New insight into formation of DNA-containing microparticles during PCR: the scaffolding role of magnesium pyrophosphate crystals

Vasily N. Danilevich*, Andrey V. Machulinb, Alexey V. Lipkinc, Tatyana V. Kulakovskaya, Steven S. Smith and Andrey L. Mulyukind

aShemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Science, ul. Miklukho-Maklaya 16/10, Moscow 117977, Russia; bSkryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia; cBeckman Research Institute and Division of Urology and Urologic Oncology, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010, USA; dWinogradsky Institute of Microbiology, Russian Academy of Science, Pr. 60-letiya Oktyabrya 7/2, Moscow 117312, Russia

Communicated by Ramaswamy H. Sarma

(Received 6 March 2015; accepted 10 April 2015)

This work aims to study molecular mechanisms involved in the formation of DNA-containing microparticles and nanoparticles during PCR. Both pyrophosphate and Mg2+ ions proved to play an important role in the generation of DNA microparticles (MPs) with a unique and sophisticated structure in PCR with Taq polymerase. Thus, the addition of Tli thermostable pyrophosphatase to a PCR mixture inhibited this process and caused the destruction of synthesized DNA MPs. Thermal cycling of Na-pyrophosphate (Na-PPi)- and Mg2+-containing mixtures (without DNA polymerase and dNTPs) under the standard PCR regime yielded crystalline oval or lenticular microdisks and 3D MPs composed from magnesium pyrophosphate (Mg-PPi). As shown by scanning electron microscopy (SEM), the produced Mg-PPi microparticles consisted of intersecting disks or their segments. They were morphologically similar but simpler than DNA-containing MPs generated in PCR. The incorporation of dNTPs, primers, or dsDNA into Mg-pyrophosphate particles resulted in the structural diversification of 3D microparticles. Thus, the unusual and complex structure of DNA MPs generated in PCR is governed by the unique feature of Mg-pyrophosphate to form supramolecular particles during thermal cycling. We hypothesize the Mg-pyrophosphate particles that are produced during thermal cycling serve as scaffolds for amplicon DNA condensation.

Keywords: polymerase chain reaction; PCR-generated DNA particles; thermal cycling; magnesium pyrophosphate microparticles; scanning electron microscopy

Introduction

The ability to adopt a compact or condensed state is a basic feature of DNA underlying functioning of all organisms. To the present, the phenomenon and mechanisms of DNA condensation in vitro have been the subject of numerous studies to gain insight into DNA packing in living systems. DNA condensates can be obtained in vitro using a wide variety of multivalent cations: polyamines (Chattoraj, Gosule, & Schellman, 1978; Vijayanathan, Thomas, Antony, Shirahata, & Thomas, 2004), cobalt hexamine – (Co(NH3)6)3+ (Arscott, Ma, Wenner, & Bloomfield, 1995; Plum, Arscott, & Bloomfield, 1990; Widom & Baldwin, 1980); histone (Hsiang & Cole, 1977; Sun, Liu, Dong, Fan, & Yao, 2013), protamine (Allen, Bradbury, & Balhorn, 1997; Maruyama et al., 2004), as well as in the presence of crowding or dehydrating agents (Arscott et al., 1995; Laemmli, 1975).

From standpoint of practical applications, DNA condensation has attracted great attention for the design and improvement of the gene delivery vehicles. Of particular interest are condensates formed upon DNA interaction with cationic amphiphilic lipids (Felgner et al., 1987, 1994) and cationic oligopeptides (Mann et al., 2011; Niidome et al., 1997), with high stability and increased transfection frequency as well as DNA condensates with polylysine (Liu et al., 2001; Stanić et al., 2008), chitosan (Gao et al., 2008; Mansouri et al., 2004), polyethyleneimine and its derivatives (Demeneix & Behr, 2005; Fischer, Bieber, Li, Elsässer, & Kissel, 1999; Neu, Fischer, & Kissel, 2005). The improvement of feasible and reliable methods to generate DNA particles with a desired size and known properties is relevant to gene therapy.

Recently, Mg2+-mediated formation of DNA-containing microparticles and nanoparticles (MPs and NPs) with unusual structure in PCR with different templates and Taq polymerases has been demonstrated (Danilevich, Barinova, & Grishin, 2009; Danilevich, Vasilenko, Pechnikova, Sokolova, & Grishin, 2012). PCR-associated
MPs and NPs were distinct in morphological diversity, complexity, and dimensions from the previously described DNA condensates with principal morphologies of toroids, rods, and spheroids (Arscott et al., 1995; Chattoraj et al., 1978; Plum et al., 1990; Widom & Baldwin, 1980).

Scanning electron microscopy (SEM) examinations showed the presence of oval microdisks tens of nanometers thick in the suspensions of DNA particles. Besides microdisks, spherical and elliptical 3D MPs with an unusual structure formed by definitely oriented micrometer disks and their segments of 30–40 nm thick were found (Danilevich, Artemov, Smith, Gainutdinov, & Mulyukin, 2014; Danilevich & Gainutdinov, 2013). Microdisks are regarded as basic structural elements of 3D MPs, and their surface consists from linear or branched cords from dozens of adhered and flattened spherical 10–15 nm NPs (Danilevich et al., 2014). Also, numerous nanosized disks (5–20 nm), the supposed elementary nanoparticles, were found in PCR mixtures (Danilevich et al., 2014).

