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Self-assembly of DNA–polymer complexes using template polymerization

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ABSTRACT

The self-assembly of supramolecular complexes of nucleic acids and polymers is of relevance to several biological processes including viral and chromatin formation as well as gene therapy vector design. We now show that template polymerization facilitates condensation of DNA into particles that are <150 nm in diameter. Inclusion of a poly(ethylene glycol)-containing monomer prevents aggregation of these particles. The DNA within the particles remains biologically active and can express foreign genes in cells. The formation or breakage of covalent bonds has until now not been employed to compact DNA into artificial particles.

INTRODUCTION

The self-assembly of artificial plasmid DNA (pDNA) containing vectors is required for the development of non-viral gene transfer methods for gene therapy (1). A critical step of this self-assembly process is the compaction of pDNA into non-aggregating particles of a size similar to those in viruses, 30–120 nm in diameter (2). Procedures to condense or compact DNA into non-aggregating particles is not only of interest for gene therapy, but also has implications for the DNA condensation processes that are integral to chromatin or virus formation (3). It is also a challenge in its own right within the context of material science and nanotechnology.

Previous efforts involved the non-covalent assembly of artificial particles by the mixing of already-formed polymers with pDNA (4). Pre-formed oligocations such as spermine, and polycations such as polylysine, polyethylenimine and polyamidoamine dendrimers condense the DNA into compact structures that are <150 nm in diameter and that are toroids or rods on electron microscopic examination (5).

In biological systems, viruses and chromatin form DNA complexes by the assembly of protein macromonomers on the DNA. Often the proteins are chemically modified during the assembly process (6). In order to enlarge the repertoire for assembling DNA–polymer complexes, this study explores the assembly of DNA–polycation complexes by template polymerization (7). DNA serves as a template on which a daughter polymer forms via the polymerization of cationic monomers. The template DNA affects the rate of polymerization as well as the structure of the daughter polymer. The formation or breakage of covalent bonds has until now not been employed to compact DNA into artificial particles.

Previous efforts using template polymerization have involved the formation of polyelectrolyte complexes such as the polymerization of N-vinylimidazole along poly(methylacrylic acid) (7). We now show that pDNA can serve as a template for polymerization, that the nascent polymer can condense the DNA into a variety of compact structures and that the DNA is still biologically active in terms of it being able to express foreign genes in mammalian cells. In addition, the complexes formed by template polymerization can be used to deliver DNA into mammalian cells.

MATERIALS AND METHODS

Monomers

For the step polymerization reactions the cationic monomer *bis*(2-aminoethyl)-1,3-propanediamine (AEPD, Fig. 1) was purchased from Aldrich Chemical Co. (Milwaukee, WI). The cationic peptide di-Cys-NLS (CGYGPKKKRKVGGC) was synthesized by Genosys Biotechnologies (The Woodlands, TX). The cross-linkers DSP, DTBP and DPDPB were purchased from Pierce (Rockford, IL).

N_2,N_2,N_3,N_3-(3′-PEG_{5000}aminopropane)-*bis*(2-aminoethyl)-1,-3-propanediammonium di-trifluoroacetate, referred to as AEPD-PEG (Fig. 1), was synthesized as follows**.** The primary amines of AEPD were protected using 2-(tert-butoxycarbonyloximino)-2-phenylacetonitrile (BOC-ON) (Aldrich Chemical Co.) at 0°C in tetrahydrofuran (THF). The secondary amines were N-alkylated using 3-bromo-1 trifluoroacidamidylpropane in dimethylformamide (DMF). Thin-layer chromatography (TLC) indicated the reaction product to be a mixture of the tri- and tetra-alkylation products. The trifluoroacidamidyl protecting groups were removed in methanolic sodium carbonate. The deprotected primary amines were pegylated using 0-[2-(N-succinimidyloxycarbonyl)-ethyl]-O′-methylpolyethylene glycol 5000 (NHS-PEG) (Fluka Chemical Co.) in DMF. NHS-PEG was added in portions; after each addition the reaction mixture was screened for the

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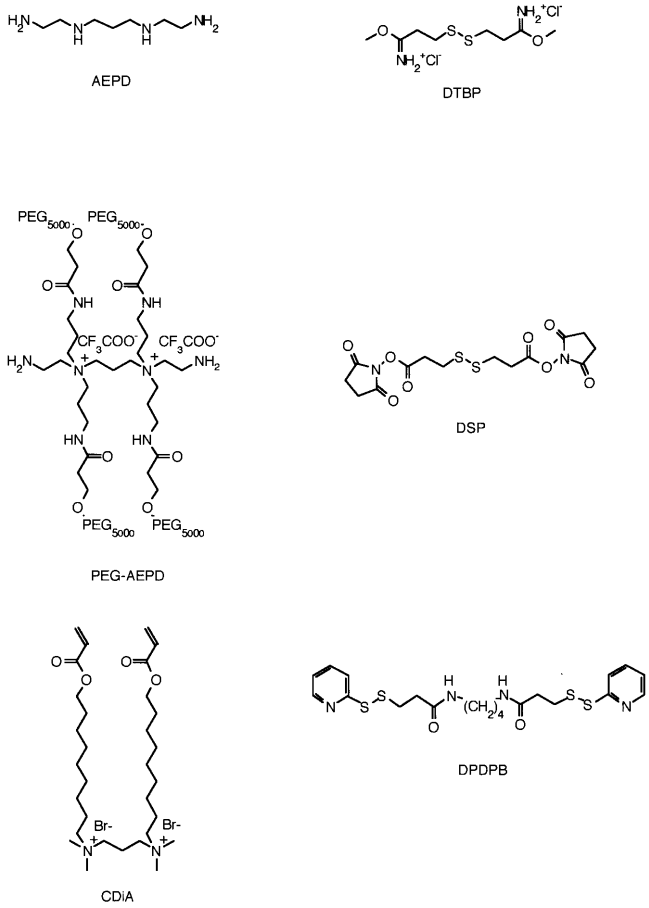

Figure 1. Chemical structures of monomers and crosslinkers used in this study.

presence of primary amines by spotting on a TLC plate and spraying with ninhydrin. No free amino groups were found in the reaction mixture after acylation with NHS-PEG. The BOC protection groups were then removed with trifluoroacetic acid yielding the product AEPD-PEG.

