Activity of site-specific endonucleases on complexes of plasmid DNA with multiwalled carbon nanotubes

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Abstract. We have synthesized and investigated structural and functional properties of plasmid DNA complexes with multi-walled carbon nanotubes (MWCNTs) for detection of changes in structural state of the plasmid DNA at its recognition by site-specific endonuclease. It has been also established that the site-specific endonuclease is functionally active on the surface of MWCNTs.

1. Introduction

Study of nuclease activity is important for understanding reasons of variability of deoxyribonucleic-acid (DNA) structure. Living cells utilize cutting and cleavage of DNA molecules by DNA-hydrolyzing enzymes: DNase, endonuclease, and other various nucleases to recognize and restrict genetic infection. Nuclease activity is proposed to use for revealing, for example, genes with single nucleotide polymorphisms through formation of a three-dimensional structure due to perfect hybridization of DNA with oligonucleotides and then subsequent cutting of the three-dimensional structure by nucleases [1]. Also, development of express methods of estimation of endonuclease activity is an actual biomedical problem of early diagnosis of cardiovascular disease [2]. For its solution the use of bionanosensors is promising [3]. However, the development of nanosensors for biochemical markers is constrained due to low sensitivity of nuclease activity detection. One solution to this problem is the use of multi-walled carbon nanotubes (MWCNTs), possessing unique electrophysical properties and biocompatibility [4]. Therefore, carbon nanotube-based sensorics of structural changes in DNA under the action of nucleases is perspective to diagnose and monitor diseases [5-7]. In connection with this, development of DNA-nanosensors based on complexes of DNA/carbon nanotube (DNA/CNT), which are designed for express DNA-diagnosis by changing of the structural state of nucleic acids under the action of nucleases, is relevant.

In this paper, to explore nuclease activity on a surface of MWCNTs the simplest model system is used, namely the site-specific restriction endonuclease (restrictase) – plasmid. In such a system, restrictase cleaves small covalently-closed circular double-stranded DNA molecules (plasmids) that coexist with a chromosome of the host bacterium.

A goal of this work is the development, synthesis and investigation of structural and functional properties of DNA/MWCNT complexes for detection of changes in structural state of plasmid DNA at its recognition by site-specific nucleases.

2. Materials and Methods

MWCNTs with diameters ranging from 2.5 to 7 nm and length of ~5–10 μm were obtained by the method of chemical vapor deposition (CVD-method) [8]. MWCNTs were covalently modified by carboxyl groups.

As a model plasmid DNA, the pBR322 plasmid (Fermentas, Lithuania) has been used, the DNA of which is characterized by homogeneity and existence of different structural forms. Plasmid pBR322 represents itself a covalently closed circular DNA molecule of 4363 bp in length. The pBR322 plasmid has several unique restriction sites indicated on the genetic map. If supercoiled pBR322 plasmid DNA was treated with a restriction enzyme which cleaves it at a single site located in one of genes for resistant to one or another antibiotics, then depending on time of restriction transition from the supercoiled to circular and then to linear double-stranded DNA takes place. Linearization (restriction) of plasmid DNA has been carried out by site-specific endonuclease EcoRI (Thermo Fisher Scientific, Germany).

2.5 μg of plasmid DNA pBR322 has been hydrolyzed by site-specific restriction endonuclease EcoRI (3 activity units) during appropriate time interval at 37 °C in 50 μl of Tango buffer, containing 33 mM Tris-HCl pH 7.9; 10 mM $MgCl_2$; 66 mM NaCl; 0.1 mg/ml BSA. One unit of activity has been defined as the amount of enzyme required to hydrolyze 1 μg of plasmid DNA in one hour at 37 °C in a 50 μl reaction mixture containing 1 × Tango buffer. At appropriate intervals, aliquots have been taken from the reaction mixture and the reaction was stopped by addition of EDTA into reaction mixture to a final concentration of 2 mM at the temperature 2 °C. Restriction products were analyzed by electrophoresis in agarose gel. Enzyme activity was estimated by electron microscopic analysis and electrophoresis of complexes plasmid DNA/MWCNT with and without the endonuclease in agarose gel.

DNA gel-electrophoresis was performed in 1% gel in 0.5 × TBE buffer 0.09 M (pH 8.0) at a voltage of 75 V and a temperature of 10-12 °C for 4 hours. As the DNA molecular weight marker, the "DNA molecular weight marker, M50Kb" (Praymteh, Belarus) has been used. The gel was stained in ethidium bromide solution (0.5 μg/ml) for 7 minutes, then washed three times in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) with subsequent review and documentation of gels on the system ImageQuant 300 (GE Healthcare, USA).