An attractive and practically valuable feature of PCR-generated MPs and NPs is that they are stable in water and in saline solutions (Danilevich et al., 2009) in contrast to DNA condensates formed with polyamines, cobalt hexammine, and crowding agents (Chattoraj et al., 1978; Laemmli, 1975; Plum et al., 1990; Widom & Baldwin, 1980). On the other hand, DNA-containing MPs and NPs readily dissociate in the presence of chelates (EDTA, citrate), yielding double-stranded (ds) DNA amplicon as the major product (Danilevich et al., 2009; Danilevich, Kadykov, & Grishin, 2010). The sensitivity to chelates pointed to a key role of Mg$^{2+}$ ions in the formation of MPs in PCR, and Mg was present in them as proved by SEM- and EDS-based elemental analysis (Danilevich et al., 2014). It is known that Mg$^{2+}$ ions solely do not cause dsDNA condensation in aqueous solutions at room temperature and during a single heating and cooling step used for DNA denaturation and renaturation (Conwell & Hud, 2004; Duguid & Bloomfield, 1995; Hud, 2009; Ma & Bloomfield, 1994). Moreover, the interaction of Mg$^{2+}$ ions with exogenous dsDNA does not yield insoluble DNA condensates during thermal cycling (Danilevich, 2012). Therefore, the other components of a PCR mixture may contribute to Mg-mediated formation of DNA microparticles. We have suggested that such important component is pyrophosphate, the common byproduct of PCR (Saiki et al., 1988).

In this report, we examined the role of pyrophosphate and the other components in the formation of condensed DNA microparticles. Our results demonstrate that pyrophosphate is a mandatory component for the formation of DNA MPs as proved by the inhibitory analysis, biochemical assays, pyrophosphatase sensitivity tests, EDS, and electron diffraction (ED) analyses. Moreover, thermal cycling of Na-pyrophosphate- and Mg$^{2+}$-containing mixtures without DNA polymerase under the conditions of routine PCR yields planar micrometer disks and 3D structures. Although the resulting magnesium pyrophosphate microparticles have a similar intersecting-disk structure, they only resemble PCR-generated condensed DNA MPs. The incorporation of dNTPs, primer oligonucleotides, or amplicon DNA into Mg-pyrophosphate particles gives a rise to structurally various and more complex 3D structures. Taken together, these results extend our knowledge on the mechanisms involved in the formation of DNA MPs in PCR and support a role of Mg-pyrophosphate nanoparticles and microparticles as structural scaffolds for DNA condensation.

**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 IST2 (1400 bp) and ISAfe1 (1250 bp) from the bacterium *Acidithiobacillus ferroxidans* (Holmes et al., 2001; Kondrat’eva, Danilevich, Ageeva, & Karavaiko, 2005; Yates, Cunningham, & Holmes, 1988). Plasmid DNA was isolated with a Wizard reagent kit (Promega, USA) according to the manufacturer’s protocol. Target sequences were amplified from plasmid DNAs using primers E2.1f and E2.2r for IST2 and E1.1f and E1.2r for ISAfe1 with the structure reported previously (Danilevich et al., 2014). PCRs were run in a MJ Research PTC-200 thermocycler (USA) using 50-μl reaction mixtures containing 2.5 U of *Taq* polymerase, each primer (10 pmol), each dNTP (0.2 mM). The template was 1 to 5 ng of plasmid DNA. PCR buffer I contained 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH$_4$)$_2$SO$_4$, 0.01% Tween 20, and 1.5 mM MgCl$_2$. PCR buffer II contained 60 mM Tris-HCl (pH 8.5), 25 mM KCl, 0.1% Triton X100, and 1.5 mM MgCl$_2$.

The standard PCR regime was the following: denaturing at 94 °C for 30 s, annealing at 63 °C for 30 s, elongation at 72 °C for 1.5 min, and the number of thermal cycles was 35. 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 microparticles with condensed DNA**

After thermal cycling, the reaction mixtures (50 μl) were diluted with MilliQ water to 400-μl volumes and then centrifuged at 8000 g (12,000 rpm) for 2 min (Eppendorf MiniSpeen centrifuge, Germany). Supernatants were
discarded carefully, and pellets of condensed DNA were washed with 400 μl of water and then suspended in 40 μl of water. Suspensions of DNA particles were subjected to analyses immediately or after storage at 4 °C for a few days.

**Thermal cycling of mixtures without DNA synthesis**
Thermal cycling of aqueous and buffer solutions, containing magnesium chloride and sodium pyrophosphate (Na₄P₂O₇·10H₂O, Sigma-Aldrich) without or together with some other components of PCR mixtures, was conducted in the same fashion as in routine PCR; the number of cycles was 35. Microparticles were pelleted by centrifugation, washed twice with water (200 μl), and suspended in 40 μl of water.

**Bright-field and fluorescence microscopy**
Suspensions of DNA condensates were incubated with propidium iodide (60 μM) at 37 °C in dark for 15 min, placed with 5-μl aliquots onto slides, and air-dried. The slides were examined under Axioplan microscope (Carl Zeiss, Germany).

**TEM and SEM**
The suspensions (5 μl) were dropped on pioloform-coated copper grids (Pelco, Sweden) and were brought to dryness at room temperature. For TEM imaging, we used a JEM 1400 microscope (Jeol, Japan). SEM was performed using a JSM-6510LV microscope (Jeol, Japan) equipped with a special grid holder. In several experiments, grids with specimens were coated with gold using a vacuum sputtering equipment JFC-1100 (Jeol, Japan) for a better resolution. The mode of secondary electron imaging at accelerating voltage 10 kV was optimal to obtain detailed information about the surface topology of microparticles. Their average size was calculated from measured dimensions of 100 particles, and the total number (per 1 μl) was estimated from counting in 10 fields on SEM images (300× magnification).

**ED and elemental analysis**
ED patterns were recorded with a Jeol JEM 2100 transmission electron microscope equipped with lanthanum hexaboride (LaB₆) gun, operating at accelerating voltage of 200 kV. Energy dispersive X-ray spectral (EDS) analysis was conducted using the same microscope with an X-max detector (Oxford instruments, UK). The atomic percentage of C, N, Mg, O, and P was determined using the INCA software.

**Pyrophosphate (PPI) and orthophosphate (Pi) assay**
The pyrophosphate (PPI) content was calculated from the concentration orthophosphate (Pi) after treatment of analyzed samples (solubilized precipitates and supernatants of PCR mixture) with Tli pyrophosphatase from Thermophilus litoralis (SibEnzyme, Novosibirsk, Russia) with the specific activity 1000 U/ml.

