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Auto-assembly of nanometer thick, water soluble layers of plasmid DNA complexed with diamines and basic amino acids on graphite: Greatest DNA protection is obtained with arginine

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ABSTRACT

We have investigated the ability of diamines as well as basic amino acids to condense DNA onto highly ordered pyrolytic graphite with minimum damage after re-dissolution in water. Based on a bibliographic survey we briefly summarize DNA binding properties with diamines as compared to basic amino acids. Thus, solutions of DNA complexed with these linkers were drop-cast in order to deposit ultra-thin layers on the surface of HOPG in the absence or presence of Tris buffer. Atomic Force Microscopy analyses showed that, at a fixed ligand-DNA mixing ratio of 16, the mean thickness of the layers can be statistically predicted to lie in the range 0–50 nm with a maximum standard deviation ±6 nm, using a simple linear law depending on the DNA concentration. The morphology of the layers appears to be ligand-dependent. While the layers containing diamines present holes, those formed in the presence of basic amino acids, except for lysine, are much more compact and dense. X-ray Photoelectron Spectroscopy measurements provide compositional information indicating that, compared to the maximum number of DNA sites to which the ligands may bind, the basic amino acids Arg and His are present in large excess. Conservation of the supercoiled topology of the DNA plasmids was studied after recovery of the complex layers in water. Remarkably, arginine has the best protection capabilities whether Tris was present or not in the initial solution.

1. Introduction

Nanometer-scale deposits of plasmid DNA (closed circular DNA double strands) which can be dissolved in water have shown considerable interest in radiobiological experiments aiming at deepening and refining understanding of the fundamental principles of how low-energy electrons, ions or VUV, X-rays and γ-rays interact with DNA [1–7]. In such experimental approaches it is vital to keep DNA plasmids as undamaged as possible (i.e. in their supercoiled topology) before treatment by radiation. Actually, when performing the plasmid relaxation assay, the percentages of supercoiled, open circular and linear forms were quantified; the two latter being the signatures of DNA single and double strand breaks respectively. It should be noted that in most studies which aimed at studying radiation effects at the molecular level, plasmid DNA thin films were used under the form of freeze-dried deposits. Sometimes however, irradiation arrangement was modified to allow humidified DNA [2–4] to be exposed to radiation under atmospheric conditions. Lyophilized plasmid DNA layers unfortunately present the disadvantage of an uneven film surface coverage and thickness [5]. In addition, with the freeze-dried layers, it is impossible to perform experiments under vacuum with so-called “pure DNA”, namely with no stabilizers added to the sample before the evacuation process. Re-dissolution of the DNA plasmids in water is a sine qua non condition if a plasmid relaxation assay is to be subsequently performed. Experimentally, the presence of a chemical buffer to stabilize the plasmid DNA topology therefore appears to be a key requirement [6]. Besides, under biological conditions, nuclear DNA does not occur in free linear strands; quite the opposite, it is highly condensed and wrapped around histones [8]. Recently, we described a way to produce nanometer-scale DNA films of uniform and directly measurable thicknesses on conductive substrates of highly ordered pyrolytic graphite (HOPG) [9]. The technique employed the aliphatic diamine, 1,3-diaminopropane in its doubly protonated form (Dap^{2+}) at neutral pH, to bind plasmid DNA molecules to each other. Diamines and polyamines are indeed actively involved in a considerable number of metabolic events, including DNA replication, protein synthesis, and cell growth [10,11]. Plasmid DNA films structured with Dap^{2+} as linkers have proven excellent capabilities under low energy electron (0–20 eV) impact with the possibility, for the first time, to measure the absolute cross-section [12] for the

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loss of plasmid DNA supercoiled topology at the resonance energy of 10 eV due to dissociative electron attachment (DEA). This was achieved thanks to the uniformity of the layers at the nanometer scale. Moreover, electron stimulated desorption (ESD) analyses [13] of such films have revealed that the presence of the Dap^{2+} molecule in the close vicinity of the phosphate DNA moieties has a profound impact on the ESD yields of anions with notably a quasi-entire suppression of the O^- desorption channel (generally observed when using "pure" or lyophilized DNA) in favor of the appearance of a high OH^- desorption yield. This indicates that the close environment of the DNA macromolecule in more realistic biological conditions should specifically affect the way in which it responds to radiation injury. It thus appears particularly relevant to search for specific (i.e. biogenic) DNA ligands that would make it possible to produce nanometer-scaled plasmid DNA layers with the property of being soluble in water with the only severe damage due to radiation effects.

In order to tightly pack the large DNA macro-anions into the nucleus of a biological cell, life has selected to use molecular tails bearing in particular protonated amino moieties (i.e. amino acids positively charged at neutral pH). In eukaryotic cell chromosomes, the basic amino acids arginine, lysine and histidine play a crucial role in packing DNA into chromatin [14,15]. It should be underlined that in addition to basic amino acids, polyamines are also involved in the mechanism of DNA folding-unfolding in biological cells [16] as well as stabilization of the negative charges of DNA or RNA [17].

In vitro condensation of DNA has been studied both theoretically [18,19] and experimentally [20] and still remains of topical interest, especially when surface-mediated delivery of DNA is foreseen [21–23]. To condense DNA in aqueous media, at least the presence of a salt is required to neutralize DNA charges and decrease DNA-DNA poly-anion repulsion. A more sophisticated and efficient way to condense DNA consists of inducing attractive interactions between the DNA macromolecules by using multivalent cationic charged ligands (multivalent metal ions, inorganic cations, polyamines, protamines, peptides, lipids, liposomes and proteins) [15].

