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Effect of pH and applied potential on the adsorption of DNA on highly oriented pyrolytic graphite electrodes. Atomic force microscopy surface characterisation

Ana Maria Oliveira Brett *, Ana-Maria Chiorcea

Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Coimbra 3004-535, Portugal

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Abstract

Single-stranded and double-stranded DNA electrochemical biosensors prepared by adsorption during 3 min on HOPG, with or without an applied potential, at pH 5.3 and 7.0, were characterised by MAC mode AFM. During adsorption DNA condenses on the substrate forming complex network films with pores exposing the HOPG surface. The thin films formed in pH 5.3 acetate buffer always presented a better coverage of the HOPG surface with DNA molecules than films formed in pH 7.0 phosphate buffer. The application of a positive potential of 300 mV during adsorption enhanced the robustness and stability of the DNA films with the formation of bigger network holes and a more condensed and compact self-assembled DNA lattice. The knowledge of the morphology of adsorbed DNA on electrode surfaces explains non-specific adsorption on the electrode surface and can be used to improve and develop DNA-electrochemical biosensors.

Keywords: DNA; DNA-electrochemical biosensor; Adsorption; AFM; MAC mode AFM

1. Introduction

DNA as carrier of genetic information is a major target for drug interaction. Many compounds bind and interact with DNA causing changes in the structure of DNA and the base sequence, leading to perturbations in DNA replication. The DNA sequences are unique to each organism and any self-replicating biological organism can be discriminated using DNA hybridisation. The need for the analysis of gene sequences, monitoring microorganisms in medical, environmental and food control, determination of the oxidative damage to DNA and understanding of DNA interactions with molecules or ions led to the development of DNA electrochemical biosensors [1–6].

A DNA electrochemical biosensor is a receptor-transducer device that uses double stranded DNA (dsDNA) and single-stranded DNA (ssDNA) immobilised on the surface of an electrochemical transducer as molecular recognition element to measure specific binding processes with DNA. The characteristics of the DNA film and the optimal electrochemical response of the DNA-biosensor depend on pH, buffer composition, ionic strength, immobilisation procedure, adsorption time, pre-treatment conditions, type and concentration of DNA. A greater understanding of the structure–function relationship of DNA interaction on transducer interfaces is necessary and the adsorption of monolayers and multilayers of DNA on the electrode needs to be investigated in order to design surfaces exhibiting greater biological activity and selectivity.

Atomic force microscopy (AFM) is an important method to study the conformation of DNA at the transducer surface. The characterisation of DNA interfacial structure is not easy because the DNA molecules do not bind strongly enough to the conducting substrates required in electrochemical experiments and the AFM tip tends to sweep away adsorbed biomacromolecules. The technique of magnetic A/C mode AFM (MAC mode AFM) enables the visualisation of the

* Corresponding author. Tel./fax: +351-239-835-295.
E-mail address: brett@ci.uc.pt (A.M. Oliveira Brett).

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molecules which are weakly bonded to the substrate material because, during scanning, the AFM tip oscillates and touches the sample surface only at the bottom of this oscillation. Therefore AFM measurements can be used to visualise the interfacial structures and gain information on lateral organisation of adsorbed layers.

In this study MAC mode AFM has been used to characterise the morphology of a DNA-electrochemical biosensor surface obtained by adsorption of dsDNA and ssDNA on a highly oriented pyrolytic graphite (HOPG) electrode. The influence of the pH of the solution on the free adsorption of DNA and adsorption of DNA under applied potential was evaluated.

2. Experimental section

2.1. Materials and sample preparation

Calf-thymus double-stranded DNA (dsDNA) (sodium salt, type I) and single-stranded DNA (ssDNA) were purchased from Sigma Chemical and were used without further purification. The electrolyte solutions used were pH 5.3 0.1 M acetate buffer and pH 7.0 0.1 M phosphate buffer, prepared using analytical grade reagents and purified water from a Millipore Milli-Q system, conductivity < 0.1 μS/cm.