To determine the orthophosphate (Pi) concentration, we used the improved colorimetric method (Baykov, Evtushenko, & Avaeva, 1988). Briefly, an analyzed sample (500 μl) was mixed with 125 μl of freshly prepared 5× malachite green solution (10 ml of 0.012% malachite green solution in 20% H₂SO₄ + 2.5 ml of 7.5% ammonium molybdate + 0.2 ml of 10% Tween 20) and allowed to stay for 15 min. The absorbance was measured at 630 nm, and the content of Pi was derived using a calibration curve for 1–20 μM K₂HPO₄.

**Treatment of supernatants and PPI assay**
To 20 μl of supernatants were added 5 μl of 10× pyrophosphatase buffer (50 mM Tris-HCl, 1 mM MgCl₂, pH 8.8), 1 μl pyrophosphatase and milliQ water to final 50 μl, and the mixture was incubated at 70 °C for 1 h. Aliquots from the mixtures (20 μl) were diluted to 500-μl volumes and used for colorimetric assays with malachite green reagent as described above. The appropriate controls were supernatant samples with pyrophosphatase buffer without the enzyme.

**Treatment of precipitates and PPI assay**
MilliQ water-washed precipitates from PCR mixtures were suspended in 50 μl of water. Then, 10 μl of the suspensions were mixed with 33 μl milliQ water and 1 μl of 50 mM EDTA, and the resultant mixture allowed to stay at 72 °C for 30 min until dissolution of microparticles. Then, 1 μl of 50 mM MgCl₂, 5 μl of 10× pyrophosphatase buffer (or 5 μl of 10× PCR buffer), and 1 μl of pyrophosphatase were added. The mixture (total volume, 50 μl) was thoroughly vortexed and incubated at 70 °C for 1 h. Sampled 20-μl aliquots were diluted to 500 μl before the colorimetric assays. The controls were 10-μl aliquots of the suspensions diluted with 40 μl of water and containing 1× pyrophosphatase buffer components and not the enzyme.

The experiments were set in three independent series. Gel electrophoresis and SEM are the data of typical experiments. Assays for PPI were conducted in four series with triplicate measurements in each.
Results

Morphology of DNA microparticles depends on the \( \text{Mg}^{2+} \) concentration in PCR mixtures

Our previous studies showed that PCR with Taq polymerase yielded two types of microparticles, 3D MPs, and oval planar microdisks (Danilevich et al., 2012), whereas Klen-Taq polymerase produced 3D MPs only (Danilevich et al., 2009, 2010). We assumed that this difference was due to higher \( \text{Mg}^{2+} \) concentrations (3.5 mM) in PCR buffer used for Klen-Taq polymerase than for Taq polymerase (1.5 mM \( \text{MgCl}_2 \)) and was not caused by the specific features of the enzymes. To verify this assumption, the IST2 amplifications were run using Taq polymerase and the buffer with KCl and varying \( \text{MgCl}_2 \) concentrations, starting from the previously used 1.5 to 3.5 mM as for the other polymerase. The number of cycles was set to 35, and the collected supernatants and precipitates were subjected for further electrophoretic analyses and SEM.

Target water-soluble DNA (1400 bp) was produced in 35-cycle PCR almost with the same yields at the \( \text{MgCl}_2 \) concentrations, ranging from 1.5 to 3.5 mM (data not shown). The total numbers of MPs in the precipitates were virtually similar (6.2 × 10³ to 5.6 × 10³ \( \mu \text{l}^{-1} \)). SEM-based examinations revealed both oval microdisks (36%) and 3D MPs (74%) in DNA precipitates formed in PCR mixtures with 1.5 mM \( \text{MgCl}_2 \) (Figure 1(A)); abundant 3D MPs and rare microdisks at 2.0 mM \( \text{MgCl}_2 \); predominant mature 3D MPs with the specific morphology (Figure 1(B)) at 3.0 and 3.5 mM \( \text{MgCl}_2 \). The formation of mature 3D MPs was also typical for Klen-Taq polymerase. The similar results were obtained for DNA microparticles produced during PCR amplification of the ISAfe1 sequence (1250 bp) using Taq polymerase in the buffer with 1.5 or 3.5 mM \( \text{MgCl}_2 \).

Thus, when \( \text{Mg}^{2+} \) ions are present at low concentrations, PCR yields diverse 3D MPs with varying complexity and microdisks in significant amounts, and the elevated level of \( \text{Mg}^{2+} \) in a PCR mixture is a prerequisite for the formation of microparticles with mature and sophisticated structure. In addition, these results are applicable for the controlled synthesis of DNA-containing microparticles with desired morphology and size.

The role of pyrophosphate in MPs formation during PCR

As known, pyrophosphate (PPi) is accumulated in sufficient amounts during PCR and inhibits DNA synthesis. To counteract the PPi inhibitory effect and to enhance DNA amplification, it is useful to supplement the reaction mixture with pyrophosphatase that hydrolyzes pyrophosphate to orthophosphate (Cunningham & Ofengand, 1990; Glukhov et al., 1991; Tabor & Richardson, 1990). Therefore, we studied the pyrophosphatase action on the yield of water-soluble DNA amplicons and DNA precipitates in PCR with the ISAfe1 or IST2 templates and Taq polymerase and buffer II with KCl. The addition of thermostable Tli pyrophosphatase (1 U per 50 µl) caused a slight increase (up to ~1.3 times) in the synthesis of target amplicons as compared to the control mixtures without the enzyme. On the other hand, precipitates with DNA were not produced in the presence of pyrophosphatase even after 40 PCR cycles, being abundant in the control. Thus, these data pointed to the involvement of PPi in the formation of MPs.

In further experiments, we treated 20-µl suspensions of MPs pellets from ISAfe1 amplicons with Tli pyrophosphatase (1 U per 50 µl, PCR buffer II) at 70 °C for 1 h with the subsequent isolation of the residue by
centrifugation, washing with water, and suspending in 20-μl volumes. SEM examinations of pyrophosphatase-treated samples revealed few intact MPs and their numerous fragments, shatters, and fine grains (Figure S1), not observed in the untreated specimens. Although native water-insoluble MPs are likely not a good substrate for pyrophosphatase, the observed destruction signs are indicative to the presence of pyrophosphate in these structures.