Transmission electron microscopic (TEM) images were obtained by means of transmission electron microscope JEM-100CX (JEOL, Japan) at accelerating voltage of 100 kV. After the objects were previously deposited on a copper grid with a formvar polymer coating, structural analysis has been performed.

3. Structural Analysis

Let us denote an open circular DNA with cleavage in one of two strands and, respectively, with one sticky end by ocDNA. A supercoiled DNA, representing itself a closed double chain, is denoted by cDNA. The linear double-stranded helical DNA and single-stranded DNA are denoted by dsDNA and ssDNA, respectively.

TEM-image of a native plasmid DNA is shown in figure 1a. A supercoiled cDNA in MWCNT-surroundings is shown in figure 1b. As one can see, cDNA molecules do not form a complex with MWCNTs. Single-stranded ssDNA, obtained by dsDNA denaturation, forms a complex ssDNA/MWCNT, shown in figure 1c. Adhesion of ssDNA nucleotides on CNT surface proceeds due to π-π stacking interactions. Denaturated dsDNA molecules link closely situated MWCNTs in figure 1c. Figure 1c demonstrates also a helicoidal part of denaturated dsDNA, which links two CNTs. At that, unwound ends of this dsDNA are fixed on the MWCNT surface, as one of arrows in figure 1c indicates. ocDNA, which has only one closed chain, can adhere by sticky end to MWCNT, as figure 1d demonstrates.

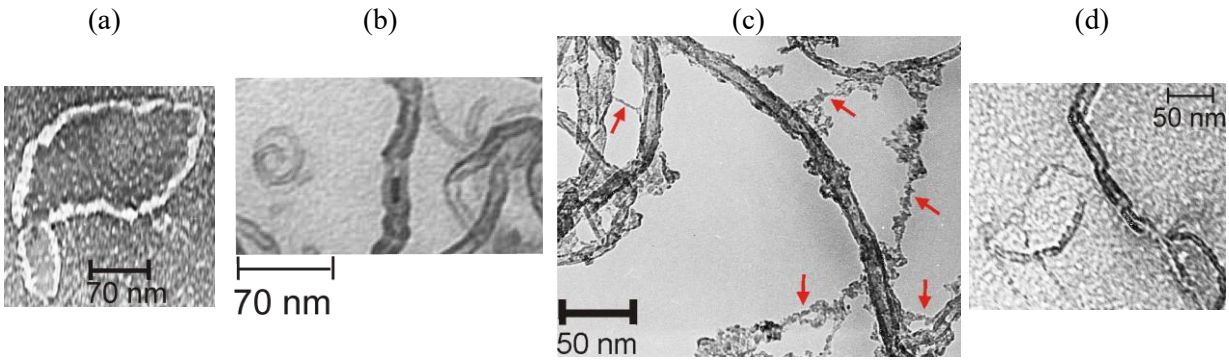

Figure 1. TEM-images of plasmid DNA molecules with different structure: (a) a native plasmid DNA; (b) a supercoiled cDNA in MWCNT-surroundings; (c) complexes ssDNA/MWCNT, arrows indicate denaturated dsDNA molecules linking closely situated MWCNTs; (d) open circular ocDNA adhered to MWCNT.

4. Electrophoretic Analysis

Electrophoresis in figure 2 allows to estimate time for which the restrictase EcoRI cleaves completely rings in closed double chain of plasmid DNA. This time is about of 1 hour. We observe that EcoRI cleaves the chemical bonds just in the ring as the concentration of linear dsDNA increases uniformly in time. The intensity of luminescence of dye-labeled cDNA and, respectively, its amount decreases uniformly in time. The intensity of luminescence of dye-labelled ocDNA and, respectively, its amount initially increases with time t, reaches a maximum at t = 15 min, and then decreases to near zero at t = 60 min. Therefore, we can assume that the strands of native plasmid DNA are cleaved one by one.

Electrophoretic lanes 3-6 in figure 3 show that the localization site of the native plasmid cDNA is narrowed in mixture of DNA with MWCNT. Since the intensity of this dye-labelled DNA from the mixture is practically independent on MWCNT concentration, it can be assumed that carbon nanotubes appear to orient DNA molecules as their localization site in lanes 4-6 is narrowed compared to lane 3. Interaction of MWCNT with the plasmid DNA affected by restrictases for 40 minutes results in narrowing of the lines and sharp decrease in luminosity of ocDNA localization cites in lane 8 in comparison with lane 7 for ethidium bromide stained gel electrophoresis in figure 3. π-π stacking interaction of ocDNA sticky end with MWCNT should lead to fixing whole dye-labelled ocDNA on the surface of the nanotube with subsequent fluorescence quenching. Decreasing the intensity of luminescence of dye-labelled ocDNA in lane 8 in comparison with lane 7 for electrophoresis in figure 3 confirms this assumption.

