DNA Block Copolymers

DOI: 10.1002/anie.200703466

Enzymatic Control of the Size of DNA Block Copolymer Nanoparticles**

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Chemists have been extremely creative in finding strategies for the preparation of organic nanoparticles. Many of these routes take advantage of polymers, including the preparation of polymer dispersions[1–3] and dendrimers,[4,5] and the aggregation of block polymers.[6,7] One interesting new example of the latter class of materials consists of linear amphiphilic DNA block copolymers (DBCs), which, induced by microphase separation, form nanoparticles with a shell of DNA and a core of the hydrophobic polymer.[8–10] Such DBC micelles have been used for antisense oligonucleotide or drug delivery,[11,12] for the assembly of thermoreversible organic/inorganic networks,[13] and as programmable nanoreactors for a variety of chemical transformations.[14] Herein we demonstrate that the size of these DBC nanoparticles can be precisely controlled by an enzymatic reaction of a non-template-dependent DNA polymerase. By varying the incubation time of spherical DBC micelles with the enzyme, the size of the nanoobjects can be adjusted with diameters of 10 to 23 nm.

Terminal deoxynucleotidyl transferase (TdT) is a template-independent DNA polymerase responsible for the generation of the random genetic information that is essential for the effective function of the vertebrate adaptive immune system.[15] The physiological role of TdT is to catalyze the addition of deoxynucleotidyl triphosphates (dNTPs) onto the 3′ hydroxy terminus of single-stranded DNA. To achieve polymerase activity, TdT requires a primer sequence at least as large as a trinucleotide, and a free 3′-OH group.[16] TdT has been used exclusively for the extension of pristine DNA[17,18] and has never been applied to DNA hybrid materials. To the best of our knowledge, controlling the size of nanoparticles with a DNA polymerase has not been explored to date.

We hypothesized that the nanoparticles formed by amphiphilic DBCs could serve as a substrate for TdT, and their size could be extended with the help of the enzyme. The block copolymers were synthesized in a fully automated fashion using a DNA synthesizer. They possess a free 3′-OH group, as the organic polymer unit is attached to the 5′ end.[19,20] The DNA-*b*-polypropylene oxide (PPO) contained a nucleic acid unit consisting of 22 nucleotides (sequence: 5′-CCTCGCTCTGCTAATCCTGTTA-3′, $M = 6670\ \mathrm{g\,mol^{-1}}$) and a synthetic polymer block with $M_w = 6800\ \mathrm{g\,mol^{-1}}$.[14] This amphiphilic block copolymer is known to form spherical micelles in aqueous solution.[9] These nanoparticles were incubated with TdT at 37 °C in the presence of Co^{2+} and dTTP (see Supporting Information for experimental details). At certain time intervals (15, 30, 60, 180, 300, 960 min), the reaction was stopped and the growth of the nanoparticles was analyzed. The increase of micelle size was assessed by three independent techniques: a) scanning force microscopy (SFM), b) fluorescence correlation spectroscopy (FCS), and c) polyacrylamide gel electrophoresis (PAGE).

To investigate how the enzymatic extension of the nucleic acid segment influences the structural features of the micelles, the resulting particles were visualized in the reaction buffer solution on a mica surface by SFM in soft tapping mode. SFM analysis before incubation with TdT revealed spherical nanoparticles (Figure 1 A). The heights of the micelles were plotted in a histogram revealing a maximum height h_{max} of (4.9 ± 1.2) nm (Figure 1 B). Figure 1 C and E show the SFM topography images of the micelles after reaction times of 60 min and 16 h, respectively. Histograms of the micelle heights were compiled for at least 100 nanoobjects, resulting in h_{max} of (6.6 ± 1.4) and (11.2 ± 1.9) nm, respectively (Figure 1 D and F). As summarized in Table 1, the height of the nanoparticles increased with reaction time. The SFM measurements show that the spherical shape of the nanoparticle does not change upon nucleotide addition through TdT. Although SFM has proven to be a powerful tool for imaging amphiphilic DBC aggregates,[9,11,13,14] it does not allow a determination of the exact dimensions of the nanoobjects. Therefore it required to examine the size of the micelles in solution by means of FCS.

