; Vijayanathan, Thomas, Antony, Shirahata, & Thomas, 2004). Here, we have used the tapping mode of AFM scanning (Hansma, Cleveland, et al., 1994; Zhong et al., 1993) to achieve high resolution of examined structures. In our study, PCR-generated MPs and NPs were suitable for direct AFM examinations without silanization of the mica surface or its modification by divalent metal cations, as well as treatment of DNA with spreading agents (Hansma, Browne, Bezanilla, & Bruce, 1994; Lyubchenko et al., 1993). We have found that strong attachment of MPs and NPs, likely, via Mg\(^{2+}\) to mica surfaces prevents lateral shifting of even the smallest structures during AFM scanning.

Precision AFM scanning showed that the surface layer of the microdisks (which are the basic element of 3D MPs), consists of linear or branched units—bundles (Figure 5). These bundles contained dozens of agglomerated NPs in the form of flattened spheres. The diameter of these NPs ranged from 10 to 15 nm, well below the reported lumens observed in coiled phage DNA (Lambert, Letellier, Gelbart, & Rigaud, 2000). Using AFM,
we also detected a vast number of single NPs (nanodisks) located near microdisks with the same lateral dimensions that the nanodisks contained in the microdisk surface. We strongly suspect that these single NPs are the building blocks from which the microparticles are built. We have also recently observed by TEM clusters of NPs with sizes ranging from 5 to 20 nm in samples IST2 condensates stained with uranyl acetate (unpublished). With regard to their size, nanodisks can be considered as elementary NPs produced via *intramolecular* Mg$^{2+}$-mediated condensation of ssDNA molecules. Indeed, the structures with such a compact DNA packing are unlikely to be formed by the continuous ds DNA given the persistence length of nearly 44 nm for double stranded DNA in the presence of magnesium (Lu, Weers, & Stellwagen, 2002). The experiments with nuclease S1 offer additional evidence that the DNA exhibits single-stranded character. Both MPs and NPs are almost completely destroyed by S1 nuclease (Figure 10) that cleaves ssDNA only. This demonstrates a role for ssDNA as the main structural element of MPs and NPs.

The granular structure of microdisks has independently been supported upon high-resolution TEM and SEM examinations of ultra-thin DNA microdisks produced in a course of asymmetric PCR amplification of DNA fragment for *gfp* gene (Danilevich et al., 2010). We observed that ultra-thin microdisks consist of spherical NPs with the average diameter 15 nm (unpublished data).

The granular structure of the condensed DNA particles was demonstrated by us for the first time. This finding is crucial for understanding of the molecular mechanisms of DNA condensation during PCR. It has been well known, that in the toroidal NPs, which are formed in the presence of cobalt hexamine as well as other ligands at room temperature, individual double-stranded DNA molecules are arranged in parallel (Widom & Baldwin, 1980; Wilson & Bloomfield, 1979; Vijayanathan et al., 2005). Toroidal structures also have DNA comprising phage particles. The smallest inner diameter of the torus has been observed in the case of the phage DNA, it is 10–15 nm (Lambert et al., 2000). Apparently, phage DNA in a B-form duplex cannot fold tighter than...
that because of its persistence length. It is important to underline that the 3D structure of MPs is not dependent from the primary structure of amplicons used in PCR. To date, we have investigated by SEM the 3D structure of the microparticles formed by four different amplicons that have no homology: IST2, ISAf1 (this work), DNA fragment carrying β–lactamase gene (800 bp) and DNA fragment for gfp gene (1600 bp) (unpublished data). They all have the same type of 3D structure and consist of intersecting disks and their segments. This means that ssDNA molecules of different amplicons are condensed (compacted) in a similar manner during thermal cycles of PCR and the formed elementary NPs interact with each other and aggregate in a similar fashion. Obviously, the specific morphology of microparticles is governed by the topology of nanodisks, their anisotropy, and their ability to assemble cord-like structures. We suggest that aggregation of elementary nanodisks in the course of thermal cycles occurs both by magnesium cations and via complementary interactions. Thus, DNA condensation in PCR is a multistage process in which nanodisks give a rise to large nano-sized aggregates and more complex MPs. Our recent studies suggest a role of pyrophosphate, which is produced from nucleoside triphosphates during DNA synthesis by thermostable DNA polymerase, in the formation of DNA condensates in the course of PCR. These data will be reported elsewhere.