For chain polymerization reactions the cationic diacrylate monomer N,N′-Dinonacrylate-N,N,N′,N′-tetramethylpropane-diammonium bromide (CDiA, Fig. 1) was synthesized and purified as follows. First, 9-bromononacrylate was synthesized by dissolving 9-bromo-1-nonanol (Aldrich) (0.939 g, 4.0 mmol) in 4.0 ml anhydrous diethyl ether in a flame dried 10 ml r.b. flask under dry nitrogen. Sodium carbonate (6.36 g, 6.0 mmol) was added to the reaction mixture. Acryloyl chloride (0.356 ml, 0.397 g, 4.2 mmol) dissolved in 3.5 ml anhydrous ether was added dropwise over a period of ~10 min. The reaction mixture was allowed to come to room temperature and stirred for 2 days. The reaction mixture was diluted to 40 ml with ether and washed three times with 10 ml 2% sodium bicarbonate to remove unreacted acryloyl chloride. The organic layer was dried over magnesium sulfate and passed through a short (~7 g) alumina column to remove unreacted alcohol. Solvent removal yielded 390 mg (35.2%) product as a clear liquid: ^{1}H-NMR ($CDCl_3$) δ 6.40 (dd, 1H), 6.12 (dd, 1H), 5.82 (dd, 1H), 4.15 (t, 4H), 3.40 (t, 2H), 1.85 (dt, 2H), 1.65 (dt, 2H), 1.35 (m, 10 H).

The purified 9-bromononacrylate (131 mg, 0.148 mmol) was dissolved with N,N,N′N′-tetramethylpropanediamine (0.0252 ml, 0.15 mmol) (Aldrich) in 0.150 ml DMF. The reaction mixture was incubated at 50°C for 5 days. The product was precipitated from the reaction mixture by the addition of ether. The resulting solid was collected and recrystallized twice from ethanol/ether yielding 56.9 mg (55.4%) product as white crystals: ^{1}H-NMR ($CDCl_3$) δ 6.40 (dd, 2H), 6.15 (dd, 2H), 5.85 (dd, 1H), 4.15 (t, 4H), 3.88 (m, 4H), 3.52 (m, 4H), 3.40 (s, 12H), 2.75 (m, 2H), 1.82 (m, 4H), 1.65 (m, 4H), 1.35 (m, 20H).

Template polymerization with AEPD monomers

All reactions were performed in 1 mM EDTA, 20 mM HEPES buffered to pH 8.0 or 8.5 (DNA concentration as indicated in figure legend) when the crosslinkers dithiobis succinimidyl propionate (DSP) or dimethyl-3,3′-dithiobis propionimidate (DTBP) (Fig. 1) were used, respectively. All buffers for reaction mixtures were filtered through a 0.2 µm filter before use. The stock solutions of AEPD and AEPD-PEG (10 mg/ml) were titrated to pH 8.5 with 4 N HCl and 4 M NaOH, respectively. The molecular weight of AEPD-PEG was assumed to be 20 kDa [as if all available amino groups on alkylated (BOC)2-PEG were acylated with PEG-NHS esters]. Samples with AEPD-PEG were polymerized at 1:20 base:AEPD total ratio (1:12 for AEPD plus 1:8 AEPD-PEG mixture) in the conditions indicated above for DTBP reaction.

All incubations were performed at room temperature for up to 3 h. Following polymerization, reaction products were analyzed via 1% agarose gel electrophoresis (TAE buffer) and stained with ethidium bromide for DNA analysis or SDS–PAGE for polymer formation (8).

Template polymerization with acrylic monomers

CDiA and DNA were mixed in 1 mM EDTA, 50 mM HEPES, pH 7.4 at various ratios. The buffer was degassed under vacuum and saturated with nitrogen gas for 20 min at room temperature. After the thermal radical polymerization initiator 2,2′-azobis(2-amidino-propane) (AAP) (Waco Biochemicals, Richmond, VA) was added to a concentration of 4 mg/ml; the mixture was incubated for 1 h at 55°C.

Benzothiazolium-4-quinolium iodide dimer (TOTO) fluorescence measurements

Degree of TOTO intercalation into DNA during the course of template polymerization was adopted as a measure of DNA condensation by the nascent counterion polymer chain (9). A sample of reaction mixture (10 µl, 0.2 µg DNA) was added to 0.5 ml of TOTO solution (0.2 µM in the same reaction mixture buffer) at various time points after polymerization reaction and incubated at least for 15 min prior to measurements. TOTO fluorescence (λ_{ex} = 490 nm, λ_{em} = 540 nm) was registered in each sample using a Shimadzu RF 1501. TOTO signal was expressed as a relative normalized value: $(F - F_0)/(F_{max} - F_0) \times 100\%$, where F_0 is the TOTO fluorescence without DNA and F_{max} is the fluorescence of unmodified DNA.

Particle sizing and ζ-potential measurements

The parameters were measured using a Zeta Plus photon correlation spectrometer equipped with 50 MW solid state laser (λ_{em} = 532 nm, Brookhaven Instruments Co., Holtsville, NY) (10). For particle size measurements each sample (0.5 ml) was

measured for at least 2 min. Immediately after mixing DNA and monomer, reaction mixture was centrifuged for 1 min at 12 000 *g* to remove dust from the sample. For ζ-potential measurements, 10 runs were performed for each sample (1.5 ml) and the average value is presented as the ζ-potential for each particular sample.

