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EXPERIMENTAL ARTICLES

# New Morphotypes of Condensed DNA Microparticles Formed in PCR with *KlenTaq* and *Taq* Polymerases and Plasmid DNA as a Template

**V. N. Danilevich[a, 1], E. A. Vasilenko[a], E. V. Pechnikova[b], and E. V. Grishin[a]**

[a] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia*
[b] *Shubnikov Institute of Crystallography, Russian Academy of Sciences, Moscow, Russia*

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**Abstract**—The specimens of DNA microparticles formed during PCR amplification of IS-elements ISAfe1 and IST2 by *KlenTaq* or *Taq* polymerases and plasmid DNA as a template under varying conditions were investigated by electron microscopy. Microparticle yield and morphology were found to depend on the level of synthesis of single-stranded DNA fragments during PCR. The conditions were studied for formation of discs (ellipsoids) several micrometers in diameter and several dozens of nanometers thick, as well as of microparticles of other morphologies, in the course of PCR with *Taq* polymerase. The structure of the microparticles produced during an asymmetric PCR, i.e., under conditions of low concentration of one of the two primers, was investigated. Morphology of the DNA micro- and nanoparticles was found to depend mainly on the DNA polymerase used in asymmetric PCR. In particular, in the presence of the *KlenTaq* polymerase, discs or ellipsoids a few dozen nanometers thick were formed, while in the presence of the *Taq* polymerase, micro- and nanospheres, heterogeneous in size with rugged surfaces, were produced. The effect of $Mn^{2+}$ cations on DNA microparticle morphology was studied. In the presence of $Mn^{2+}$, microparticle morphology changed dramatically; in PCR mixtures containing *KlenTaq* polymerase supplemented with $Mn^{2+}$, DNA microspheres with fringed surfaces were formed; in the presence of *Taq* polymerase, microparticles in the form of short, rounded rods were produced. In light of these data, the molecular mechanism of micro- and nanoparticle formation in the course of PCR is discussed.

*Keywords*: polymerase chain reaction (PCR), asymmetric PCR, *Taq* polymerase, *KlenTaq* polymerase, $Mg^{2+}$-mediated DNA condensation, microparticles and nanoparticles of condensed DNA, effect of $Mn^{2+}$ cations, electron microscopy.

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DNA condensation in vitro is a good model for investigation of the mechanisms of DNA condensation in live systems. Investigation of these mechanisms is important for understanding the molecular mechanisms of regulation of such biological processes as replication and transcription. Potential application of condensed DNA nanoparticles for transfection and in gene therapy is of growing interest.

Condensation of double-stranded DNA in vitro at room temperature may be induced by various ligands, particularly polyamines (spermine, spermidine) [1, 2] or cations of trivalent metals, for example, by cobalt hexamine $Co(NH_3)_6^{3+}$ [3–7].

It was shown that, in contrast to trivalent cations, bivalent cations (including $Mg^{2+}$) are unable to induce condensation of linear double-stranded DNA at room temperature [8]. The only exception is $Mn^{2+}$ cations capable of inducing toroidal condensation of superhelical but not linear plasmid DNA in aqueous solutions [8]. At the same time, $Mg^{2+}$ and other bivalent cations may act as condensing agents in solutions with low dielectric constants, such as in water–alcohol mixtures [7]. The mechanism of ligand-induced DNA condensation is presently well-studied [9–11].

We have previously described the phenomenon of the formation of condensed DNA micro- and nanoparticles during polymerase chain reaction (PCR) [12]. Micro- and nanoparticles are formed during the concluding stages of PCR involving gene-specific oligonucleotide primers with microbial genome DNA or plasmid DNA molecules as templates [13–16].

Initially, we used *KlenTaq* polymerase to obtain microparticles [17]. *KlenTaq* polymerase lacks the N-terminal fragment of 235 amino acid residues determining the 5' ⟶ 3' exonuclease activity of the enzyme [18].

DNA microparticles formed during the PCR thermal cycles are rather stable in water [13, 14]. They are, however, easily destroyed in the presence of low

[1] Corresponding author; e-mail: dan@mx.ibch.ru

amounts of EDTA (1 mM) [13, 14]. The major dissociation product is linear double-stranded DNA flanked by the primers (that is, the DNA of amplicon). These data indicate the importance of $Mg^{2+}$ cations contained in the PCR buffer for formation of microparticles and their structural stabilization. Moreover, the presence of single-stranded DNA, along with $Mg^{2+}$ cations, is essential for DNA condensation during PCR [15, 19].

The microparticles of condensed DNA formed during PCR possess a number of characteristic features, particularly unique morphology and ultrastructural organization. Thus, the microparticles obtained in PCR with yeast genomic DNA using *KlenTaq* polymerase were electron-dense microspheres ~1 μm in diameter, with numerous spikes [15]. If plasmid DNA was used as a template, large microspheres (about 3 μm in diameter) of several morphotypes were formed in PCR with *KlenTaq* polymerase [16].

Previous studies demonstrated that *Taq* polymerase was significantly less efficient in terms of microparticle formation than *KlenTaq* polymerase [13, 14]. However, efficient formation of micro- and nanoparticles of DNA in the presence of *Taq* polymerase was recently shown as well [19]. In the work [19], various morphotypes of microparticles formed in the presence of *Taq* polymerase and plasmid templates were described. It was noticed that the efficiency of microparticle formation and their morphology depended upon the rate of nonspecific synthesis of single-stranded DNA fragments [19].

The present work pursued the study of formation of condensed DNA during PCR. Using the same plasmid templates and buffer solutions, we compared the efficiency of micro- and nanoparticle formation and their morphology upon PCR with either *KlenTaq* or *Taq* polymerase. The morphology of DNA microparticles was found to depend not only upon the PCR buffer composition and the primer oligonucleotide ratio, but also to be determined to a considerable extent by the presence of $Mn^{2+}$ cations and the DNA polymerase used in PCR. The goal of the present work was to describe the new morphotypes of microparticles formed in the presence of *KlenTaq* or *Taq* polymerase under various conditions.

## MATERIALS AND METHODS

**Template DNA samples.** Plasmids pBS::IST2 and pBS::ISAfe1, derived from pBlueScriptIIsk+, with insertion elements IST2 and ISAfe1 from *Acidithiobacillus ferrooxidans* were used in the work. Their sizes were 1400 and 1250 bp, respectively [20]. Plasmid DNA preparations were obtained using the Wizard reagent kit (Promega, United States) according to the manufacturer's protocol. Plasmid DNA preparations (about 500 ng/mL) were diluted 200-fold in distilled water, and 1-μl aliquots of the dilutions were used per 50 μl of the PCR reaction mixture.

**PCR procedure.** PCR was performed on an MJ Research PTC-200 thermocycler (United States). The reaction mixture (50 μL) contained PCR buffer, four dNTPs (0.2 mM each), oligonucleotide primers (10–15 pmol each), 1–10 ng plasmid DNA, and 2.5 U *Taq* polymerase or 10 U *KlenTaq* polymerase.