Noteworthy, DNA filaments represent another interesting form of DNA condensates. According to AFM data, filaments have a specific structure and resemble flat tapes of thinnest nanofibrils. The nanofibrils are more rigid and conformationally stable (Figures 6–8) than ssDNA and dsDNA and they do not form elements of the tertiary structure, for example, lumps on mica surface (Hansma et al., 1992; Lyubchenko et al., 1992). It is possible that rigidity of nanofibrils is a result of cross-linking of several ssDNA molecules via Mg<sup>2+</sup> bridges. We assume that nanofibrils (Figures 6 and 7) are produced in the result of lateral intermolecu-

![Figure 10. SEM images of rare condensed DNA particles undergoing destructive changes during treatment with S1 nuclease for 1.5 h at 37 °C. Starting DNA condensates were produced after 35 thermal cycles of PCR amplification of IST2 sequence with Taq polymerase. (A) Microdisks (1.5 μm × 1 μm) and (B) filaments possessed signs of erosion and defragmentation. According to independent microscopy examinations, 90% of microparticles disappeared completely after this treatment.](image)

| Basic shape | Dimensions of a selected particle | Volume | Suggested number of ss DNA molecules |
|-------------|---------------------------------|--------|-------------------------------------|
| **Microparticles** | | | |
| Spherical 3D MP (from 5 microdisks 1) | Radius 750 nm | $5.5 \times 10^9$ nm<sup>3</sup> (for completely packed sphere) | $1 \times 10^5$ |
| | | $1.8 \times 10^8$ nm<sup>3</sup> | |
| Microdisc 1 | 1500 nm × 1000 nm × 30 nm | $3.5 \times 10^7$ nm<sup>3</sup> | $2 \times 10^4$ |
| Microdisc 2 | 4000 nm × 3000 nm × 50 nm | $4.7 \times 10^8$ nm<sup>3</sup> | $2.5 \times 10^5$ |
| **Nanoparticles** | | | |
| Nanofibril | 2000 nm × 11 nm × 2 nm | $4.4 \times 10^4$ nm<sup>3</sup> | ND** |
| Elementary nanodisc | 7 nm × 7 nm × 2 nm (AFM) | $3.1 \times 10^2$ nm<sup>3</sup> | 1–2 |

In calculations, we divided the volume ratio (microparticles: elementary discs) by packing density factor ($F = 3$) and a factor (3) for the underestimated thickness of nanodisc due to the known errors of AFM (Hansma et al., 1992; Lyubchenko et al., 1992).

*Long and short diameters are indicated for discs.

**ND, impossible to evaluate.
lar DNA condensation and represent sheaves or tapes from at least four ssDNA molecules in a row. Nanofibrils are much more stable than DNA helices (B form DNA) and preserve their structure during heating at 94 °C as already demonstrated (Danilevich et al., 2010). More complex filaments are agglomerates from nanofibrils which are possibly interlinked with lateral Mg$^{2+}$-bridges and/or via complementary interactions. The fine structure of nanofibrils still remains unclear and should be studied by high-resolution microscopy methods.

Hence, this study demonstrates unique morphology of 3D MPs and provides new insights into mechanisms of DNA condensation in PCR. The smallest nanodisks and nanofibrils, discovered in this work, represent the new forms of DNA and will be subject of further studies.

Conclusions

Recently we have described the phenomenon of the formation of condensed DNA MPs and NPs during PCR. This study demonstrates the unique morphology and fine structure of PCR-generated condensed DNA particles using electron and atomic force microscopy. SEM examinations have revealed a new structural type of 3D microparticles formed irrespectively of the nucleotide sequence of amplicons. Microparticles are constructed from numerous de-adenated DNA structures. As visualized by AFM, the surface of microdisks possesses a finely granular structure and consists of adhered and flattened spherical nano-sized particles. Besides microdisks, long filamentous structures (as tapes from thinnest nano-sized fibrils) are found among DNA condensates. Single microparticles are surrounded by numerous NPs and the smallest ones (10–15 nm) are considered as elementary structures. NPs with this compact structure cannot be formed by dsDNA due to intrinsic rigidity of the double helix. We hypothesize that the formation of DNA nanofibrils and nanodisks in PCR occurs due to Mg$^{2+}$-mediated intermolecular (lateral) and intramolecular condensation of ssDNA. Both MPs and NPs are almost completely destructed by S1 nuclease that cleaves ssDNA only. This proves the role of ssDNA as their main structural element. Hence, this study demonstrates unique morphology of 3D MPs and provides new insights into mechanisms of DNA condensation in PCR.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2013.848411.

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