Aggregation of the reaction mixtures was assessed using the intensity of scattered light measured at 90° angle in a Shimadzu RF 1501 spectrofluorometer operated at λ_{ex} = 500 nm and λ_{em} = 500 nm (11).

Electron microscopy

Polymerization mixtures were placed onto glow-discharged Formvar-coated 200-mesh grids for 1 min followed by staining with 1% uranyl acetate for 30 s (12). The grids were blotted dry with filter paper and examined using a Jeol JEM 100S electron microscope.

Transfections

Transfections were performed using NIH 3T3 cells in 35 mm wells as previously described (13). Briefly, for transfection of template polymerization products, 2 µg of the reporter plasmid pCILuc (14) encoding the firefly luciferase cDNA was complexed with the di-Cys NLS peptide both with and without addition of the crosslinker 1,4-di[3′,2′-pyridyldithio-(propionamido)butane] (DPDPB). Complex formation and template polymerization was performed as described in Figure 2C. Reaction products were then mixed with 4 µg of the endosome disrupting cationic lipid ODAP (13) and the resulting complexes were added to 35 mm wells containing NIH 3T3 cells at ~60% confluence. Transfected cells were harvested 48 h after transfection and cells were lysed and analyzed for luciferase activity using a Lumat LB 9507 luminometer (EG&G Berthold). For transfection of dithiothreitol (DTT) treated samples, both control pDNA (pCILuc) and pDNA containing complexes (pDNA/AEPD/DSP, AEPD/DSP + pDNA) were treated with 100 mM DTT and then dialyzed into buffer (10 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl). Dialyzed pDNA was then mixed with 6 µg of the cationic lipid Lipofectamine (Life Technologies, Gaithersberg, MD) and the resulting complexes were added to NIH 3T3 cells in 35 mm wells. Transfected cells were incubated for 48 h, harvested and cell extracts were assayed for luciferase expression (13).

RESULTS

Step and chain polymerization

A variety of monomers using different polymerization processes were assessed for their ability to undergo polymerization in the presence or absence of DNA, a critical test for template polymerization (7). Initial studies utilized agarose gel electrophoresis as a simple and indirect indication of whether polymer formed and retarded the migration of pDNA.

In one type of step polymerization, the growing chain contains alternating crosslinker and cationic monomer. As an example of step template polymerization, the cationic monomer AEPD (Fig. 1) containing ~2.5 positive charges per molecule at pH 8 was first mixed with DNA (1:2 AEPD:nucleotide, mol/mol), followed by the addition of the homobifunctional amine reactive crosslinker DSP at a 2:3.3 AEPD:DSP ratio. A large reduction in the mobility of the pDNA was observed when the polymerization

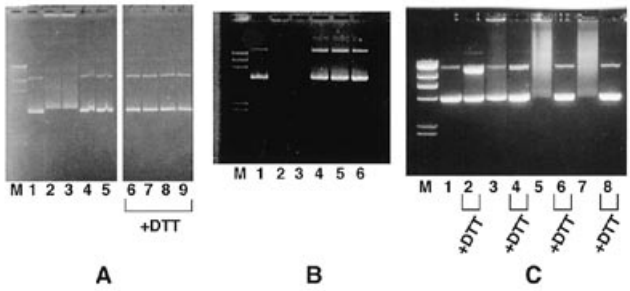

Figure 2. Agarose (1%) gel electrophoresis of template polymerization products before or after treatment with 100 mM DTT. Ratios indicated are monomer and crosslinker per base of DNA. 'M' lanes indicate λ/*Hin*dIII ladder (Life Technologies) (**A**) AEPD/DSP/pDNA (2:3.3:1 molar ratio) polymerization reaction containing 330 µg/ml of pDNA in 20 mM HEPES, 1 mM EDTA at pH 7.5 for 60 min at room temperature. Each sample was analyzed before and after removal of insoluble precipitates (5 min centrifugation at 1800 *g*) designated as total reaction products (tot) and soluble reaction products (s), respectively. Lane 1, unreacted pDNA; lanes 2 (tot) and 3 (s), AEPD and DSP mixed in the presence of pDNA; lanes 4 (tot) and 5 (s), pDNA added after 1 h reaction of AEPD and DSP (no template control); lanes 6–9, same reactions as lanes 2–5 respectively, after DTT treatment and dialysis. (**B**) AEPD/DTBP/pDNA (2:3.3:1 molar ratios) polymerization reaction containing 330 µg/ml of pDNA in 150 µl of 20 mM HEPES, pH 7.5, 1 mM EDTA for 60 min at room temperature and then dialyzed. Lanes 1 and 6, unreacted pDNA; lanes 2 (tot) and 3 (s), AEPD and DTBP mixed in the presence of pDNA; lanes 4 (tot) and 5 (s), pDNA added after 1 h reaction of AEPD and DTBP (no template control) and then kept at room temperature for another 20 min before dialysis. (**C**) Di-Cys-NLS peptide as the cationic 'macromonomer' and 0.6 mM of DPDPB crosslinker (3.3:1 molar ratio of DPDPB:peptide). After incubating at room temperature 267 µg/ml of peptide and 330 µg/ml of pDNA together (0.2 NLS:1 base) in 20 mM HEPES pH 7.5, 1 mM EDTA for 10 min, crosslinker was added and the mixture was incubated for another 1 h at room temperature. Lane M, λ/*Hin*dIII digested marker DNA; lane 1, unreacted pDNA; lane 2, unreacted pDNA after 1 mM DTT treatment; lane 3, peptide and pDNA alone; lane 4, peptide, pDNA and 1 mM DTT; lane 5, peptide, pDNA and 0.6 mM DPDPB (total reaction products); lane 6, reaction from lane 5 after 1 mM DTT treatment; lane 7, peptide, pDNA and 0.6 mM DPDPB (soluble reaction products); lane 8, reaction from lane 7 after 1 mM DTT treatment.