PCR amplification of the IS elements ISAfe1 and IST2 was carried out with the primers E1.1f + E1.2r and E2.1f + E2.2r, respectively [14]. The structures of the primer oligonucleotides were reported earlier [14]. 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 varied from 30 to 40. Variations in the number of cycles and PCR modes are reported in the Results section. Linear double-stranded DNAs, PCR products, as well as plasmid DNAs, were analyzed by electrophoresis in 0.8–1.0% agarose gel [19].

**PCR in the presence of $Mg^{2+}$ and $Mn^{2+}$ cations.** In the experiments, microparticle samples were prepared by PCR amplification of ISAfe1 and IST2 elements with *KlenTaq* and *Taq* polymerases. Two buffer systems were used: with potassium chloride (buffer I) and ammonium sulfate (buffer II). Buffer I contained 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5–2.0 mM $MgCl_2$, and 0.01% Tween 20. Buffer II contained 67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, and 3.3 mM $MgCl_2$ [13]. Prior to PCR, 0.5 or 1 mM $MnCl_2$ was added to the reaction mixtures. No $MnCl_2$ was added to the control samples.

**The procedure of DNA microparticles isolation and purification** was described in detail previously [13].

**Staining of DNA microparticles with fluorescent dyes.** The microparticles obtained in PCR were stained with DNA-intercalating fluorescent dyes, usually with propidium iodide (PI). The staining procedure was described earlier [13].

**Fluorescence and light microscopy.** Microparticle samples were studied under an Olympus CK40 epifluorescence microscope (Germany). The details were reported previously [13].

**Electron microscopy** of the total samples of DNA nano- and microparticles was performed on a Tecnai $G^2$ spirit twin microscope (FEI Company, Netherlands) equipped with a Block Mega View III digital camera. For sample preparation, 5-μl aliquots of microparticle aqueous suspensions were applied to copper grids with pioloform supports and dried at room temperature to complete removal of water. The samples were viewed at the accelerating voltage of 120 kV.

## RESULTS

### *Morphology of the Microparticles Obtained in PCR with Klentaq and Taq Polymerases Under Standard Conditions*

In order to compare morphologies of the microparticles formed during PCR with the two related DNA polymerases, experiments on amplification of the ISAfe1 and IST2 elements with *KlenTaq* and *Taq* were carried out in two independent buffer systems: potassium chloride and ammonium sulfate buffers. PCR with *KlenTaq* polymerase was performed in the presence of 3.3 mM $Mg^{2+}$. For PCR with *Taq* polymerase, 1.5 mM $Mg^{2+}$ was used. The number of cycles was 30. In all PCR mixtures, the fragments of 1250 and 1400 bp were elaborated matching the size of IS elements ISAfe1 and IST2 [20]. After centrifugation of the PCR mixtures, condensed DNA precipitate could be observed in all test tubes. Aqueous suspensions of the microparticles thus obtained were studied by electron microscopy.

Electron microscopy images of the microparticles obtained during PCR amplification of the ISAfe1 and IST2 elements with *KlenTaq* polymerase are presented in Figs 1a and 1b. Several microparticle types differing by morphology and ultrastructure were revealed in the samples. Some of them were of spherical or ellipsoid shape with smooth edges, with the major axis varying between 2 and 4 μm. Microparticles of another type were characterized by high electron density; they were of ellipsoid shape, with rugged edges and spikes, 2 to 6 μm in size. Electron-dense microparticles of the third type (star-shaped) had big, sharp spurs. In the samples under study, aggregates of microspheres 7 μm or more in size were also present (data not shown). Another type of microparticles detected in the samples were the so-called three-dimensional network structures of irregular shape described previously [16, 19]. The network size varied in the range from 0.3 to 4 μm. The same microparticle morphotypes were registered upon electron microscopy of the samples obtained by PCR amplification of the IST2 element with *KlenTaq*. In general, morphology of the microparticles formed during PCR amplification of ISAfe1 and IST2 elements corresponded to the one observed in the case of PCR amplification of the β-lactamase gene with *KlenTaq* [16].

In the PCR mixtures obtained with *Taq* polymerase in KCl buffer, microparticles of several morphotypes were revealed: (1) cone-shaped, 3 to 7 μm in diameter; (2) flattened ellipsoids of varying thickness, 3–4 μm along the major axis; (3) spherical with small projections and/or spikes; and (4) star-shaped with sharp and extending spurs, 2.5–5 μm in size (Figs 1c and 1d).

In the samples obtained upon amplification of ISAfe1 and IST2 elements in ammonium sulfate buffer, microparticles similar to those observed in potassium chloride buffer, although somewhat larger, were present. Some of the microparticles reached 10 μm along the minor axis and 15 μm along the major one [19].

Thus, the microparticles formed in PCR with *Taq* polymerase were of somewhat different morphology and larger in size than those produced in PCR with *KlenTaq*.

### *Ellipsoid Types and Conditions of Their Formation in the Standard PCR Procedure with Taq Polymerase*

Earlier, it was found that in PCR amplification of the IST2 element (35 cycles) with *Taq* polymerase under conditions of low rate of non-specific synthesis (low background), most microparticles (about 80%) were semi-electron-dense flattened ellipsoids. Their major axis did not exceed 1.5 μm (Fig. 2a) and they were several dozen nanometers thick. The number of microparticles in a sample was over $1 \times 10^7$ per 50 μL of the reaction mixture [19]. Numerous nanofilaments were present in the sample. To decrease the rate of non-specific synthesis in the experiment, components of the PCR mixture (dNTP and primer solutions) were freshly-prepared. (Earlier we observed that repeated (over five times) freezing and thawing of the primer oligonucleotide solutions resulted in their decay, leading to a sharp increase in the rate of non-specific synthesis in PCR.)

After three weeks, we repeated the experiment on PCR amplification of IST2 with *Taq* polymerase under the same conditions and with the same concentrated stock solutions. The number of PCR cycles was increased up to 40, since little or no precipitate of condensed DNA was observed after 35 cycles. Electrophoresis of the aliquots of PCR mixture revealed the presence of a single intense band matching the IST2 amplicon in size. Background fluorescence was visible, albeit low. According to electron microscopy, flat ellipsoids approximately 4 μm long and 100 nm thick were formed in the reaction, their titer being an order of magnitude lower than in the first experiment ($\sim 1 \times 10^6$ per 50 μL of the reaction mixture; Fig. 2). Electron-dense microparticles of irregular shape, single or in association with ellipsoids, were also revealed by EM observations (Figs. 2b and 2c). In the samples under study, there was considerable amount of particles (approximately 3% to the number of ellipsoids) in the form of small rods of medium electron density about 500 nm thick and 2.5–3 μm long, sometimes over 5 μm long (Fig. 2c). Agglomerates of nanoparticles of spherical and irregular shape were also present. The amount of nanoparticles exceeded the number of ellipsoids several times. In contrast to the previously studied samples obtained in the first experiment, the number of DNA nanofilaments was somewhat lower (Fig. 2d).