Using the soft adsorption (drop-casting) protocol developed for fabricating Dap-DNA layers deposited on HOPG [12], we studied the ability of aliphatic diamines of ethylene diamine (Eda), 1,4-diaminobutane (putrescine or Put), 1,5-diaminopentane (cadaverine or Cad) as well as basic amino acids histidine (His), arginine (Arg) and lysine (Lys) to condense plasmid DNA into dense layers on the surface of HOPG in the presence but also in the absence of the Tris-EDTA buffer. Atomic Force Microscopy (AFM) will be used to check and characterize the deposits formed on freshly cleaved HOPG with a defined mixing ratio R (R = [Ligand] / [DNA-phosphate]). The relationship thickness (in nanometers) of the layer versus C_{DNA} at a fixed mixing ratio will be established for Dap and for Arg which gave the best deposit qualities (the cases of His, Lys, Eda, Put, Cad nevertheless are also discussed). Special attention will be paid to the integrity of the initial proportion of supercoiled topology of the plasmids over all the different experimental steps using gel electrophoresis measurements, including the last step that consists in recovering the plasmids in water for further analysis. The proportion of ligands (diamines and amino acids) remaining in the layers was then determined by means of X-ray Photoelectron Spectroscopy (XPS). The relationship between the type of ligand binding site and the stability of the complexes, especially maintenance of the supercoiled topology of the DNA plasmids is discussed.

2. Materials and methods

2.1. Chemicals and materials

2.1.1. Preparation of [DNA·ligand] complexes

Ethylene diamine dihydrochloride (≥98%), 1,3-diaminopropane dihydrochloride (≥98%), 1,4-diaminobutane dihydrochloride (≥98%), 1,5-diaminopentane dihydrochloride (≥98%) and protonated basic amino acids of arginine hydrochloride (≥98%), histidine hydrochloride (≥98%), and lysine hydrochloride (≥98%) were all purchased from Sigma-Aldrich (France) and kept at 4 °C. Plasmid DNA (pUC21, 3266 bp length and with a molecular weight of 2.13×10^6 Da) at an initial concentration of 1 mg/mL in water for injection (WFI) was purchased from Plasmid Factory GmbH & Co. KG (Germany). The pUC21 mother solution was free of Tris buffer. Without further purification, stock solutions of this DNA (>95% supercoiled) were prepared by dilution with ultrapure water having a resistivity of 18.2 MΩ cm. The stock solutions were stored at −20 °C. DNA concentrations were determined by measuring the absorbance at 260 nm using the molar extinction coefficient $\varepsilon_{260} = 5.3 \times 10^7\ cm^{-1}\ M^{-1}$. Solutions of plasmid DNA were mixed with an equal volume of a solution containing protonated ligand molecules to attain the desired ratios R = 16 at a final DNA concentration (C_{DNA}) in the range 100–500 ng μL^{-1}. R, the mixing ratio, is defined as the ratio of the molar concentration of the cations to that of anionic phosphate moieties of plasmid DNA in the solution, namely R = [cation]/[PO_4−]. When the Tris buffer had to be present in the solution, the pUC21 plasmid DNA mother solution was first diluted in Tris/EDTA so that the final concentration of Tris is 10 mM Tris-HCl and 1 mM EDTA, i.e. pH = 7.6. Solutions were freshly prepared before each experiment.

2.2. Layer preparation and AFM investigation

The DNA-ligand deposits were prepared by soft adsorption: A droplet of 50 μL of the [DNA·ligand] mixture was deposited onto the surface of freshly cleaved graphite (HOPG, ZYA grade, NT-MDT, NanoAndMore, France) and incubated for 15 min. Next, the surplus solution was removed using filter paper. This protocol ensures that a circular smear of constant diameter 8 ± 1 mm is created on the freshly cleaved HOPG surface (it can be observed by the naked eye). Then, the surface was dried in clean air for 3 min performed.

AFM images were made using a Molecular Imaging scanning probe microscope (Agilent, USA). The topography of the surface was recorded in air, at standard temperature and pressure, with silicon nitride tips coated with aluminum (NanoAndMore, France) at a resonant frequency of 300 kHz in the tapping mode. The way the thickness measurements were performed at the nanometer scale was presented in detail elsewhere [9,13]. Let us briefly recall here that, unlike the situation where the tapping mode is used to observe the layers, when working in the contact mode, the layers can be swept away leaving a tear over the entire scanned field revealing the naked HOPG surface (see Fig. 4, inset). An additional scan in the tapping mode over a greater field makes the thickness of the layer accessible to AFM measurement.

2.3. Instruments and methods

Gel electrophoresis measurements were based on 1% agarose in TAE buffer at 6.7 V cm^{-1} for 7 min and 5.0 V cm^{-1} for 70 min. Both the gel and DNA-ligand mixtures were pre-strained with SYBR® Safe (Life Technologies SAS, France) (1× for gel and 20× for DNA respectively). The equivalent of 100 ng of DNA was loaded per well. Gels were scanned with Bio-Rad Gel DocTM XR (Bio-Rad, France) using blue fluorescence mode at an excitation wavelength of 302 nm. The relative amounts of each form of DNA were obtained from image analysis by Quantity One software (Bio-Rad, France).

XPS spectra were recorded with a PHI Versaprobe 5000 (PHI Sales - France, NanoAndMore) apparatus using monochromatic Al K_α X-ray (1486.6 eV). The average circular spot size is 200 μm diameter. High-resolution (Pass energy = 58 eV) 45° emission angle integrated scans were acquired. Measurements were carried out at room temperature inside an ultra-high vacuum compartment (base pressure of 8.10^{-7} Pa). Low-energy (<10 eV) electron flood and ion gun were used to neutralize the surface charge caused by photoelectron emission. The XPS data were calibrated according to the adventitious carbon 1s