reaction took place in its presence (Fig. 2A, lanes 2 and 3). The crosslinker DSP contains a reducible internal disulfide bond and treatment of the polymerized complex with 100 mM DTT resulted in the pDNA returning to its normal supercoiled position following electrophoresis (Fig. 2A, lanes 6 and 7). This shows that the observed pDNA mobility shift is a result of AEPD polymerization. In addition, no reduction in the mobility of pDNA was observed when it was added 1 h after the AEPD and DSP were mixed (Fig. 2A, lanes 4 and 5). Presumably, the succinimide reactive group of the DSP crosslinker is hydrolyzed after 1 h and can no longer react with the amines on the AEPD monomer. These results indicate that the DNA template is required for polymer formation under these conditions.

A second homobifunctional crosslinker, DTBP, was tested for its ability to polymerize AEPD monomers along the pDNA template (Fig. 2B). When pDNA was present during the polymerization reaction, no pDNA was observed in the lane following ethidium bromide staining, indicating either that the pDNA had not entered the gel or was completely protected from ethidium bromide staining (15) (Fig. 2B, lanes 2 and 3). This effect is probably due to the fact that the reaction of AEPD with DTBP yields a polymer with a greater charge density than that obtained from the reaction of AEPD and DSP. After DTT treatment of these AEPD/DTBP reactions, pDNA was accessible

to ethidium bromide staining and migrated at its supercoiled position (data not shown). If the pDNA was added after the polymerization reaction, then the migration of staining of pDNA was not altered (Fig. 2B, lanes 4 and 5). Thus, the pDNA was also required for polymerization using these concentrations of AEPD and DTBP.

The template dependent polymerization process was also tested using a 14mer peptide encoding the nuclear localizing signal (NLS) of SV40 T antigen (CGYGPKKKRKVGGC) as a cationic 'macromonomer' (Fig. 2C). Cysteine residues on the termini were added to facilitate crosslinking with the sulfydryl reactive homobifunctional crosslinker DPDPB. Agarose gel electrophoresis of polymerization products revealed a crosslinker (DPDPB) dependent shift in pDNA mobility (Fig. 2C, lanes 5 and 7) characterized by pDNA smearing up to the well and no pDNA migrating at the supercoiled position. In the control reaction, the majority of the pDNA migrated at the supercoiled position (Fig. 2C, lane 3). The small amount of DNA retardation (i.e. smearing) in this control lane (lane 3) is not seen if the sample has been treated with DTT (lane 4) suggesting that this results from interaction of the pDNA with a small percentage of dimers that are present in the di-Cys NLS peptide stock solution. These results indicate that even when using different cationic 'monomers' it is possible to alter the migration pattern of plasmid DNA in a crosslinker dependent manner.

A second type of template dependent polymerization involving chain polymerization was explored using a cationic diacrylate monomer that polymerizes following free radical initiation (Fig. 1). The diacrylate monomer (CDiA) containing two positive charges was mixed with DNA and the initiator AAP was added to start the reaction. Following template dependent polymerization, pDNA was not detectable by ethidium bromide staining in the reactions in which monomer (CDiA), pDNA and initiator (AAP) were present (data not shown). When initiator was not present (pDNA + CDiA) pDNA migrated at its supercoiled position.

More direct evidence of DNA template polymerization was obtained by using SDS–PAGE to determine the approximate size of the formed polymers (Fig. 3). For AEPD template dependent polymerization facilitated by DSP, complexes were formed and run on an SDS–PAGE both with and without reducing agents (Fig. 3A). If pDNA was present during the reaction then a smear was observed indicating the presence of AEPD polymers of increasing size (Fig. 3A, lane 1). Without pDNA present, this smear representing large molecular weight polymer was not observed (Fig. 3A, lane 2). DTT treatment (100 mM) of the samples prior to electrophoresis resulted in the disappearance of the smear in the template polymerized samples (Fig. 3A, lane 3).

Template polymerized NLS peptide/DPDPB/pDNA complexes were also analyzed by SDS–PAGE (Fig. 3B). When pDNA was present during the reaction, the electrophoresis revealed a ladder of bands starting from peptide dimers and ranging up to multimers of 10–20 peptides in length (Fig. 3B, lane 4). The addition of reducing agent (100 mM DTT) resulted in the elimination of the ladder of multimers and only monomer sized peptides were observed (Fig. 3B, lane 5). Only peptide monomers and dimers were observed with peptide alone (Fig. 3B, lane 1), peptide and pDNA alone, (Fig. 3B, lane 2) or when pDNA was added after the polymerization reaction (Fig. 3B, lane 3).

The chain CDiA polymerization products were also analyzed by SDS–PAGE (Fig. 3C). Large molecular weight polymer was obtained when pDNA was present during the reaction (Fig. 3C, lane 1) but not when it was absent (lane 2). The approximate size of the polymer appears much larger in the chain polymerization than the step polymerization reaction.