Thus, in the repeated experiment on PCR amplification of IST2 element with *Taq* polymerase under conditions of low background, large ellipsoids were

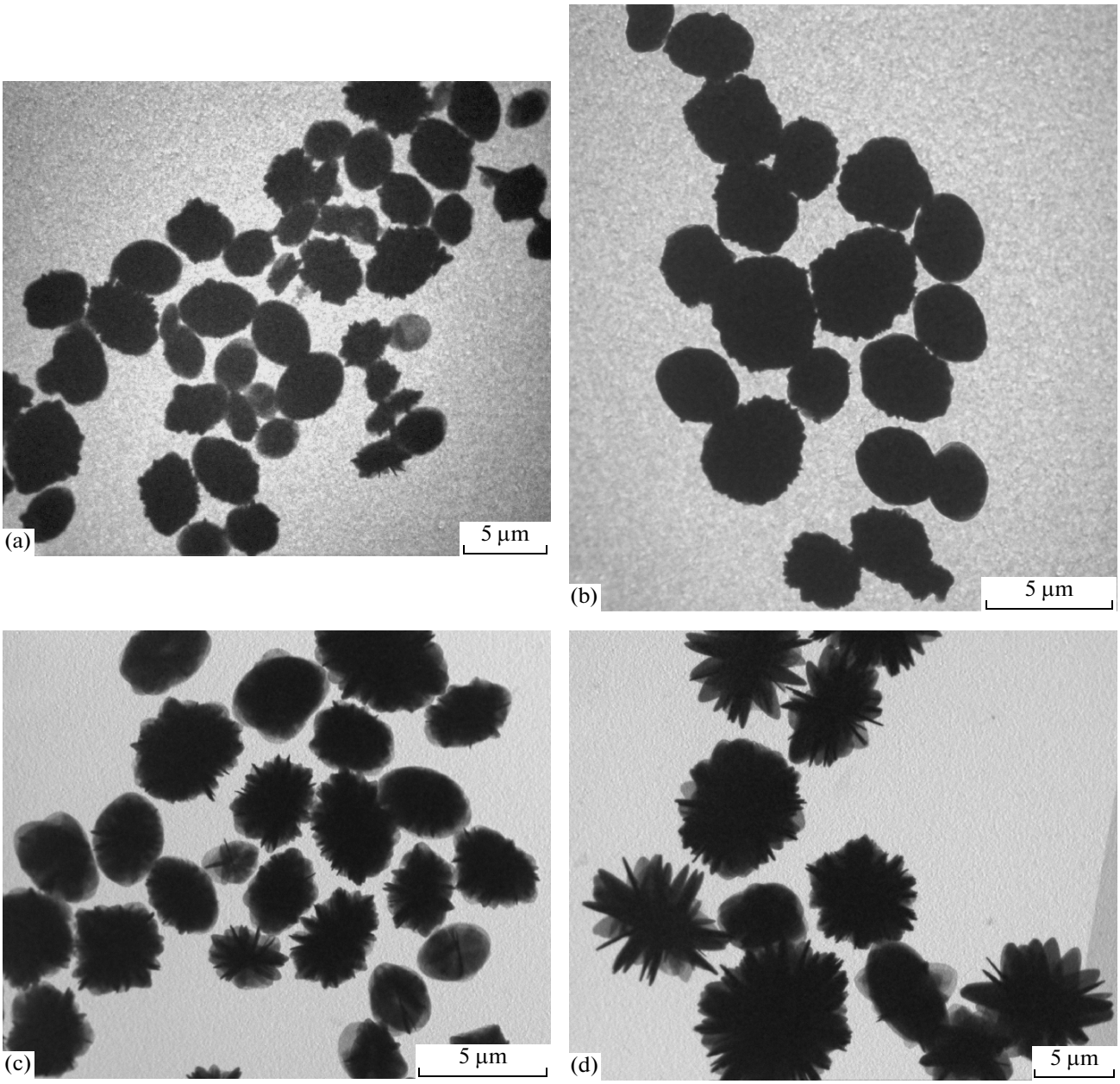


**Fig. 1.** Electron microscopic (EM) images of the microparticles obtained during PCR amplification of the ISAfe1 (a) and IST2 (b) elements with *KlenTaq* polymerase in KCl buffer and in PCR amplification of IST2 with *Taq* polymerase in KCl (c) and ammonium sulfate buffer (d). Microparticles of various types can be seen.

produced. Most likely, under otherwise equal conditions ellipsoid size depends upon the number of PCR cycles.

### *Microparticle Degradation upon Storage*

DNA microparticles formed during PCR are rather labile structures decomposing upon storage at both +4°C and –20°C. We used EM to study the changes microparticles undergo during storage. Samples obtained in PCR amplification of IST2 with *Taq* polymerase and containing mainly large ellipsoids (see Fig. 2) were used in the experiment.

Microparticle suspensions were stored for one month at 4°C before samples for electron microscopy were prepared. In the samples, large semi-electron-dense ellipsoids were revealed. In contrast to the freshly-prepared ellipsoids, those stored for one month were more transparent and aggregated, and formed large shapeless agglomerates (data not shown). Interestingly, some of the ellipsoids were eroded, while some contained holes. Apart from the ellipsoids, some amount of electron-dense microparticles of spherical