FCS is an ultrasensitive analysis method introduced in the early 1970s to study chemical kinetics at very dilute concentrations in biological systems.[21] The technique has developed into a powerful tool in analytical chemistry and biological research. For instance, DNA hybridization events have been detected at the single-molecule level.[22] Furthermore, FCS

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[**] This work, as part of the European Science Foundation EUROCORES Program BIONICS, was supported by funds from the DFG and the EC Sixth Framework Programme. Additional funding was provided through the ERA-CHEMISTRY program and the DFG-Sonderforschungsbereich 625 "From Single Molecules to Nanostructured Materials". The authors would like to thank Daphne Diemer and Andrew Musser for their help.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

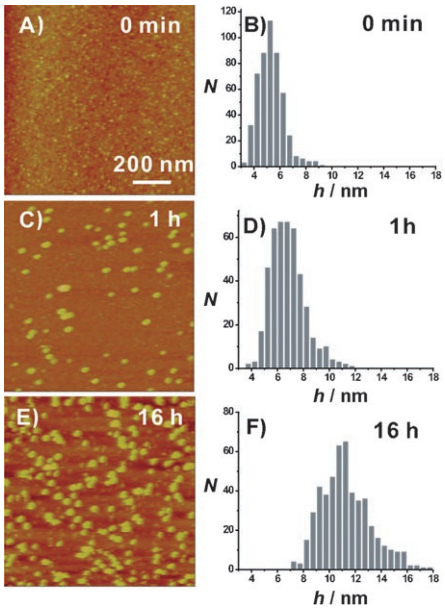

Figure 1. SFM analysis of DNA block copolymer nanoparticles that were extended by adding thymidines using the TdT enzyme. A, C, and E show the SFM topographical images at different reaction times, and presented at the same scale. B, D, and F illustrate the corresponding histograms of the nanostructure heights *h*.

Table 1: Characterization of DNA block copolymer micelles that were enlarged by TDT reaction.

t [min]	Diameter[a] [nm]	h_{max}[b] [nm]	T segments added[c]
0	9.6 ± 0.9	4.9 ± 1.1	–
15	9.9 ± 1.1	5.1 ± 1.4	6 ± 4
30	10.8 ± 1.6	5.2 ± 1.3	11 ± 3
60	12.4 ± 0.8	6.6 ± 1.4	22 ± 5
180	13.7 ± 1.3	7.2 ± 1.5	35 ± 8
300	17.5 ± 1.4	8.3 ± 1.6	43 ± 7
960	23.0 ± 0.8	11.2 ± 1.9	62 ± 11

[a] Based on FCS analysis, [b] derived from SFM measurements, and [c] determined by PAGE.

has been employed to detect the mobility of proteins and DNA- or RNA-fragments within cytosol or other cell organelles. FCS can be used to monitor the change of DBC micelle shape from spherical into rod-like aggregates.[9] The transit times of the freely diffusing fluorescent micelles through the excitation volume of 4.5 fL were measured in buffer solution by using a confocal microscope setup.[23] The translational diffusion coefficients were calculated from the mean diffusion times. As the diffusion coefficient D is related to the frictional coefficient f of the hydrated micelles, the shape information of the immobilized DBC aggregates could be used to calculate the radius r_0 of the spherical micelles from the FCS diffusion data (see the Supporting Information). Prior to TDT reaction, the DBC micelles were labeled with the complementary sequence that was functionalized with Alexa488 (Invitrogen, USA) at the 5′ end by hybridization. 1% of the DNA-*b*-PPOs were tagged with the chromophore so that the predominant form of DNA within the corona remained single stranded. In the presence of TdT and dTTP, the enzymatic extension of the single stranded DNA chains resulted in an increase in hydrodynamic radius of the micelles, as calculated from the diffusion times. After 16 h of reaction with TdT, the FCS curve showed a 2.6-fold increase in the mean diffusion time τ_D relative to unreacted micelles. However, for a detailed analysis of the autocorrelation functions, a fit for two diffusing components had to be used.[24,25] The longer diffusion times of the labeled micelles varied from $\tau_{D2} \approx 280$ μs for a TDTase reaction time of 15 minutes up to $\tau_{D2} \approx 650$ μs for a 16-hour reaction time. The short diffusion time component with $\tau_{D1} \approx (60 \pm 12)$ μs was similar to the diffusion time of the reference dye rhodamine 110 ($\tau_D = (43 \pm 1)$ μs) and most likely originated from fluorescent impurities. Mean diameters of (9.9 ± 1.1), (10.8 ± 0.6), and (12.4 ± 0.8) nm were found for the DBC micelles after 15, 30, and 60 min of incubation with TDT, respectively. With increasing reaction time, the micelles enlarged further to diameters of (13.7 ± 1.3) nm (3 h) and (17.5 ± 1.4) nm (5 h). Extension with TdT for 16 h resulted in a micelle diameter of (23.0 ± 0.8) nm (Figure 2A and Table 1). These values are in good agreement with trends established from the AFM in the growth of the micelles (Figure 2B). It can be concluded from the FCS data that the micelles are flattened by immobilization arising from interaction with the surface and/or the SFM imaging process.

To determine the number of nucleotides added with increasing enzyme incubation times, DBCs extended with thymidine (T) residues were analyzed by PAGE (Figure 3).