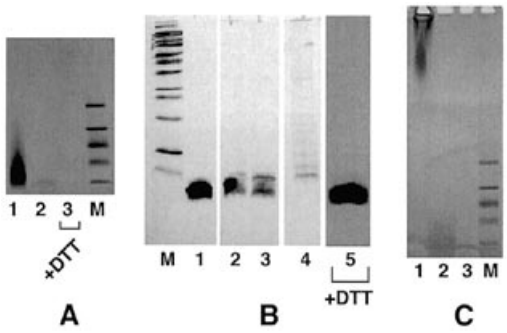

Figure 3. SDS–PAGE (10–20% gradient) analysis of products after staining with Coomassie Blue at [DNA] = 20 µg/ml. M, protein standards (range 3.5–26.6 kDa). (**A**) AEPD/DSP/pDNA (20:20:1 molar ratios) products: lane 1, pDNA is present; lane 2, pDNA is absent; lane 3, reaction from lane 1 after 1 mM DTT treatment. (**B**) di-Cys NLS peptide/DPDPB/pDNA (peptide:DNA 0.2:1 base, 2 mM DPDPB) products: lane 1, peptide alone; lane 2, peptide and pDNA alone; lane 3, pDNA added 1 h after reaction of peptide and DPDPB; lane 4, pDNA present during reaction with peptide and pDNA; lane 5, reaction from lane 4 after 1 mM DTT treatment. (**C**) CDiA monomer/AAP/pDNA base (6:100:1 molar ratios) products: lane 1, pDNA present; lane 2, pDNA absent; lane 3, CDiA monomer alone.

In summary, the agarose gel electrophoresis and SDS–PAGE results show that under specific conditions polymers form when pDNA is present but not absent, indicating that DNA can act as a template for both step and chain polymerizations.

DNA remains intact and expressible after polymerization

A key tenet of this work with regard to potential gene therapy applications is whether the DNA remains both intact and expressible following the template polymerization process. The results described above indicate that after DTT treatment the pDNA template used in the reactions does in fact appear intact (i.e. supercoiled) when observed on agarose gel electrophoresis (Fig. 2). To look specifically at the ability of DNA to be expressed after template polymerization, post-polymerization reaction products of AEPD, DSP and pDNA (pCILuc) (as in Figs 2 and 3) were first treated with DTT and subsequently dialyzed. The DTT treatment breaks down the polymer into constituent cationic 'monomers' by reducing the disulfide bond in the crosslinker. The pCILuc recovered from the DTT treated complexes after dialysis was able to express luciferase at levels comparable to pCILuc that had not undergone template polymerization but was treated with DTT and dialyzed (Fig. 4A). These results indicate that for step polymerization pDNA is not adversely affected by the template polymerization process.

Furthermore, significant increases in transfection efficiency were observed when the template polymerization products of pCILuc, di-Cys NLS peptide and DPDPB (as in Figs 2 and 3) were exposed to NIH 3T3 cells in the presence of an amphipathic polyamine ODAP (13) (Fig. 4B). Complexes formed when pCILuc was present during the polymerization reaction (i.e. template polymerization) enabled 100-fold higher luciferase expression than complexes formed when pCILuc was added after the reaction of peptide and DPDPB.

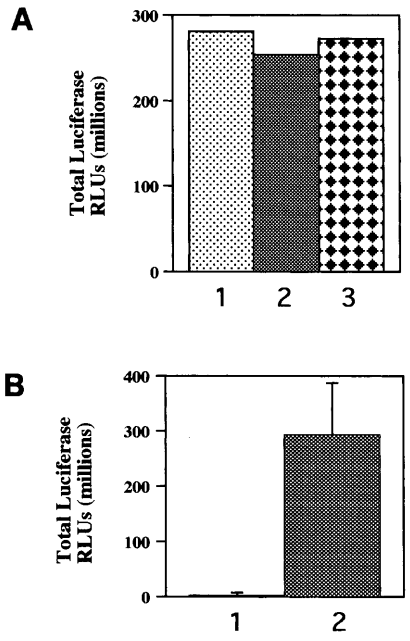

Figure 4. Transfection of plasmid DNA (pCILuc) into NIH 3T3 cells. (**A**) Plasmid DNA remains expressible after template polymerization. Control pDNA and template polymerized pDNA were treated with DTT (100 mM) and dialyzed. Lane 1, pDNA (pCILuc) treated with 100 mM DTT and dialyzed; lane 2, AEPD/DSP/pDNA polymerization complexes treated with 100 mM DTT and dialyzed; lane 3, AEPD/DSP polymerization with pDNA added after the polymerization treated with 100 mM DTT and dialyzed. Each column represents an N of 2. (**B**) Transfection of template polymerization complexes using the macromonomer NLS peptide and the crosslinker DPDPB. Lane 1, peptide/DPDPB reacted together with pDNA added after reaction; lane 2, peptide/DPDPB/pDNA template polymerization complexes. Each column represents an N of 4. T bars represent the standard error. Columns represent total luciferase activity per 35 mm well.

Condensation of DNA

As DNA becomes tightly packed during condensation it becomes resistant to intercalating dyes. To determine if the polymerization reaction initiated condensation of DNA, fluorescence was measured after mixing the template polymerized DNA with the intercalating dye TOTO (Molecular Probes). When the AEPD was polymerized with DTBP in the presence of DNA, TOTO protection was achieved when using molar ratios of AEPD to DNA base >20:1 (Fig. 5A). When the DNA was added 1 h after mixing of AEPD and DTBP, TOTO protection was substantially diminished, providing additional evidence for the template dependence of polymerization. Depolymerization of the polymer by DTT resulted in the DNA becoming fully accessible to TOTO intercalation, indicating that decondensation had occurred. Mixing of AEPD and DNA without crosslinker also resulted in the DNA being fully accessible to TOTO intercalation.