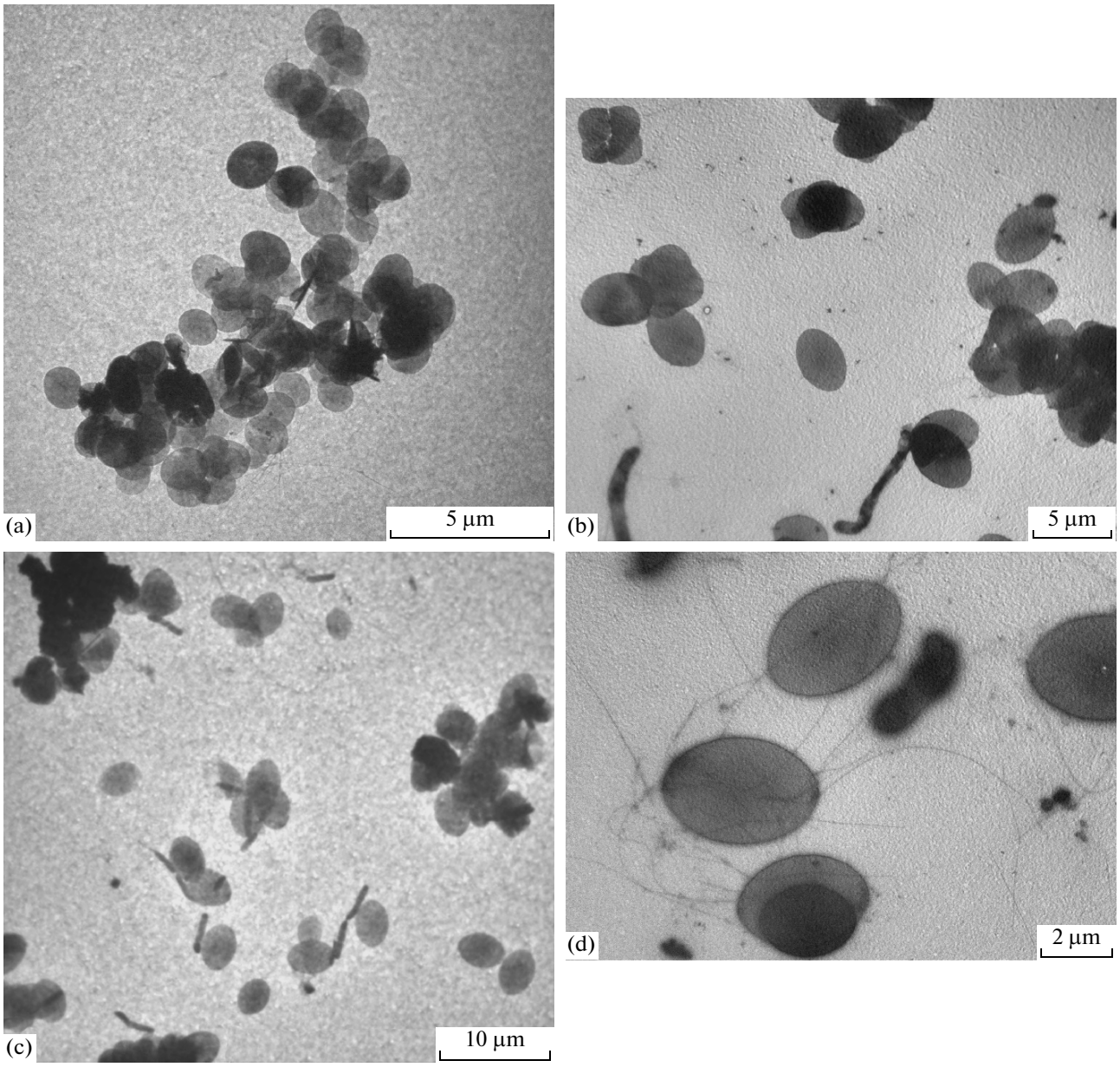


**Fig. 2.** EM images of ellipsoids and other particles obtained during PCR amplification of the IST2 element with *Taq* polymerase in potassium chloride buffer (low background experiment). Cluster of small ellipsoids, 35 cycles (a); cluster of large ellipsoids obtained in a repeated experiment, 40 cycles (b)–(d), where (b) and (c) are different view fields of the same sample. Numerous nanoparticles are seen in panel (b) as electron-dense dots. Besides ellipsoids, rod-shape microparticles and aggregates of electron-dense microparticles are present (c). Panel (d) presents nanofilament knots at high magnification.

or of other shape 0.7 to 1.5–2 μm in diameter, as well as rods approximately 0.5 μm thick and 2 μm long, were observed. Most particles lost contrast. Rare blurry filaments and agglomerates of compact nanoparticles of varying electron density 30–50 nm big were observed. The number of nanoparticles decreased two- to threefold.

Thus, even a short-term storage of the microparticle aqueous suspension at 4°C resulted in their considerable degradation. We assume that the process occurs as a result of $Mg^{2+}$ washing-out of the particles, since these cations play an essential role in formation of micro- and nanoparticles during PCR [13, 14].

### *Microparticles Produced in an Asymmetric PCR*

The samples of microparticle suspensions formed during asymmetric PCR were studied. During asymmetric PCR, preferential accumulation of single-stranded DNA fragments complementary to the

primer present at lower concentration is known to occur. We found it interesting to study how asymmetric conditions would affect the structure of microparticles.

In the experiment, ISAfe1 element was amplified with either *KlenTaq* or *Taq* polymerase. Concentration of either E1.1f or E1.2r primer was decreased 10- or 25-fold. In control samples, both primers were present at concentration of 10 μM. PCR was performed under standard conditions, the number of cycles was 35.

It turned out that even under conditions of a primer deprivation, considerable amount of DNA amplicon was synthesized (Fig. 3). Moreover, fluorescence intensity of the bands produced upon 25-fold dilution of E1.2r was even higher (by a factor of two) than that of the bands produced upon 10-fold dilution of the primer (Fig. 3). This controversial result may be explained by the fact that primers E1.1f and E1.2r possess similar structures (several nucleotide pairs are different) and the primer present at higher concentration might be annealing, although less efficiently, at the complementary DNA strand. In the control samples, fluorescence intensity of the bands corresponding to the DNA of amplicon was two (or sometimes three) times higher than in the experimental samples. In all the samples, background fluorescence was present in gels, indicating accumulation of single-stranded DNA fragments during PCR. Upon centrifugation of the PCR mixtures, microparticle precipitates were clearly observed only in the case of the control samples and in sample 1 (E1.1f/E1.2r = 0.1). No precipitate was observed in other samples.

The control samples (obtained with *Taq* or *KlenTaq* polymerases), as well as the total preparations of microparticles of joint samples 1+2 and 3+4 obtained with *KlenTaq* polymerase and sample 6+7 obtained with *Taq* polymerase, were studied by electron microscopy (see Fig. 3).

Sample 1+2 was found to contain mainly semi-electron-dense discs from 2.5 to 4 μm in diameter (Fig. 4a). Electron density varied across the individual discs. Some discs were associated with compact electron-dense particles of varying form and size (250–500 nm). Along with the discs, considerable amount of electron-dense microparticles of spherical or irregular shape, 1 to 4 μm large, were present in the sample, together with numerous individual spherical nanoparticles 50 to 250 nm in diameter (Fig. 4b).

Sample 3+4 obtained with *KlenTaq* polymerase at lowered E1.2r concentration contained mainly semi-electron-dense ellipsoids 1 to 3 μm along the major axis (Fig. 4c). In the sample, electron-dense microparticles of spherical or irregular shape (in small amounts), 0.7 to 1.5–2.0 μm in diameter were also revealed. Numerous short filaments and considerable amount of rods 0.5 μm thick and 2 μm long were observed (data not shown). The control sample

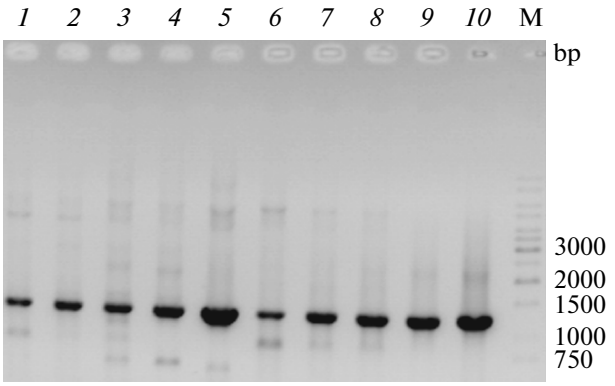


**Fig. 3.** Agarose gel electrophoresis of the products of PCR amplification of the ISAfe1 element with *KlenTaq* polymerase (*1–5*) and *Taq* polymerase (*6–10*). In lanes *1* and *2*, the concentration of E1.2r primer is 10 and 25 times lower, respectively, and in lanes *3* and *4*, the concentration of E1.1f primer is 10 and 25 times lower, respectively. In the control lanes *5* and *10,* the primer ratio E1.1f/E1.2r = 1. Concentration of the primers E2.1f and E2.2r follows the same pattern in lanes *6–10*. M is the DNA molecular mass marker (1-kb DNA ladder).