To support the above conclusion, we determined the PPi content using malachite green reagent (Baykov et al., 1988) in the precipitates and supernatants obtained upon PCR amplifications of IST2 with *Taq* polymerase (buffer II with KCl and 1.5 mM MgCl₂) for 25, 30, 35, and 40 cycles. Prior to pyrophosphatase addition, MPs were solubilized with EDTA (see Materials and Methods).

As shown in assays with malachite green reagent, the PPi content ranged from 232 to 262 μM in the supernatants with water-soluble DNA amplicons produced at varying PCR cycles. It is noteworthy that the PPi content in solubilized DNA microparticles increased sharply with prolongation of PCR from 25 to 30 cycles and reached a plateau during further thermal cycling (Table 1). The total content of PPi in the supernatants and precipitates at 35 and 40 cycles was of approximately 500 μM, less than the initial dNTP content (800 μM PPI). Taken together, the obtained data support that pyrophosphate is the major structural component of DNA MPs produced in PCR.

Regarding that ammonium polyphosphate might be present in PCR mixtures, we also used buffer I with (NH₄)₂SO₄ in order to rule out its role in MPs formation. In this case, no differences in their morphology and shapes were found. Furthermore, polyphosphates were absent in supernatants and MPs produced in a course of PCR with this buffer as revealed in assays with polyphosphatases PPN1 (Andreeva, Trilisenko, Kulakovskaya, Dumina, & Eldarov, 2015) and PPX1 (Lichko, Eldarov, Dumina, & Kulakovskaya, 2014) (data not shown).

### Table 1. The pyrophosphate concentration in the supernatants and suspended precipitates produced in PCR with *Taq* polymerase and the IST2 sequence (buffer II with KCl, 1.5 mM MgCl₂) over different thermal cycles.

| The number of cycles | Pyrophosphate (μM) |
|----------------------|---------------------|
|                      | Supernatant | Precipitate* | Total   |
| 25                   | 262 ± 13     | 52 ± 7       | 314 ± 10 |
| 30                   | 232 ± 9      | 234 ± 12     | 466 ± 11 |
| 35                   | 257 ± 11     | 245 ± 10     | 502 ± 11 |
| 40                   | 235 ± 12     | 289 ± 14     | 524 ± 13 |

*Precipitates were suspended in 50 μl of water (as the volume of supernatants) before treatments with EDTA and pyrophosphatase and further colorimetric assays. Results of a typical experiment are shown.

**Concentration-dependent effect of Na-pyrophosphate on DNA synthesis in PCR**

Our experiments showed a complete inhibition of DNA synthesis by 1 mM NaPPi added to PCR mixtures. We clarified an issue on pyrophosphate effects on the yield of water-soluble DNA and MPs in routine PCRs (35 cycles, ISAf1 as the amplified sequence, *Taq* polymerase, 1.5 mM MgCl₂ in buffer II with KCl) at various NaPPi concentrations from 0.1 to 1 mM. After completion of PCR, the pellet material was separated from all samples, and the precipitates and supernatants were assayed using electrophoresis, microscopy with PI staining, and SEM.

Target water-soluble DNA amplicons were detectable in both supernatants and precipitates from PCR mixtures produced at 0.1 and 0.2 mM NaPPi, although in somewhat lesser amounts than in the control. It is noteworthy that only a small part of total amplicon DNA was present in precipitates. At concentrations higher than 0.3 mM, NaPPi completely inhibited DNA synthesis in PCR as judged from electrophoretic analysis of the supernatants and pellets (Figure 2). The minimum inhibitory concentration of NaPPi was in the range from threshold 0.2 to 0.3 mM. We supposed that pelleted material from thermocycled mixtures with ≥0.3 mM NaPPi consisted of magnesium pyrophosphate with low solubility in water (see also results below). Although being different in their composition, all the precipitates showed similar fluorescence due to the presence of DNA amplicons (produced at the appropriate NaPPi concentrations) or primer oligonucleotides in the case of DNA synthesis inhibition. Indeed, we showed the inclusion of primers into MPs in special experiments.

As revealed upon SEM examinations, the precipitates contained sufficient amounts of oval microdisks and 3D MPs but their average size (d, 2 μm) was approximately four times less than that in the control condensates produced without NaPPi added (Figure 3(A–C)). It is noteworthy that 3D MPs which contained or not DNA amplicons had a similar architecture of intersecting disks. With increasing of NaPPi concentrations from 0.3 to 0.7 mM, the dimensions of MPs slightly declined, and their number increased from 5 × 10⁴ to 1 × 10⁵ μl⁻¹. In addition, we observed a similar NaPPi effect and morphological types of formed MPs in the case of PCR runs with the IST2 sequence.

It is worth mentioning that the 3.5 mM MgCl₂ concentration in PCR mixture was optimal for the formation of 3D MPs only, the size of which sharply declined with increasing of NaPPi concentration (Figure 3(D) and (E)) simultaneously with an increase of their number (up to 2 × 10⁶ μl⁻¹). In this case, DNA synthesis occurred at 0.1–0.2 mM NaPPi, as shown above for 1.5 mM MgCl₂ and short primer-derived products incorporated into MPs (figures not shown).
Inorganic microparticles formed during thermal cycling of MgCl₂ and NaPPi solutions have the similar architecture as DNA-containing MPs. Aqueous solutions. Reaction mixtures of MgCl₂ (1.5 mM) and NaPPi (0.1–0.9 mM) were subjected to 35 thermal cycles as in the standard PCR. The precipitates were isolated from the samples with NaPPi >0.2 mM and contained microparticles (Figure 4). Both the size and yield of MPs were dependent on the NaPPi concentration. Thus, most 3D structures had the average diameter 2–4 μm. Some 3D MPs and planar disks were of approximately 8 μm at 0.3 mM NaPPi; their total number reached 3.2 × 10⁴ μl⁻¹ (Figure 4(A)). With increasing of NaPPi levels, microdisks were no longer produced, and 3D MPs became abundant (8.1 × 10⁴ μl⁻¹ at 0.5 mM NaPPi and 5 × 10⁶ μl⁻¹ at 0.9 mM NaPPi) and had a reduced average size of 1 and 0.5 μm, respectively, (Figure 4(C) and (D)). The similar results were obtained upon thermal cycling of aqueous solutions containing 3.5 mM MgCl₂ and 0.1–0.9 mM NaPPi. In contrast to 1.5 mM MgCl₂, only 3D MPs (3–4 μm) were produced at 0.3 mM NaPPi, and singular microdisks (8 μm) were absent (Figure 4(B)). Both the size and morphology of 3D MPs produced at higher NaPPi concentrations were similar to the above described structures. Hence, magnesium pyrophosphate may form particles from intersecting disks. The structure of smallest MPs is shown in Figure 5. It is noteworthy that small 3D MPs possessed a distinct morphology of spherulites composed from flat sheets.