To look more closely at the kinetics of DNA condensation during the step polymerization reaction, a range of AEPD:DNA ratios were assayed for TOTO protection in a time dependent manner (Fig. 5B). At a 4:1 AEPD:DNA base ratio there is no appreciable reduction in TOTO binding after a 180 min polymerization reaction, while at a 20:1 ratio an ~75% reduction occurs by 60 min. The rate of reduction in TOTO binding was slower with a 10:1 ratio but the final reduction in TOTO signal was similar to that in the 20:1 ratio. As expected, the addition of

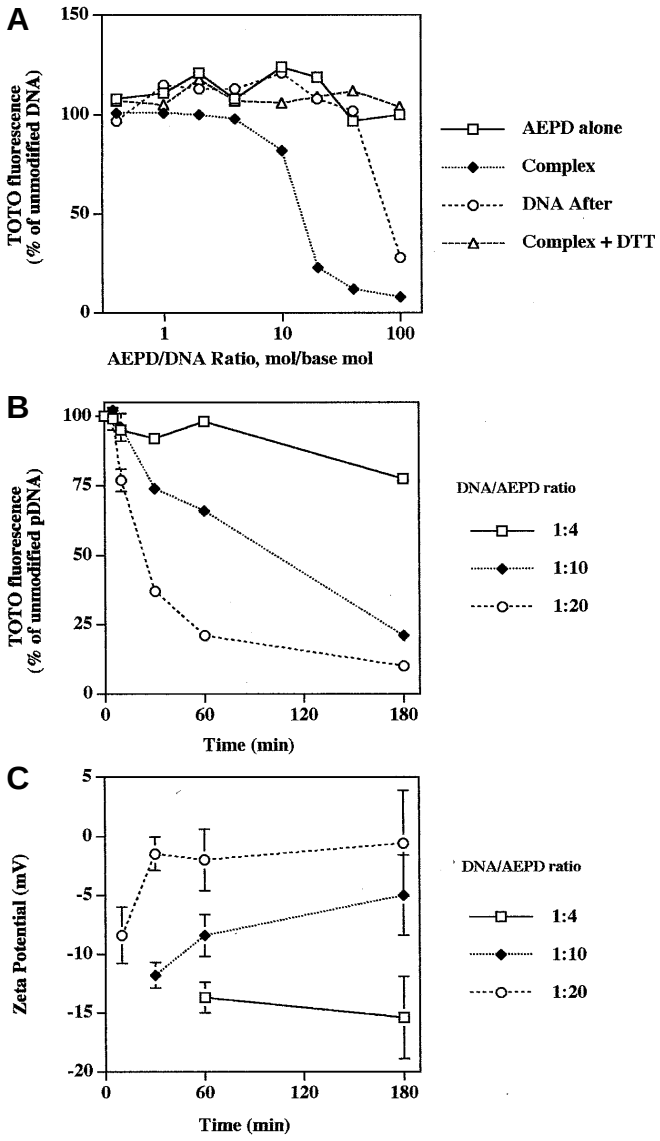

Figure 5. Condensation of the pDNA into particles during AEPD/DTBP template step polymerization. (**A**) The relationship between the AEPD:DNA base molar ratio and TOTO protection when the DNA was added before or after a 1 h AEPD and DTBP reaction (APED/DTBP 1:1 molar ratio). (**B**) The relationship between TOTO protection and the AEPD:DNA base molar ratio over time. (**C**) The relationship between the ζ-potential and the AEPD:DNA base molar ratio over time. pDNA concentration was 20 µg/ml in 20 mM HEPES, 1 mM EDTA, pH 8.5. The values in (B) and (C) represent the mean of three independent trials. T bars indicate their standard deviations.

20 mM glycine (monofunctional amine) arrested polymerization at 5 min and substantially attenuated the decrease in TOTO signal (data not shown).

Under all the above conditions, the decreases in TOTO signal under the above conditions were accompanied by increases in the scattered light intensity (data not shown). This suggested that the condensation of the DNA was accompanied by aggregation.

Particle formation

Dynamic light scattering was employed to further explore the relationship between the molar ratio of AEPD:DNA base and the