(Fig. 3, lane *5*) contained the typical set of microparticles of four morphotypes (see above).

A completely different picture was observed in the case of joint sample 6+7, obtained with *Taq* polymerase. Particles of a different and unique kind were observed by EM, namely, micro- and nanospheres highly heterogeneous in size, with their diameter varying from 200 nm and 7 μm (Fig. 5). The pictures presented in Figure 5 made it possible to evaluate the ratio between the particles of different sizes in the studied sample. Micro- and nanospheres were highly electron- dense, lacking spikes or other projections, although with rough surface (see Fig. 5b).

Thus, semi-electron-dense discs and ellipsoids were mainly produced in the course of asymmetric PCR with *KlenTaq* polymerase, while completely different structures were formed in the presence of *Taq* polymerase, namely, heterogeneous electron-dense micro- and nanospheres with rough surface.

### *Microparticles Produced in PCR in the Presence of $Mn^{2+}$ Cations*

Ignatov (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow) reported (personal communication) that complete replacement of $Mg^{2+}$ cations of the PCR mixture with $Mn^{2+}$ cations was possible. Optimal concentration of $Mn^{2+}$ for PCR with *Taq* polymerase was approximately 0.9 mM. In PCR with $Mn^{2+}$, only short DNA fragments (less than 0.5 kb) were successfully amplified. Considering the differences in the properties of cations of the two metals (as DNA-condensing ligands [8]) it was interesting to study the morphology of micro- and nanoparticles

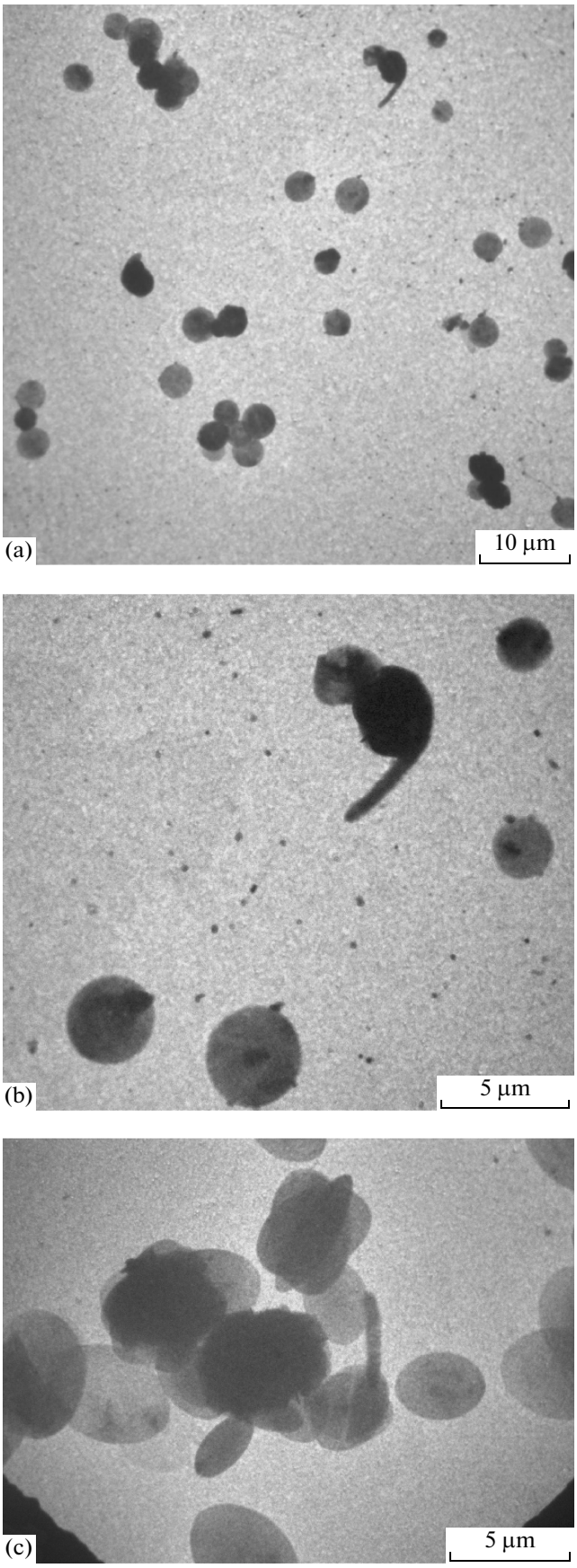


**Fig. 4.** EM images of micro- and nanoparticles obtained by PCR amplification of the ISAfe1 element (35 cycles) with *KlenTaq* polymerase at lowered concentration of E1.1f primer, joint samples 1+2 (a), (b) and at lowered concentration of E1.2r primer, joint samples 3+4 (c).

Panoramic view of ultrathin discs (a), high magnification view of electron-dense particles associated with the discs together with spherical nanoparticles (b), and a cluster of ultrathin ellipsoids and particles of other morphology (c).

formed in PCR in the presence of $Mg^{2+}$ supplemented with small amounts of $Mn^{2+}$.

In the experiments, microparticle samples were obtained in PCR amplification of ISAfe1 and IST2 elements with *KlenTaq* or *Taq* polymerase. Potassium chloride and ammonium sulfate buffers were used for *KlenTaq* and *Taq* polymerase, respectively. Prior to the reaction, 0.5 or 1 mM $MnCl_2$ was added to the reaction mixture. Manganese was not added to the control samples. The number of cycles was 35 for samples 2 and 6, and 30 for the rest of the samples. Increase in the number of cycles in several samples resulted in increased production of microparticles. Electrophoresis picture of the PCR products of ISAfe1 amplification in agarose gel is shown in Fig. 6. As follows from the figure, along with the band corresponding to the DNA amplicon, background fluorescence was high in all samples evidencing accumulation of single-stranded DNA fragments. The presence of $Mn^{2+}$ cations at concentration of 0.5 mM did not significantly influence the yield of the ISAfe1 DNA amplicon in PCR with either polymerase. However, increase in $Mn^{2+}$ concentration to 1 mM had a considerable effect on the amplicon yield: in PCR with *KlenTaq*, DNA yield decreased approximately twofold. DNA amount was evaluated visually by comparison of the intensities of lanes *2* and *3* (Fig. 6); in the case of *Taq* polymerase, the PCR product was practically absent (Fig. 6, lane *7*). Similar results were obtained in PCR amplification of IST2 element by the two DNA polymerases in the presence of $Mn^{2+}$ cations (data not shown). Apparently, DNA polymerase efficiency was determined by the ratio of $Mn^{2+}$ to $Mg^{2+}$ concentrations. In the case of *Taq* polymerase, the ratio was much higher than in the case of *KlenTaq* (1/1.5 and 1/3.5, respectively).