**PCR buffers.** Thermal cycling of buffers I or II with MgCl₂ and NaPPi resulted in the formation of morphologically diverse microparticles (Figure 6) that resemble DNA-containing MPs. Irrespectively of buffer used, the same particles were found in the samples subjected to thermal cycling at other equal conditions (MgCl₂ and NaPPi content). Thus, thermal cycling of buffers I and II with 1.5 mM MgCl₂ and 0.2 mM NaPPi mixtures yielded lenticular disks (long axis, 8–10 μm) and 3D MPs (Figure 6), distinct from the above structures from aqueous solutions.

With increasing of NaPPi concentration (0.3–0.7 mM) at the same 1.5 mM MgCl₂ content, 3D microparticles became dominating and complex, and their size decreased to approximately 5 μm (Figure 6). Analogously with the above noted, the total number of 3D MPs began increasing at higher NaPPi concentrations (2.7 × 10³ μl⁻¹ at 0.3 mM; 3.4 × 10³ μl⁻¹ at 0.5 mM; 1.5 × 10⁴ μl⁻¹ at 0.7 mM). With increasing of MgCl₂ concentration (3.5 mM), 3D MPs were the only structural type (Figure S2), but their size reduced sharply to 500 nm with increasing of NaPPi content (0.7 mM).

**Morphological changes caused by incorporation of dNTPs, primer oligonucleotides, or amplicon DNA into MgPPi microparticles.** The addition of 0.8 mM dNTPs (as in standard PCRs) to buffer I with (NH₄)₂SO₄ containing 1.5 mM MgCl₂ and 0.2–0.7 mM NaPPi with further thermal cycling gave a rise to more complex MPs than pure magnesium pyrophosphate structures. Thus, 3D MPs that were produced with dNTPs showed an entire variety of morphological types with sophisticated organization, similarly to normal PCR-generated DNA-containing MPs (Figure 7); large structures of 50 μm in the length, consisting from fused MPs, were present in some samples with 0.3 and 0.5 mM NaPPi (Figure 7(C) and (E)). Planar microdisks displayed some alterations in the shape (lenticular – round – oval) with increasing NaPPi concentrations (Figure 7). Under the same conditions at
3.5 mM MgCl₂, only 3D MPs that resembled PCR-generated structures were produced, and their size decreased with elevated NaPPi content (Figure S3). Large aggregates with the size several tens of microns, consisting from fused MPs, were also found in some samples (Figure 7).
When primer oligonucleotides (10 pmol per 50 μl) were added to the same inorganic mixtures, instead of dNTPs, the produced MPs showed no significant differences in their size and complexity as compared to the control samples containing NaPPi and Mg2+ only (images not shown). Indeed, it was expectable that little concentrations of primers would not cause a visible effect on MPs morphology. It is important that oligonucleotides incorporated into inorganic MgPPi structures as proved by staining with PI.

In special experiments, the amplicon ISAfe1 DNA (~1.5 μg; purified by precipitation with ethanol) was added to the MgCl2–NaPPi solutions followed by thermal cycling. The produced MPs had a similar morphology and formed with the similar yields as in the above cases. Electrophoretic analysis revealed the incorporation of approximately 50% of total amplicon DNA into MPs produced at 1.5 mM MgCl2 and 0.7 mM NaPPi and 100% loading in the case of 3.5 mM MgCl2 and 0.7 mM NaPPi (Figure S4). The efficient DNA inclusion to MPs was also supported by intense red fluorescence after staining with PI, which was absent in the control particles without DNA. Hence, it is possible to control the formation of microparticles with desired size, containing required DNA amounts.

**Elemental composition and crystal structure of inorganic MgPPi, dNTP-MgPPi, and PCR-generated DNA-containing MPs**

Using energy dispersive X-ray spectroscopy (EDS) and ED high-resolution TEM, we detected the constituting elements of MPs generated during PCR or thermal cycling of inorganic phase without/with dNTP addition and analyzed their crystallinity properties. Inorganic MgPPi particles displayed the magnesium, oxygen, and phosphorus peaks; a minor C peak was due to a supporting pioloform film (Figure 8(A)); the estimated atomic...

---

**Figure 4.** SEM images of MPs formed upon thermal cycling (35 cycles) of aqueous solutions, containing: (A) 0.3 mM NaPPi and 1.5 mM MgCl2; (B) 0.3 mM NaPPi + 3.5 mM MgCl2; (C) 0.5 mM NaPPi + 3.5 mM MgCl2; (D) 0.9 mM NaPPi + 3.5 mM MgCl2. Both the size and yield of MPs produced at 0.5 mM NaPPi and 0.9 mM NaPPi were not dependent on the MgCl2 concentration.
percentages in these particles were of 20% Mg, 53% O, 27% P, similar to the calculated values for Mg$_2$P$_2$O$_7$·$n$H$_2$O (Souhassou, Lecomte, & Blessing, 1992). The disks from MgPPi with incorporated dNTPs contained Mg, O, and P at similar atomic percentages (15.4, 68.9, 15.7%) and C from dNTPs and supporting film (Figure 8(B)). EDS spectra of disks generated in PCR had the peak of C (the element in DNA, dNTPs, and pioloform film) and Mg, O, P (16.3, 66.4, 17.3%) (Figure 8(C)). The N peak was not resolved in thin disks in contrast to voluminous 3D MPs (Danilevich et al., 2014). Indeed, individual disks contained less amounts of DNA and had weaker fluorescence after DAPI staining than 3D MPs. As seen from ED patterns, inorganic MgPPi- and PCR-generated disks possessed the crystalline structure (Figure 8).