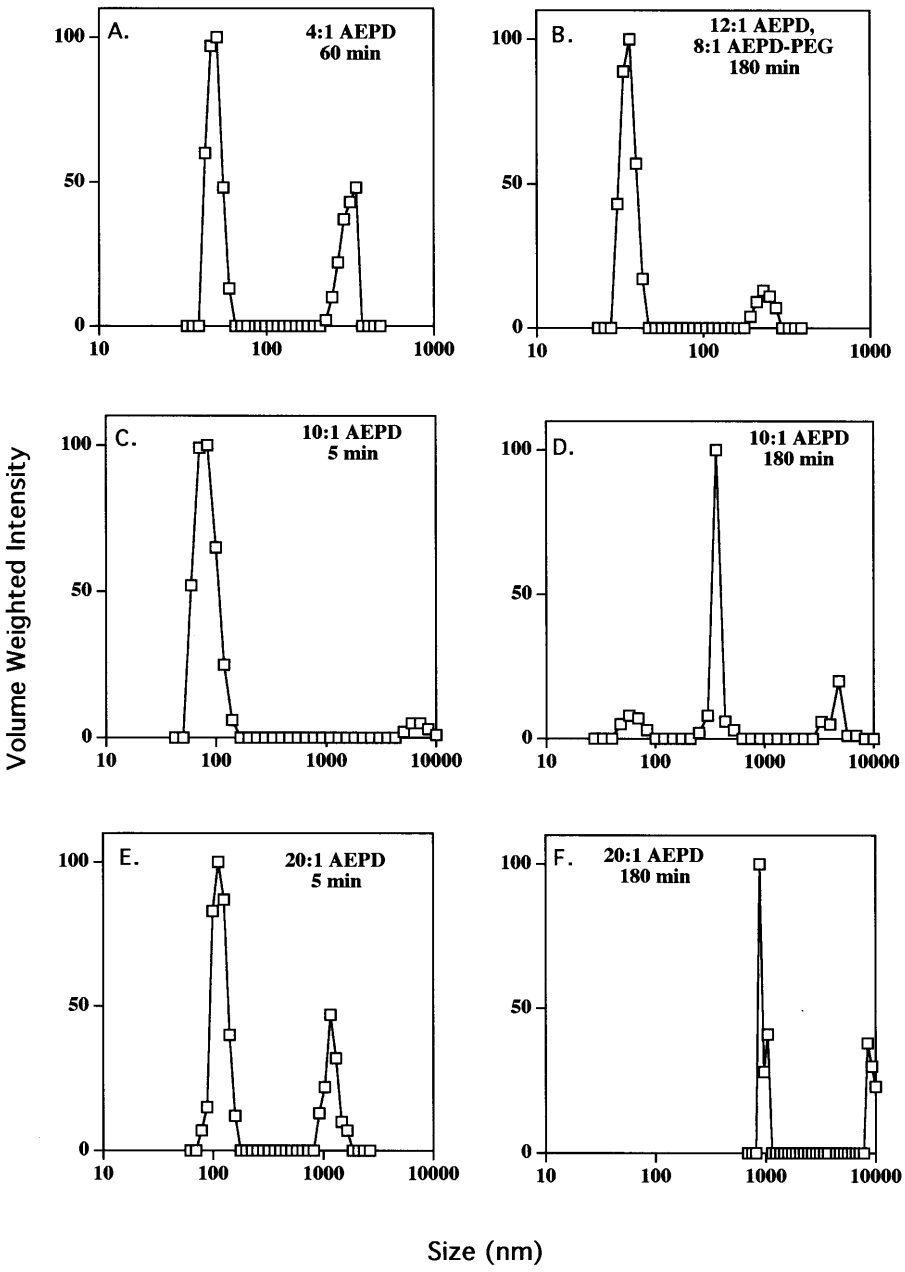

Figure 6. Particle size distribution at various times after template polymerization with AEPD or AEPD and AEPD-PEG using the DTBP crosslinker. Reaction conditions as in Figure 5. Ratios of the monomer to nucleotide base are indicated in the upper right-hand corners.

kinetics of particle formation (Fig. 6). The time course of particle formation paralleled that of TOTO protection. At a 20:1 AEPD:DNA base ratio, particles <150 nm formed by 5 min but then quickly aggregated into particles >150 nm (Fig. 6E and F). At a lower 10:1 AEPD:DNA base ratio particles <150 nm also formed by 5 min but then more slowly aggregated into particles >150 nm (Fig. 6C and D). At a 4:1 AEPD:DNA ratio, no particles formed until 60 min (Fig. 6A). Inclusion of the AEPD-PEG monomer into the reaction mixture (8:1 ratio for AEPD-PEG and 12:1 ratio of AEPD) prevented aggregation and enabled ~40 nm particles to persist (Fig. 6B) for at least 180 min.

The effect of the polymerization process over time on the ζ-potential of the particles were also studied (Fig. 5C). At all the ratios employed the particles remained negative. At a 20:1 AEPD:DNA base ratio the ζ-potential quickly approached 0 mV by 5 min while at a lower 10:1 AEPD:DNA base ratio the ζ-potential became more than –5 mV only after 180 min. At a 4:1 AEPD:DNA ratio, the ζ-potential remained at about –15 mV. The ζ-potential of the AEPD-PEG/AEPD/DTBP reaction mixture was found to be –0.6 ± 0.9 mV after 180 min.

Transmission electron microscopy revealed that rod-shaped particles were the predominant morphology for AEPD-based

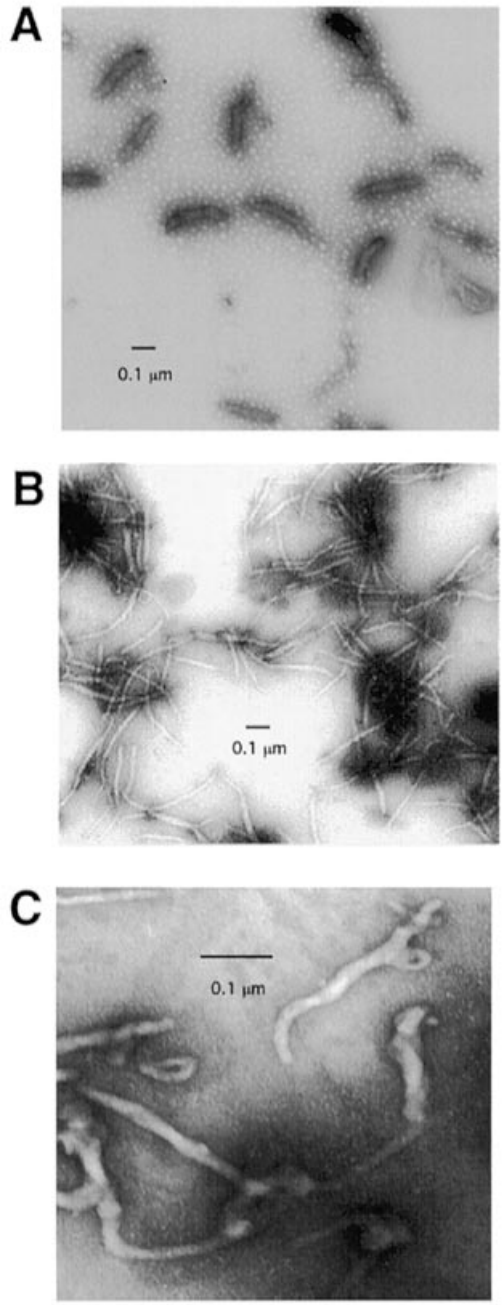

Figure 7. Transmission electron microscopy of pDNA particles prepared via AEPD polymerization with DSP (**A**), DTBP (**B**) and PEG-AEPD polymerization with DTBP (**C**).

polymerization reactions involving AEPD/DSP, AEPD/DTBP and AEPD-PEG/AEPD/DTBP (Fig. 7). While rods were observed in all three samples, their morphology was different. AEPD/DSP samples had small rods, AEPD/DTBP samples had longer and thinner rods, and AEPD-PEG/AEPD/DTBP samples had 'worm-like' structures. A limited number of toroids could be found in all three samples.