Upon centrifugation and washing, the samples were studied with electron microscopy. EM images of microparticles obtained in PCR amplification of ISAfe1 element with *KlenTaq* polymerase in the presence of 0.5 mM $Mn^{2+}$ are presented in Figs. 7a and 7b. As follows from the pictures, these were microparticles of a new type of spherical or nearly spherical shape and high electron density. Their size varied between 3 and 5 μm. Numerous small fringes were found on the surface.

Thorough analysis of the samples under study did not reveal microparticles of other shapes. Nanoparti-

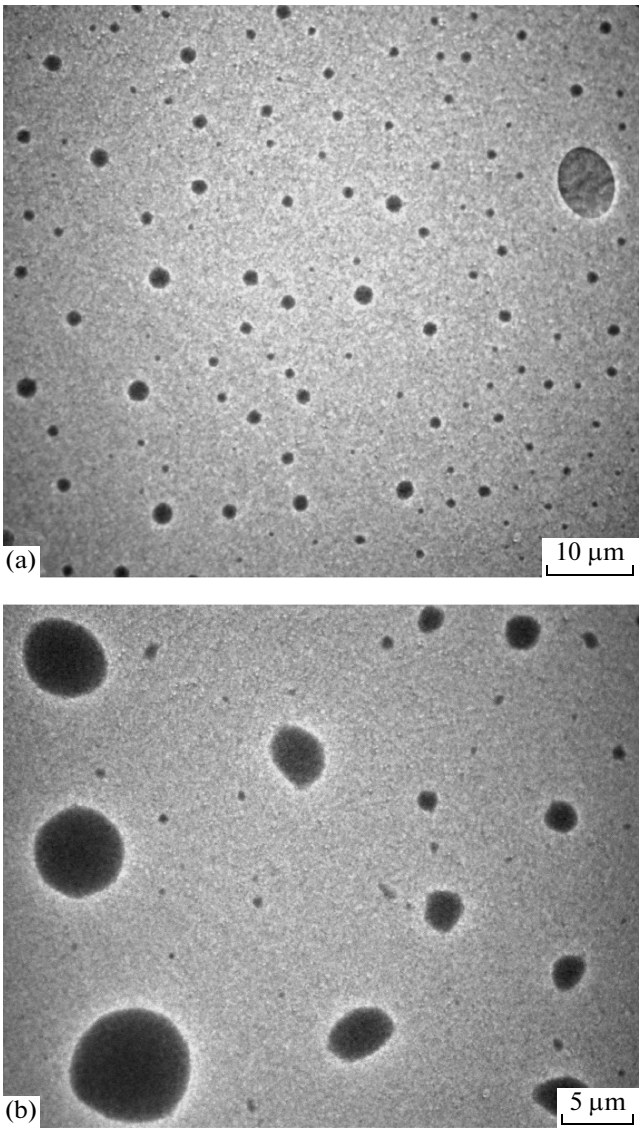


**Fig. 5.** EM images of condensed DNA particles obtained by PCR amplification of the ISAfe1 element (35 cycles) with *Taq* polymerase at lowered concentration of the E1.1f primer (joint sample 6+7): a panoramic view (a) and a high magnification view (b) of micro- and nanospheres of various diameters.

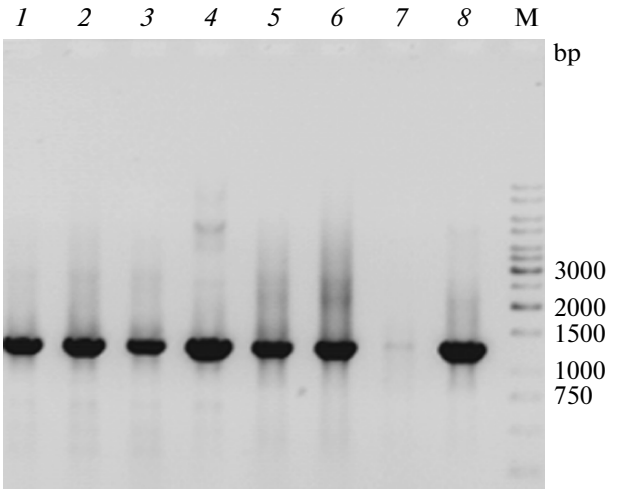


**Fig. 6.** Gel electrophoresis picture of the products of PCR amplification of the ISAfe1 element with *KlenTaq* polymerase (*1–4*) and *Taq* polymerase (*5–8*) in the presence of $Mn^{2+}$ cations. Concentration of $Mn^{2+}$ was 0.5 mM in samples *1, 2, 5*, and *6*, and 1 mM, in samples *3* and *7*. Control samples *4* and *8* did not contain $Mn^{2+}$. The number of cycles was 35 in samples *2* and *6*, and 30 in the rest of the samples. M is the DNA molecular mass marker (1-kb DNA ladder).

cles were also absent. While an increase in $MnCl_2$ concentration up to 1 mM resulted in almost the same picture, heterogeneity in the particle size was more pronounced: together with rather small particles (2.5 μm), large ones up to 8 μm in diameter were often observed (data not shown).

Microparticles of similar morphology were also revealed in the samples obtained by PCR amplification of another element, IST2, under the same conditions (*KlenTaq* polymerase, 0.5 mM $Mn^{2+}$) (Fig. 7c).

A completely different picture was observed in the case of the samples obtained by PCR amplification of IST1 element with *Taq* polymerase in the presence of 0.5 mM $Mn^{2+}$. Microparticles with fringes were absent in the samples, while microparticles of a new morphotype, oval in shape, were revealed instead. These particles have not been observed previously and are therefore unique. The new particles varied greatly in size with the length from 5 to 10.5 μm and width of 3 to 5 μm (Fig. 8). As is demonstrated by Fig. 8, the microparticles varied also in their electron density: both electron-transparent and electron-dense particles occurred. In three dimensions, these particles were most probably short, rounded rods or flattened rods of varying thickness. Much less frequent were the microparticles similar to ellipsoids and flattened ellipsoids. Rare structures shaped as rods 0.25 μm in diameter and approximately 6.5 μm long are presented in Fig. 8d.

Apart from the structures described above, electron-dense microparticles of unusual shape with a "ridge" (a small number of thin sharp thorns and/or ripples) were present in the samples, (Figs. 8b and 8c).

Electron microscopy of the samples obtained by PCR amplification of IST2 element with *Taq* polymerase in the presence of 0.5 mM $Mn^{2+}$ revealed microparticles of the same types as in the case of IST1 amplicon (data not shown).

Interestingly, the control sample (containing no $MnCl_2$) with pBS::IST2 template yielded mainly semi-electron-dense ellipsoids, as well as electron-dense particles of other shape (data not shown). However, the microparticle titer was low, $\sim 1 \times 10^4$.