The electrophoresis lanes 3-5 in figure 4 show mobility of DNA subjected to restrictase digestion and then treated with increased amounts of MWCNTs. According to these lanes, the probability of binding with MWCNT increases not only for ocDNA, but for the cDNA and dsDNA as well, since the phenomenon of their fluorescence quenching by MWCNTs is observed. dsDNA has sticky ends that bind it to MWCNT. This explains the fluorescence quenching of dye-labelled dsDNA MWCNTs. The fluorescence of dye-labelled cDNA, not cleaved with a restrictase after incubation, is quenched by MWCNTs. It allows to suggest changes in the structure of supercoiled DNA due to its local destabilization by the enzyme. This destabilization facilitates the exit of the nucleotide bases and, respectively, leads to the formation of a complex between the cDNA and MWCNT. To prove the latter assumption, a short-term thermal destabilization of the native plasmid DNA, which was previously subjected to restrictase digestion, has been carried out.

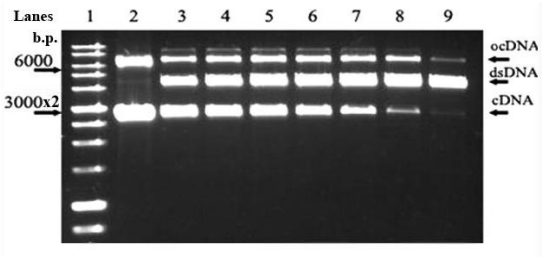

Figure 2. Electrophoregram of the plasmid-DNA hydrolysis with site-specific endonuclease EcoRI for different restriction time. Lanes: 1 – M50kb marker; 2 – native plasmid DNA (control); 3-9 – mixture of ocDNA, cDNA, and dsDNA, the restriction time equals to 5, 10, 15, 20, 30, 40, and 60 minutes, respectively.

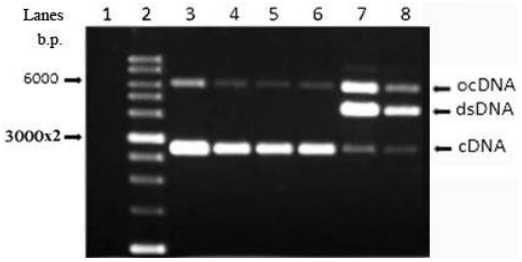

Figure 3. Electrophoregram of mixture from MWCNTs and native plasmid DNA. Lanes: 1 – MWCNTs; 2 - M50kb marker; 3 - native plasmid DNA (control 1); 4-6 – cDNA + MWCNT for nanotube concentration C_{MWCNT} 50, 100, and 200 μg/ml, respectively; 7 – mixture of cDNA, ocDNA, and dsDNA, the restriction time is 40 min (control 2); 8 – mixture of cDNA, ocDNA, and dsDNA+ MWCNTs , C_{MWCNT} =50 μg/ml.

Lanes 6-8 of electrophoresis in figure 4 gives information about the adsorption ability of thermally destabilized mixture of plasmid DNA with the endonuclease EcoRI on the surface of MWCNT. Comparison of lane 6 with lanes 7 and 8 in figure 4 gives the following. Denatured (single-stranded) DNA molecules in the free state have been not detected in the mixture of fermented DNA + MWCNTs already at concentration MWCNT C_{MWCNT} equal to 50 μg/ml (lane 7, figure 4). Thermally destabilized ocDNA is practically all linked at C_{MWCNT} = 100 μg/ml (lane 8, figure 4). Similarly, since the emission intensity I_{cDNA} for the free cDNA (lane 8, figure 4) at C_{MWCNT} = 100 μg/ml is significantly less I_{cDNA} (lane 7, figure 4) at C_{MWCNT} = 50 μg / ml, the thermal destabilization of cDNA leads to increase in the probability of the cDNA/MWCNT complex formation. Comparison of lanes 4 and 5 with lanes 7 and 8 in figure 4 for dsDNA shows that the thermal destabilization does not practically change the probability of dsDNA/MWCNT complex formation. Therefore, the electrophoretic analysis shows that the lifetime of thermally destabilized state of dsDNA is much smaller than for ocDNA and cDNA due restrictase linked to the latter.

Thus, the electrophoretic analysis shows, on the one hand, a large value of the formation probability for complex of carbon nanotubes with denatured DNA compared to other types of DNA-structures and, on the other hand, thermal enhancement of restrictase destabilization of cDNA and ocDNA.