DISCUSSION

This study demonstrates that a variety of monomers can be used for either chain or step template polymerization on DNA. AEPD or peptide monomers were used for step polymerization while CDiA served as an example for chain polymerization. One criterion for template polymerization is that the template increases the size and yield of the nascent polymers (16). Polyacrylamide gel electrophoresis indicated that DNA catalyzed the formation of polymers (Fig. 3). Another criterion for template polymerization is that the daughter polymer forms an interpolymer complex (17). The formed polymers bound the DNA sufficiently to either retard its electrophoretic mobility (Fig. 2) or prevent its interaction with the intercalator dye TOTO (Fig. 5A). When the DNA was present during polymerization it was compacted into particles (Figs 6 and 7) but if the DNA was added after the reaction, particles did not form (data not shown). This observation provides support for the template dependence of polymerization. In addition, unpolymerized AEPD cannot condense DNA even at very high monomer/base ratios (up to 1:100). This indicates that polymerization-induced condensation is not simply a result of a high local concentration of cationic monomers interacting with the DNA (Fig. 5A).

The process of DNA compaction and condensation into particles was studied in greater detail using the step polymerization of AEPD. DNA condensation occurs when >90% of the phosphate's negative charges are neutralized by a counter ion (4). With template polymerization this condensation can be modeled as occurring in three phases. The time of these phases was inversely proportional to the monomer concentration (Figs 5B and 6).

During the initial phase of polymerization, <90% of the DNA's negative charge was neutralized, and electron microscopic analysis revealed a particle with a centralized globular core with a halo of DNA strands (data not shown). The negative ζ-potential of these particles is consistent with the presence of non-condensed DNA possibly organized as extended loops, as has been recently demonstrated (18).

In the second phase, the DNA was almost completely condensed and the ζ-potential was greater than –10 mV (Figs 5 and 6). With a 1:20 molar ratio of DNA base:AEPD monomer, this transition occurred at ~5 min, while at a 1:10 ratio it occurred between 30 and 60 min. The addition of glycine stopped this transition, indicating that it prevented further polymerization. Electron microscopy indicated that the particles formed during this phase were toroids and rods which are similar to the condensed DNA particles obtained by the addition of a charge excess of pre-formed polycations to DNA (5). Dynamic light scattering analysis indicated that the majority of the particles were relatively small (<150 nm) and non-aggregated (Fig. 6). Electron microscopy studies indicated that the length of the rod-shaped particles is >150 nm. This apparent discrepancy with dynamic light scattering data can be explained by the fact that the correlation procedure from the latter method calculates size assuming a spherical shape of the particles (19).

In the third phase, electron microscopy and particle sizing indicated that the particles formed aggregates (Fig. 6). The ζ-potential of the particles remained near neutral (Fig. 5C) which explains the aggregation according to colloid stabilization theory (20). A positive ζ-potential from an excess of cations would have charge stabilized the particles and prevented the aggregation. This type of charge stabilized particle occurs when a charge

excess of polycation is mixed in low salt aqueous solutions with DNA (5). The neutralization and condensation of the DNA during template polymerization most likely inhibited further template dependent polymerization by preventing interaction of monomer with the DNA and growing polymer chain. As a result at the advanced stages of the template polymerization, extensive aggregation of charge neutralized DNA particles occurs as the stabilizing excess of free DNA (negative charge) is neutralized by the growing chains of polymeric counterion and an excess of polymeric cationic charge cannot be generated. Thus template polymerization facilitates the generation of individual uncharged particles (ζ-potential close to 0 mV) but another mechanism of condensed DNA particle stabilization is required to maintain them in solution (e.g. such as steric stabilization by PEG chains attached to one of the monomers).

Inclusion of the AEPD-PEG comonomer in the AEPD template polymerization did enable the formation of non-aggregating DNA particles (Fig. 6). Incorporation of the PEG into the polymer sterically stabilized the DNA particles even though the ζ-potential was near zero, thus providing evidence that template polymerization can be used to form non-aggregating, uncharged particles. The inclusion of AEPD-PEG caused the particles to form 'worm'-like structures that have previously been observed with DNA complexes formed from block co-polymers of polylysine and PEG (15,21).

Previous efforts using template polymerization have involved the formation of polyelectrolyte complexes such as the polymerization of N-vinylimidazole along poly(methylacrylic acid) (7). Concerning DNA, Kosturko *et al.* used DNA as a template for the polymerization of acrylamide derivatives containing intercalators (22). Another study initiated aziridine polymerization in the presence of polyadenylic acid from a 3′ amino group on an oligothymidine (23).

An important question for DNA template polymerization was whether the template DNA remained biologically active. The chemical processes that enable polymerization could have also chemically modified the DNA rendering it biologically inert. Although Kosturko *et al.* used DNA as a template for polymerization, the same DNA molecules that served as a template were not assessed for biological activity (22). In the Kolb and Orgel study, the aziridine polymerization chemically modified the nucleotide bases (23). In the current study, the pDNA present during template step polymerization was able to express the reporter enzyme luciferase demonstrating a critical test for the biological activity of the template DNA. DNA chemical modification inhibits expression presumably by blocking transcription (24).

The DNA complexes formed by template polymerization were also able to deliver the DNA into cells in culture (Fig. 4B). DNA template polymerization will be a very powerful approach for developing virus-like particles that can be used for gene therapy. Besides DNA delivery, it could also be used for the preparation of materials that will be of use for polynucleic acid purification and diagnostics. The use of a mix of comonomers provides a rich combinatorial approach to prepare and select for materials that can serve these functions. It also enables the production of polymers that would be difficult to interact correctly or at all with DNA unless they were formed *in situ* in the presence of DNA.

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