Thus, the presence of $MnCl_2$ in the PCR buffer results in formation of microparticles of two new mor-

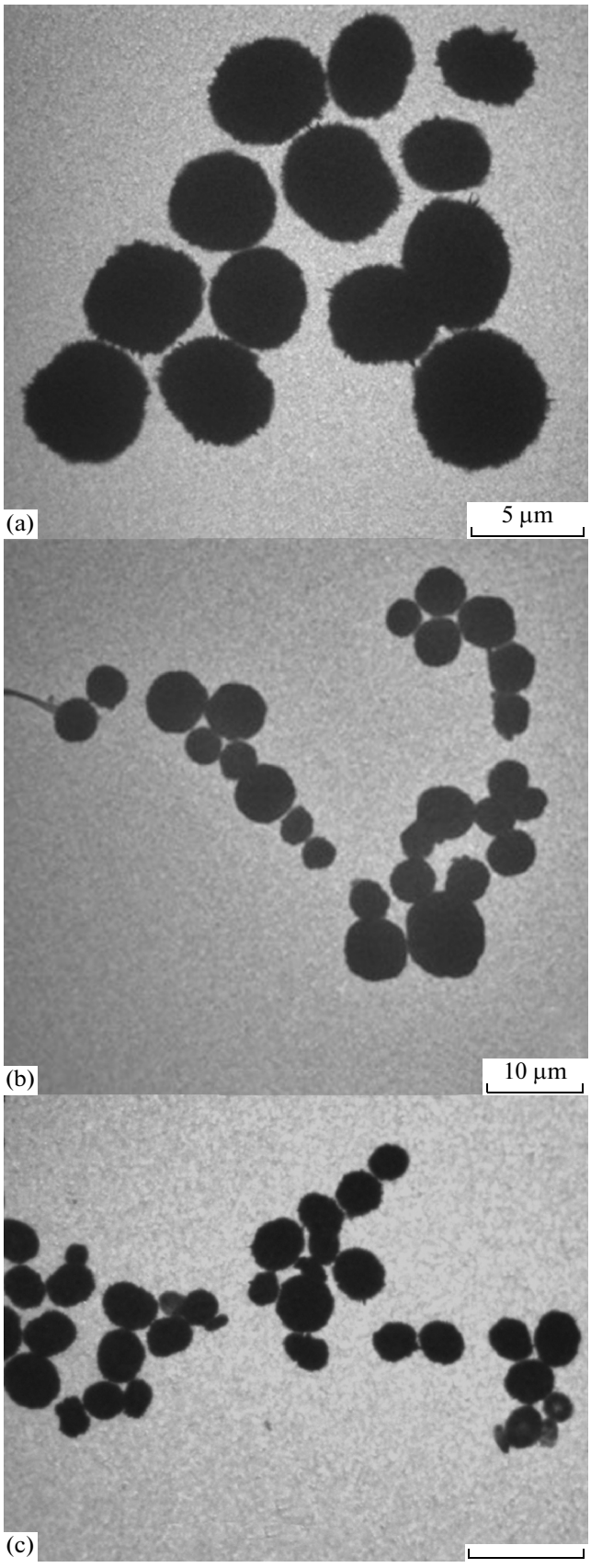


**Fig. 7.** EM images of the microparticles obtained during PCR amplification of the ISAfe1 (a, b) and IST2 (c) elements with *KlenTaq* polymerase in the presence of $Mn^{2+}$ cations. Concentration of $Mn^{2+}$ was 0.5 mM in panels (a) and (c) and 1 mM in panel (b). The microparticles are in the shape of electron-dense microspheres with small fringes. Scale bar on panel (c) is 10 μm.

photypes, namely electron-dense spherical microparticles with small fringes, and microparticles shaped as short rounded rods of high electron density.

## DISCUSSION

A comparative study of microparticles formed during PCR with two related polymerases, *Taq* and *KlenTaq*, was performed. Initially, the microparticles were obtained in a PCR with *KlenTaq* polymerase and yeast genome DNA used as a template [13]. Those microparticles were electron-dense microspheres with multiple spikes, 0.5 to 3 μm in diameter [15]. Then, it was found that applying the plasmid DNA as a template in PCR resulted in formation of larger microparticles of several morphotypes [16].

It was found that another enzyme, *Taq* polymerase, was also capable of micro- and nanoparticle generation. In PCR mixtures produced with *Taq* polymerase in a routine PCR amplification of the ISAfe1 and IST2 elements, several microparticle morphotypes were revealed: (1) cone-shaped, (2) with extended sharp spines (star-shaped), (3) smooth ellipsoids, and (4) of an intermediate type. Interestingly, the size of the microparticles obtained with *Taq* polymerase using KCl buffer exceeded almost 1.5 times the size of the microparticles obtained using the same templates but with *KlenTaq* polymerase. At the same time, the size of microparticles obtained with *Taq* polymerase in an ammonium sulfate buffer was two to three times higher than the mean size of the microparticles formed in the presence of *KlenTaq* (see also [19]).

Interestingly, condensed DNA particles of several morphological types could be formed in PCR with *Taq* polymerase under the same conditions and the same PCR mode, namely, the four morphotypes or ellipsoids [19]. The latter ones were semi-electron-dense particles of micrometer range in diameter and few nanometers thick. These structures may be considered as nanoparticles since their thickness does not exceed 100 nm. Apparently, the morphology of the microparticles produced during PCR is determined to a considerable extent by the rate of synthesis of non-specific single-stranded DNA fragments on a plasmid template. Indeed, ellipsoid formation during amplification of IST2 with *Taq* polymerase occurred only in the case when background fluorescence of gels (indicating accumulation of single-stranded DNA fragments resulting from non-specific primer annealing on the template) was relatively low. On the contrary, in the case of high background noise, a typical set of large

electron-dense microparticles was formed. Ellipsoid size was variable. Initially, in the PCR experiments performed under the conditions of low background signal, small ellipsoids less than 1.5 μm long were formed. In further experiments performed using the same stock solutions, ellipsoid size increased up to several micrometers, and their titer decreased by an order of magnitude. The reason for this phenomenon is not clear yet.

The data obtained show that the microparticle morphology changes in the case of asymmetric PCR under conditions of limitation of one of the primers. Electron microscopy of the samples of condensed DNA obtained upon PCR amplification of the ISAfe1 element with *KlenTaq* polymerase demonstrated that at the primer ratio E1.1f/E1.2r = 1/10, mostly the semi-electron-dense discs of nanometer range thickness and 2.5 to 4 μm in diameter were formed (Fig. 4a). In the samples obtained at the primer ratio E1.1f/E1.2r = 10/1, mostly the semi-electron-dense ellipsoids 1 to 3 μm along the major axis were formed (Fig. 4c). Therefore, the ellipsoids could be formed in abundance both in routine PCR with *Taq* polymerase and in asymmetric PCR with *KlenTaq* polymerase at a certain primer ratio. The explanation for the phenomenon is not yet clear so far.