Discussion
Notwithstanding progress in studies on the morphology and fine structure of DNA-containing particles produced in PCR (Danilevich et al., 2014), many issues concerning the molecular mechanisms of their formation have remained unclear. The leitmotif of the present work was to understand these mechanisms better in order to improve a method for producing DNA particles with required properties and to shed new light on the role of magnesium pyrophosphate in DNA assembling in sophisticated 3D structures.

First, results reported here show that the yield and morphology of DNA-containing MPs generated in PCR are controllable due to selection of the appropriate MgCl$_2$ concentration (Figure 1). Second, pyrophosphate turned out to be the mandatory component of
DNA-containing MPs as proved by the inhibitory effect of pyrophosphatase on the formation of precipitated DNA-containing MPs as well as its destructive action on produced microparticles (Figure S1). The observed stimulation of amplicon DNA synthesis by pyrophosphatase is in consistence with (Blakesley, 2004; Glukhov et al., 1991) and results from the elimination of pyrophosphate accumulating during DNA or RNA synthesis with various polymerases (Abbotts & Loeb, 1985; Cunningham & Ofengand, 1990; Glukhov et al., 1991; Tabor & Richardson, 1990). In addition, pyrophosphate was found closely associated with MPs.

Here, we demonstrated the dose-dependent effect of sodium pyrophosphate on DNA amplification with the minimum inhibitory concentrations (0.2–0.3 mM) (Figure 2), similarly to those determined for TTh polymerase (Glukhov et al., 1991).

It is of interest that microdisks and 3D MPs are produced under the complete inhibition of DNA synthesis in PCR and resembled DNA-containing MPs (Figure 3). The demonstrated formation of microparticles from inorganic components (MgCl₂ and NaPPi) during thermal cycling contribute to better understanding of mechanisms involved in the generation of DNA-containing MPs in PCR. It is should be emphasized that inorganic microparticles produced by thermal cycling (Figures 4, 6, and S2) are morphologically similar but simpler than DNA-containing MPs. Moreover, inclusion of dNTPs, oligonucleotide primers, or amplicon DNA is followed by structural diversification (Figures 7 and S3). The ED patterns, characteristic for monocrystalline structures (Zou & Hovmöller, 2008), were inherent to both inorganic and DNA-containing microdisks (Figure 8). Noteworthy, similar morphology and crystalline properties were demonstrated for composite MgPPi-RNA microsponges produced during isothermal synthesis using phage T7 RNA polymerase (Shopsowitz, Roh, Deng, Morton, & Hammond, 2014). The formation of
Mg-pyrophosphate precipitates during loop-mediated isothermal amplifications was reported and proposed for real-time monitoring for DNA synthesis (Mori, Nagamine, Tomita, & Notomi, 2001; Notomi et al., 2000); however, their structure remains unclear. Taken together, results of this study suggest the role of magnesium pyrophosphate structures as scaffolds for DNA package in composite microparticles. As for DNA localization in MPs and compaction, it is still an open question to be solved in further studies.

Figure 7. The effect of dNTPs on MPs morphology. SEM images of microparticles produced upon thermal cycling (35 cycles) of 1× PCR buffer with (NH₄)₂SO₄, 0.8 mM dNTPs, 1.5 mM MgCl₂, and sodium pyrophosphate: 0.2 mM (A); 0.3 mM (B, C); 0.5 mM (D, E); 0.7 mM (F). Large aggregates from microparticles were formed in the presence of 0.3 and 0.5 mM NaPPi (C, E). Besides 3D MPs, microdisks (~8 μm) were present, and increasing of the NaPPi concentrations caused changes in their shape.
Finally, results of this study have opened possibilities to manipulate the size and structure of microparticles and nanoparticles. It is an attractive feature that PCR-generated or DNA loaded microparticles and nanoparticles are stable to specific and nonspecific endonucleases as shown in our ongoing research (unpublished). As for potential applications, DNA- and RNA-containing particles should meet many of the requirements of gene

Figure 8. EDS spectra (left) and ED patterns (right) of microdisks. (A) a lenticular disk from pure magnesium pyrophosphate; (B) a disk produced in the presence of 0.8 mM dNTPs; (C) a disk produced in PCR with ISA\textsuperscript{fe1} DNA. The peaks corresponding to O, Mg, and P (elements of Mg-pyrophosphate) were detectable; the peak of C (the DNA element) was of high amplitude in disks with loaded dNTP and PCR-generated DNA-containing disk.
therapy, such as the uniform size (~100 nm), and ability to overcome numerous biological barriers after local or systemic administration. As such, the particles appear to provide a non-toxic tool for protecting DNA against DNase degradation in mammalian blood plasma for a necessary period. Further, they are biocompatible, high penetrable though cell membranes and are possible dissolved in the cytoplasm for DNA release (Hart, 2010; Itaka & Kataeoka, 2009; Mansouri et al., 2004; Neu et al., 2005; Niidome et al., 1997; Rao, 2010; Xie, Chen, Sun, & Ping, 2014). DNA-containing particles may be used in the transfection experiments without additional protection with polymers (Roy, Mitra, Maitra, & Mozumdar, 2003; Xie et al., 2014). Importantly, the efficiency of DNA incorporation into magnesium pyrophosphate particles during thermal cycling can reach 50 and 100%, much higher than that in PCR. The issues of possible practical applications of microparticles and nanoparticles will be subject of our further reports. For practical purposes, we have also found the conditions for a controlled synthesis of nanoparticles up to 100 nm in size and are planning to use them in further transfection studies.