Further, we examine the effect of MWCNTs on enzymatic activity of site-specific endonucleases and show that dsDNA is formed by transition of double destabilized (unwound) chain of dsDNA-molecule in a low-energy state of the double helix. According to comparison of lanes 7-9 in figure 2 with 3-4 in figure 5, restriction-nuclease concentration increase from 3 to 6 activity units is equivalent to increasing of restrictase-digestion time from 30 to 60 minutes. Since the fluorescence intensity for free dye-labelled cDNA from the mixture of cDNA + MWCNTs subjected to restrictase digestion (lane 8, figure 5) is decreased compared to the control (lane 5, figure 5), the restrictase functionality is retained in the mixture of cDNA + MWCNTs. Electrophoresis lanes 5-7 and 8-10 in figure 5 show a dependence of motilities on amounts of MWCNTs for complexes of cDNA with MWCNT and for complexes between MWCNT and cDNA subjected to restrictase digestion, respectively.

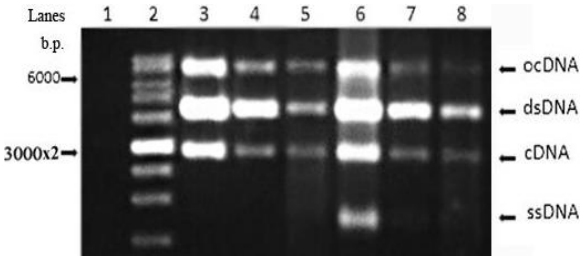

Figure 4. Electrophoregram of complexes ssDNA/MWCNT. Lanes: 1 – MWCNTs; 2 – M50kb marker; 3 – mixture of cDNA, ocDNA, and dsDNA, the restriction time is 30 min (control 1); 4 and 5 – mixture of cDNA, ocDNA, and dsDNA + MWCNT, C_{MWCNT} = 50, 100 μg/ml (control 2); 6 – mixture of sDNA, ocDNA, dsDNA, and ssDNA, the restriction time is 30 min; 7 and 8 – mixture of cDNA, ocDNA, dsDNA, and ssDNA + MWCNT, C_{MWCNT} = 50, 100 μg/ml.

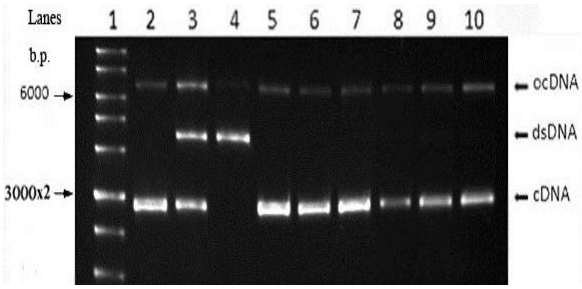

Figure 5. Electrophoregram of mixture from MWCNTs and native plasmid DNA after restriction enzyme treatment. Lanes: 1 – M50kb marker; 2 – native plasmid DNA (control 1); 3, 4 – mixture of cDNA, ocDNA, and dsDNA, restriction-enzyme concentration 3 and 6 activity unit respectively, the restriction time is 30 min (control 2); 5-7 – native plasmid DNA + MWCNT at C_{MWCNT} equal to 50, 100, and 200 μg/ml, respectively; 8-10 – mixture from native plasmid DNA and MWCNTs after treatment with a restriction enzyme at C_{MWCNT} = 50, 100 and 200 μg/ml, respectively; the restriction time is 45 min.

We observe an increase in the emission intensity of free dye-labelled cDNA and ocDNA in the mixture of cDNA + MWCNT subjected to restrictase digestion with an increase of MWCNT concentration (lanes 8-10, figure 5). It can be explained by an inactivation of the restrictases during adhesion to the surface of the nanotubes.

The observed absence of free dsDNA in the mixture of cDNA + MWCNTs which is subjected to restrictase digestion, regardless of MWCNT concentration (lanes 8-10, figure 5) is explained by sufficiently long lifetime of unstable DNA-structure formed immediately after the cleavage by restrictase of the second ringed DNA- chain. This time is sufficient for formation of the complex of destabilized linear dsDNA with MWCNT.

5. Discussion and Conclusions

So, the investigation performed demonstrates that supercoiled DNA molecules do not form a complex with MWCNTs. Endonucleases are functionally active on the surface of MWCNTs. An interaction of plasmid DNA with restrictase on nanotube surface amplifies the dsDNA binding with MWCNT. The changes in the structure of dsDNA are due to its local destabilization by the enzyme and adsorption of "sticky" ends of the dsDNA on the surface of MWCNTs on account of π–π-stacking interaction. However, increasing of MWCNT concentration leads to decrease of luminescence intensity of free open-circular and supercoiled DNA molecules in the gel. It can be explained in the following way. Restrictases change a conformation on the surface of MWCNTs and, therefore, are inactivated. Thus, plasmid DNA in various conformational forms (supercoiled, open circular, double-stranded and single-stranded linear (denatured)) can form thermodynamically stable complexes with MWCNTs in the presence of endonucleases only.

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