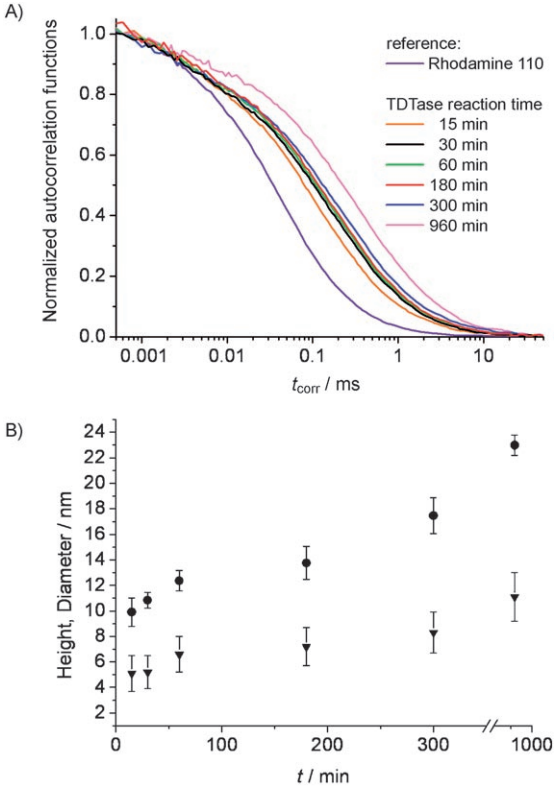

Figure 2. A) The autocorrelation functions of the DBC nanoparticles after different incubation times with the TDT enzyme. B) The diameters obtained by FCS of the nanoparticles (●) and the mean maximum heights (▼) of the micelles obtained from SFM.

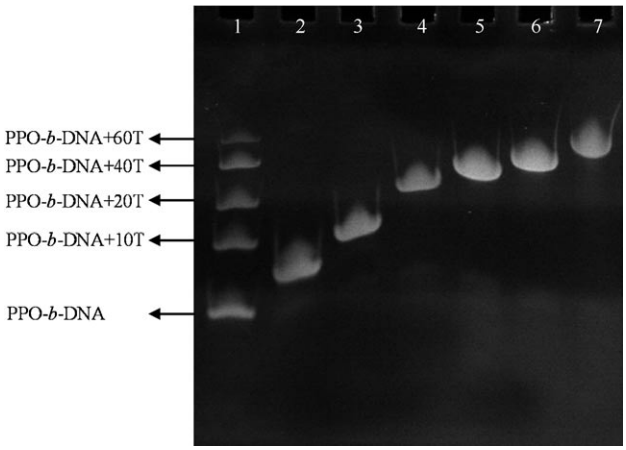

Figure 3. Gel electrophoretic analysis of the DBCs after different reaction times with TdT. Lanes are as follows: DNA-*b*-PPO molecular weight standards (1), and the products of incubation with TdT for 15 min (2), 30 min (3), 60 min (4), 3 h (5), 5 h (6), and 16 h (7).

To achieve the most accurate assessment, this required the synthesis of DBC molecular weight standards. A variety of DNA-*b*-PPO block copolymers were prepared consisting of the 22-mer sequence and additional thymidine segments of variable length attached at the 3′ end (0, 10, 20, 40 and 60 T residues). These molecular weight markers were synthesized as described previously.[14] From the gel electrophoretic analysis it can be concluded that incubation with TDT for 15, 30, 60, 180, 300, and 960 min resulted in the addition of 6 ± 4, 11 ± 3, 22 ± 5, 35 ± 8, 43 ± 7, and 62 ± 11 T residues, respectively. Furthermore, this comparison allowed a correlation of the size of the micelles with the increase in length of the nucleic acid segments of the DBCs achieved by the TdT enzyme (Table 1). For instance, upon addition of on average 62 T residues to the DNA micelle corona, the diameter of the aggregates increased by 13.4 nm.

The synthesis of linear block copolymers by combining conventional polymerization techniques with enzymatic polymerizations has been demonstrated.[26–28] One block was generated by atom-transfer radical polymerization, and the other block was synthesized by lipases, producing polyester segments. These block copolymers were characterized regarding their chemical composition but a further analysis of the aggregation behavior of these materials was not undertaken. Usually the size of the resultant micelles is determined by the lengths of the constituent polymer blocks. In the work presented herein, the post-synthetic extension of block copolymer aggregates was achieved. This method offers easy control over the growth of nanoparticles by employing a template-independent DNA polymerase under mild isothermal conditions in an aqueous medium. It was possible to significantly increase the micelle size by a factor of up to 2.4. Further studies will focus on the use of TdT on other nanoparticle systems such as DNA nanoparticles with an inorganic core.

Received: July 31, 2007
Published online: December 20, 2007

Keywords: bionanotechnology · block copolymers · DNA · enzymes · nanostructures

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