HOPG, grade ZYH from Advanced Ceramics, UK was used as a substrate.

DNA samples were prepared by free adsorption placing 100 μl of ssDNA and dsDNA solutions of different concentration, on a freshly cleaved HOPG surface and incubating for 3 min. The excess of DNA was gently cleaned with a jet of Millipore Milli Q water and the HOPG surface with adsorbed DNA was then dried with nitrogen.

DNA samples were prepared under an applied potential of +300 mV (vs. Ag wire) during 3 min, placing 500 μl of ssDNA and dsDNA solutions of different concentrations in the electrochemical cell, holding the HOPG working electrode on the bottom. The HOPG with adsorbed DNA was rinsed with a jet of Millipore Milli Q water and dried with nitrogen.

2.2. Instrumentation

All voltammetric experiments were done using a PalmSens potentiostat, running with PalmScan version 1.11, from Palm Instruments BV, The Netherlands and were carried out in a one-compartment Teflon electrochemical cell of approximately 12.5 mm internal diameter, Molecular Imaging, USA. The HOPG substrate was the working electrode, a Pt wire counter electrode and an Ag wire, as quasi-reference electrode, were placed into the cell.

AFM was performed with a Pico SPM microscope, with a CS AFM S scanner, controlled by a magnetic AC mode (MAC mode) module and interfaced with a PicoScan controller from Molecular Imaging, USA. Silicon type II MAClevers 225 μm length, 2.8 N/m spring constant and 60–90 kHz resonant frequencies (Molecular Imaging, USA) were used in MAC mode AFM. The images were taken at room temperature, scan rates 1.0–1.3 lines/s. The images were processed by flattening in order to remove the background slope and the contrast and brightness were adjusted. All images were visualised three-dimensionally using the Scanning Probe Image Processor, SPIP, and version 2.3011, Image Metrology ApS, Denmark.

Section analysis of the surfaces with adsorbed DNA molecules and films was performed with PicoScan software version 4.19, Molecular Imaging. The mean values of the heights were calculated using 50–70 measurements from different scanned images. Origin version 6.0 from Microcal Software, USA, was used to calculate the standard deviation of the DNA film thickness.

3. Results and discussion

3.1. Effect of pH on dsDNA films on HOPG

The pH and the composition of the buffer electrolyte solution influences DNA free adsorption and adsorption of DNA under applied potential as well as the coverage and robustness of the DNA-electrochemical biosensor, as will be described below.

Adsorption studies were carried out at two different pHs: physiological pH 7.0, and a more acid pH 5.3, where dsDNA has an increased binding efficiency on hydrophobic surfaces [7].

3.1.1. Free adsorption of dsDNA

The surface morphology of a dsDNA-based biosensor prepared on the HOPG electrode by free adsorption from 60 μg/ml dsDNA solutions and two different pHs according to the procedure described in the experimental section was investigated. Although dsDNA is a negatively charged, hydrophilic polyanion and HOPG has a hydrophobic surface, the spontaneous adsorption of DNA in a buffered solution on the HOPG surface occurs, stabilised by a continuous dissociation–association of the bases at the DNA double helix extremities [8]. Additionally, it was observed that hydrophobic surfaces can induce destabilisation and even local denaturation of DNA at room temperature [7,9], increasing the number of bases exposed to the surface.