Quite a different picture was observed in the study of the samples obtained in asymmetric PCR with *Taq* polymerase. In this case, at primer ratio E1.1f/E1.2r = 0.1, the particles of unique morphology (highly heterogeneous micro- and nanospheres with rough surface) 200 nm to 7 μm in diameter were revealed (Fig. 5). Differences in the morphology of the microparticles formed in the presence of the two related polymerases were possibly determined by the differences in the structure of the single-stranded DNA molecules elaborated in asymmetric PCR and the ratio between double- and single-stranded DNA in the mixture.

We also studied the influence of admixtures of $Mn^{2+}$ cations on the morphology of the microparticles formed in PCR. It was found that upon PCR amplification of the ISAfe1 and IST2 elements with *KlenTaq* polymerase in the presence of 0.5 mM $Mn^{2+}$, microparticles of a new type were formed, spherical or nearly spherical in shape, and of high electron density, with diameter varying between 2.5 and 8 μm depending on the DNA amplicon structure. The surface of the microspheres was covered with numerous small fringes.

Study of the samples obtained in PCR amplification of the ISAfe1 and IST2 elements with *Taq* poly-

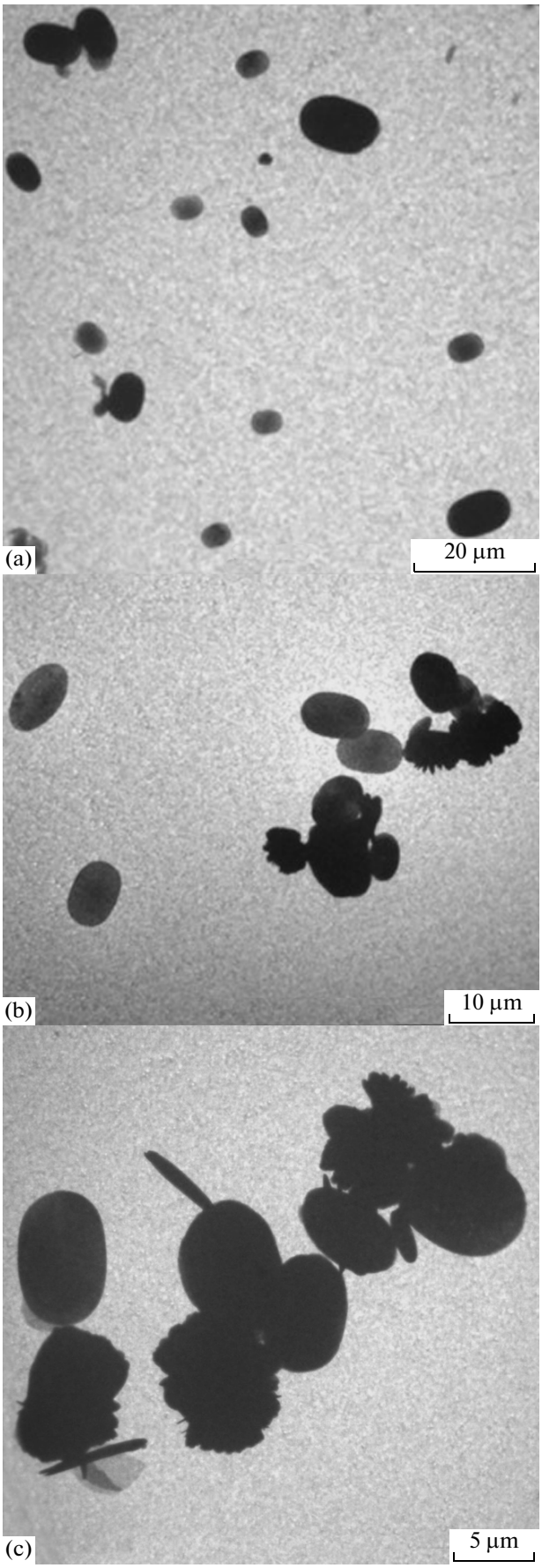


**Fig. 8.** EM images of the microparticles obtained during PCR amplification of the ISAfe1 element with *Taq* polymerase in the presence of 0.5 mM $Mn^{2+}$ (30 cycles). A panoramic view (a), oval-shaped microparticles (b), and microparticles of different types at high magnification (c). The images are different fields of the same sample.

merase in the presence of 0.5 mM $Mn^{2+}$ revealed microparticles shaped as ovals or short rounded rods 5 to 10.5 μm long and 3 to 5 μm wide. Apart from the rod-shaped structures, rare electron-dense microparticles of unusual shape with crest-like structures were revealed in the samples (Fig. 8).

Thus, morphology of the microparticles formed in PCR depends on multiple factors including the DNA polymerase used, amplicon nature, the number of PCR cycles, duration of the PCR stages, primer ratio, presence of other cations ($Mn^{2+}$), and many other factors yet to be discovered.

Among the numerous types of the described microparticles, ellipsoids of nanometer-range thickness deserve special attention since they are the simplest built. Since the studied discs, ellipsoids, and short rounded flattened rods are topologically closely related structures, they probably share a common mechanism of formation. Recently, ultrastructure of the ellipsoids formed in PCR with *Taq* polymerase was studied by atomic force microscopy (AFM). They turned out to be large aggregates comprising many smaller nanoparticles (data not shown). AFM also revealed large agglomerates of nanoparticles approximately 10 nm in diameter and 2 nm thick. Supposedly, the nanoparticles are formed as a result of intramolecular $Mg^{2+}$-mediated condensation of single-stranded DNA fragments. Indeed, due to their rigidity, double-stranded DNA helices are incapable of formation of such compact particles. Cations of $Mg^{2+}$ neutralize the negative charge of the phosphate groups and thus decrease electrostatic repulsions between the closely located fragments of single-stranded DNA, which is a prerequisite for formation of compact nanoparticles.

There are therefore considerable differences between the mechanisms of previously studied ligand-mediated DNA condensation in vitro and its condensation in the course of PCR. In the former case, DNA condensation occurs at the physiological temperature, with double-stranded DNA or RNA as the starting material and rods and toroids as the resulting structures. The micro- and nanoparticles of DNA we described formed in the process of PCR thermal cycles, and are characterized by much higher diversity of shapes. Various morphotypes of individual microparticles, as well as their aggregates, together with numerous and diverse particles of nanometer range are elaborated. Micro- and nanoparticles under study are formed in the presence of $Mg^{2+}$ cations. It was shown previously that $Mg^{2+}$ is not able to induce the condensation of double-stranded DNA at room temperature [7]. The microparticles formed during PCR contain both double- and single-stranded DNA fragments. Using the S1 nuclease, we have previously shown that the latter ones were essential for microsphere formation, together with $Mg^{2+}$ cations [15, 19]. Therefore, this is a unique mechanism of DNA condensation mediated by $Mg^{2+}$ cations and involving single-stranded DNA fragments.

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