**Supplementary material**

The supplementary material for this paper is available online at [http://dx.doi.org/10.1080/07391102.2015.1040842](http://dx.doi.org/10.1080/07391102.2015.1040842).

**Acknowledgments**

The authors thank Prof. V.I. Duda for consults and discussion, Dr V.V. Sorokin, and A.S. Shebanova for assistance in electron microscopy studies.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by Russian Foundation for Basic Research [grant number 14-08-00585].

**References**

Abbott, J., & Loeb, L. A. (1985). DNA polymerase α and models for proofreading. *Nucleic Acids Research*, 13, 261–274.

Allen, M. J., Bradbury, E. M., & Balhorn, R. (1997). AFM analysis of DNA-protamine complexes bound to mica. *Nucleic Acids Research*, 25, 2221–2226.

Andreeva, N., Trilisenko, L., Kulakovskaya, T., Dumina, M., & Eldarov, M. (2015). Purification and properties of recombinant exopolysphatase PPN1 and effects of its overexpression on polysphosphate in *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering*, 119, 52–56.

Arscott, P. G., Ma, C., Wenner, J. R., & Bloomfield, V. A. (1995). DNA condensation by cobalt hexaamine(III) in alcohol-water mixtures: Dielectric constant and other solvent effects. *Biopolymers*, 36, 345–364.

Baykov, A. A., Evtushenko, O. A., & Avaeva, S. M. (1988). A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. *Analytical Biochemistry*, 171, 266–270.

Blakesley, R. W. (2004). Methods for preventing inhibition of nucleic acid synthesis by pyrophosphate. *U.S. Patent No. 6,764,839 B2*.

Chattooraj, D. K., Gosule, L. C., & Schellman, J. A. (1978). DNA condensation with polyamines. II. Electron microscopic studies. *Journal of Molecular Biology*, 121, 327–337.

Conwell, C. C., & Hud, N. V. (2004). Evidence that both kinetic and thermodynamic factors govern DNA toroid dimensions: Effects of magnesium(II) on DNA condensation by hexamine cobalt(III). *Biochemistry*, 43, 5380–5387.

Cunningham, P. R., & Ofengand, J. (1990). Use of inorganic pyrophosphatase to improve the yield of *in vitro* transcription reactions catalyzed by T7 RNA polymerase. *BioTechniques*, 9, 713–714.

Danilevich, V. N. (2012). Rapid and efficient technique for the production of condensed DNA and RNA nanoparticles using thermal cycling. *Doklady Biochemistry and Biophysics*, 443, 71–75.

Danilevich, V. N., Artemov, V. V., Smith, S. S., Gainutdinov, R. V., & Mulyukin, A. L. (2014). The structural peculiarities of condensed DNA micro- and nanoparticles formed in PCR. *Journal of Biomolecular Structure & Dynamics*, 32, 1979–1992.

Danilevich, V. N., Barinova, E. S., & Grishin, E. V. (2009). Microparticles from condensed DNA formed in the process of polymerase chain reaction. *Russian Journal of Bioorganic Chemistry*, 35, 207–218.

Danilevich, V. N., & Gainutdinov, R. V. (2013). Morphology and ultrastructure of condensed DNA microparticles formed during PCR. *Doklady Biochemistry and Biophysics*, 450, 134–139.

Danilevich, V. N., Kadykov, V. A., & Grishin, E. V. (2010). Condensed DNA particles formed in a PCR with plasmid templates: An electron microscopy study. *Russian Journal of Bioorganic Chemistry*, 36, 497–507.

Danilevich, V. N., Vasilenko, E. A., Pechnikova, E. V., Sokolova, O. S., & Grishin, E. V. (2012). Micro- and nanoparticles of condensed DNA formed in PCR with *Taq* polymerase and plasmid DNA as a template. *Microbiology*, 80, 420–431 [in Russian].

Demeneix, B., & Behr, J. P. (2005). Polyethyleneimine (PEI). *Advances in Genetics*, 53, 217–230.

Duguid, J. G., & Bloomfield, V. A. (1995). Aggregation of melted DNA by divalent metal ion-mediated cross-linking. *Biophysical Journal*, 69, 2642–2648.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., …, Danielsen, M. (1987). Lipofection: A highly efficient, lipid-mediated DNA transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 7413–7417.

Felgner, H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., …, Felgner, P. L. (1994). Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *Journal of Biological Chemistry*, 269, 2550–2561.
Fischer, D., Bieber, T., Li, Y., Elssässer, H., & Kissel, T. (1999). A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: Effect of molecular weight on transfection efficiency and cytotoxicity. *Pharmaceutical Research, 16*, 1273–1279.

Gao, Y., Xu, Z., Chen, S., Gu, W., Chen, L., & Li, Y. (2008). Arginine-chitosan/DNA self-assemble nanoparticles for gene delivery: *In vitro* characteristics and transfection efficiency. *International Journal of Pharmaceutics, 359*, 241–246.

Glukhov, A. I., Trofimova, M. E., Gordeev, S. A., Grebennikova, T. V., Vinogradov, S. V., Kiselev, V. I., & Kramarov, V. M. (1991). Amplification of the phage lambda DNA sequence by polymerase chain reaction using thermostable DNA polymerase. *Molekularnaja Biologija (Moscow)*, 25, 1602–1610.

Hart, S. L. (2010). Multifunctional nanocomplexes for gene transfer and gene therapy. *Cell Biology and Toxicology, 26*, 69–81.

Holmes, D. S., Zhao, H.-L., Levican, G., Ratouchniak, J., Bonnefoy, V., Varela, P., & Jedlicki, E. (2001). ISAfe1, an ISL3 family insertion sequence from *Acidithiobacillus ferrooxidans* ATCC 69859. *Journal of Bacteriology, 183*, 4323–4329.

Hsiang, M. W., & Cole, R. D. (1977). Structure of histone H1–DNA complex: Effect of histone H1 on DNA condensation. *Proceedings of the National Academy of Sciences of the United States of America, 74*, 4852–4856.

Hud, N. V. (Ed.). (2009). *Nucleic acid – Metal ion interactions* (p. 448). Cambridge: Royal Society of Chemistry.

Karavaiko, G. I. (2005). Identification of IS elements in *thermostable DNA polymerase*. *Journal of Biological Chemistry, 272*, 15307–15312.

Kondrat’eva, T. F., Danilevich, V. N., Ageeva, S. N., & Karavaiko, G. I. (2005). Identification of IS elements in *thermostable DNA polymerase*. *European Journal of Pharmaceutics and Biopharmaceutics, 71*, 475–483.

Laemmli, U. K. (1975). Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine. *Proceedings of the National Academy of Sciences of the United States of America, 72*, 4288–4292.

Li, H., Molas, M., Grossmann, G. A., Pasumarthi, M., Perales, J. C., Cooper, M. J., & Hanson, R. W. (2001). Biological properties of poly-L-lysine-DNA complexes generated by cooperative binding of the polycation. *Journal of Biological Chemistry, 276*, 34379–34387.

Ma, C., & Bloomfield, V. A. (1994). Condensation of supercoiled DNA induced by MnCl2. *Biophysical Journal, 67*, 1678–1681.

Mann, A., Thakur, G., Shukla, V., Singh, A. K., Khanduri, R., Naik, R., ..., Ganguli, M. (2011). Differences in DNA condensation and release by lysine and arginine homo-peptides govern their DNA delivery efficiencies. *Molecular Pharmaceutics, 8*, 1729–1741.

Mansouri, S., Lavigne, P., Corsi, K., Benderdour, M., Beaumont, E., & Fernandes, J. C. (2004). Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: Strategies to improve transfection efficacy. *European Journal of Pharmaceutics and Biopharmaceutics, 57*, 1–8.

Maruyama, K., Iwasaki, F., Takizawa, T., Yanagie, H., Niidome, T., Yamada, E., ..., Koyama, Y. (2004). Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar containing polyanion ternary complex. *Biomaterials, 35*, 3267–3273.

Mori, Y., Nagamine, K., Tomita, N., & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications, 289*, 150–154.

Neu, M., Fischer, D., & Kissel, T. (2005). Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *The Journal of Gene Medicine, 7*, 992–1009.

Niidome, T., Ohnori, N., Ichinose, A., Wada, A., Michara, H., Hirayama, T., & Aoyagi, H. (1997). Binding of cationic alpha-helical peptides to plasmid DNA and their transfer abilities into cells. *Journal of Biological Chemistry, 272*, 15307–15312.

Ogier, J. (2008). Filamentous condensation of plasmid DNA with a thermostable DNA polymerase. *Nucleic Acids Research, 28*, e63.

Perales, J. C., Cooper, M. J., & Hanson, R. W. (2001). Biochemistry (Moscow), 79, 1211–1215.

Plum, G. E., Arscott, P. G., & Bloomfield, V. A. (1990). Condensation of DNA by trivalent cations. 2. Effects of cation structure. *Biopolymers, 30*, 631–643.

Rao, N. M. (2010). Cationic lipid-mediated nucleic acid delivery: Beyond being cationic. *Chemistry and Physics of Lipids, 163*, 245–252.

Roy, L., Mitra, S., Maitra, A., & Mozumdar, S. (2003). Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. *International Journal of Pharmaceutics, 250*, 25–33.

Salminen, M., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., ..., Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science, 239*, 487–491.

Shopsowitz, K. E., Roh, Y. H., Deng, Z. J., Morton, S. W., & Hammond, P. T. (2014). RNAi-microsponges form through self-assembly of the organic and inorganic products of transcription. *Small, 10*, 1623–1633.

Souhassou, M., Lecomte, C., & Blessing, R. H. (1992). Crystal chemistry of Mg2+PO34–-H2O, n = 0, 2 and 6: Magnesium-oxygen coordination and pyrophosphate ligation and conformation. *Acta Crystallographica Section B Structural Science, 48*, 370–376.

Stanić, V., Arntz, Y., Richard, D., Affolter, C., Nguyen, I., Crucifix, C., ..., Ogier, J. (2008). Filamentous condensation of DNA induced by pegylated poly-l-lysine and transfection efficiency. *Biomacromolecules, 9*, 2048–2055.

Sun, S., Liu, M., Dong, F., Fan, S., & Yao, Y. (2013). A histone-like protein induces plasmid DNA to form liquid crystals in vitro and gene compaction *in vivo*. *International Journal of Molecular Sciences, 14*, 23842–23857.

Tabor, S., & Richardson, C. C. (1990). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Effect of pyrophosphorylation and metal ions. *Journal of Biological Chemistry, 265*, 8322–8328.

Vijayanathan, V., Thomas, T., Antony, T., Shirahata, A., & Thomas, T. J. (2004). Formation of DNA nanoparticles in the presence of novel polyanime analogues: A laser light scattering and atomic force microscopic study. *Nucleic Acids Research, 32*, 127–134.
Widom, J., & Baldwin, R. L. (1980). Cation-induced toroidal condensation of DNA: Studies with Co(NH$_3$)$_6^{3+}$. *Journal of Molecular Biology, 144*, 431–453.

Xie, Y., Chen, Y., Sun, M., & Ping, Q. (2014). A mini review of biodegradable calcium phosphate nanoparticles for gene delivery. *Current Pharmaceutical Biotechnology, 14*, 918–925.

Yates, J. R., Cunningham, R. P., & Holmes, D. S. (1988). IST2: An insertion sequence from *Thiobacillus ferrooxidans*. *Proceedings of the National Academy of Sciences, 85*, 7284–7287.

Zou, X., & Hovmöller, S. (2008). Electron crystallography: Imaging and single-crystal diffraction from powders. *Acta Crystallographica Section A Foundations of Crystallography, 64*, 149–160.