MAC mode AFM images in air revealed a very good coverage of the surface of dsDNA adsorbed in pH 5.3 acetate buffer, Fig. 1(A). The dsDNA molecules self-organise at the electrode in a tight film, with

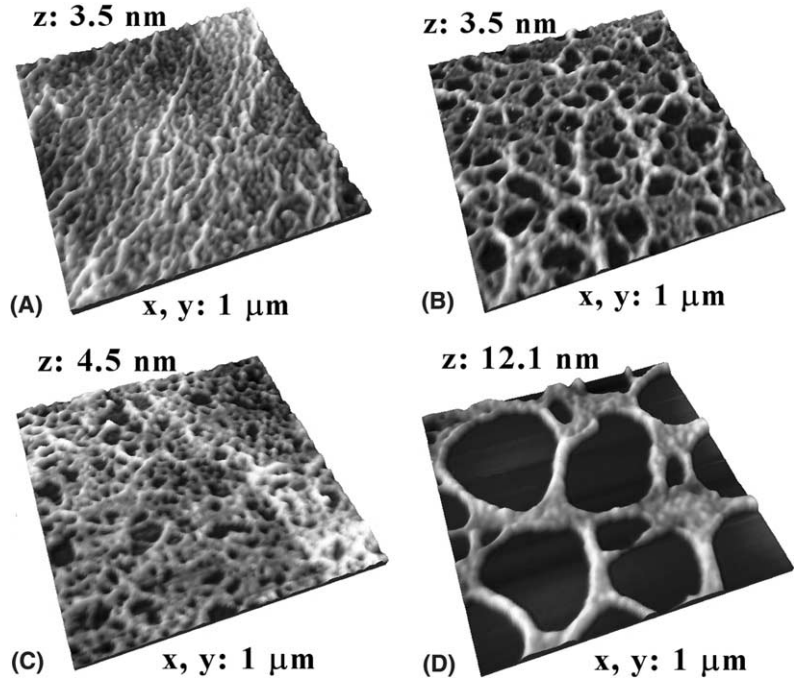

Fig. 1. MAC mode AFM topographical images in air of the DNA-biosensor surface prepared onto HOPG from a 60 μg/ml dsDNA after 3 min in 0.1 M electrolyte solution: (1) free adsorption: (A) pH 5.3 acetate buffer and (B) pH 7.0 phosphate buffer; (2) at a deposition potential of +300 mV (vs. Ag wire): (C) pH 5.3 acetate buffer and (D) pH 7.0 phosphate buffer.

1.26 ± 0.27 nm thickness, that almost do not reveal the electrode surface. Overlapped and superposed molecules and at some points straight dsDNA were observed.

Fig. 1(B) shows the dsDNA film grown in pH 7.0 phosphate buffer electrolyte. The dsDNA film appears as a well spread two-dimensional network and the images revealed coiled and twisted dsDNA structures. The dsDNA network was slightly irregular, and section analysis performed inside various images permitted the determination of two typical heights: of 0.99 ± 0.25 nm and 2.15 nm $\pm$ 0.25 nm, respectively. dsDNA did not cover the HOPG surface completely; dark regions in the images correspond to holes in the DNA film leading to exposed HOPG surface at their bottom.

The values of the dsDNA film thickness measured at both pHs are higher than the expected height of the diameter of A-DNA upon dehydration [8], as reported in typical AFM studies in air [9], which suggest the formation of complex structures, dsDNA aggregation phenomena and overlapping of dsDNA molecules.

3.1.2. dsDNA adsorbed under applied potential

One of the key factors in the biosensor design is the development of immobilisation methodology that strongly stabilises the DNA on the transducer surface. The immobilization of DNA by free adsorption involves weak interactions with HOPG and the DNA lattices are rather unstable, although it is a fast and easy procedure for preparing a DNA-electrochemical biosensor. Electrochemically assisted adsorption of DNA on the HOPG electrode surface leads to strong and stable DNA layers, by taking advantage of the electrostatic interaction with the nucleotide phosphate backbone. The surface morphology of a dsDNA-based biosensor prepared from 60 μg/ml dsDNA solutions at pH 5.3 and 7.0, under an applied potential of +300 mV (versus Ag wire), which is not sufficiently high to oxidise the DNA bases [10].

The dsDNA-electrochemical biosensor surface obtained at pH 5.3 is shown in Fig. 1(C). The dsDNA network loops were intertwined very tightly. The height of the DNA film was 1.41 ± 0.39 nm, slightly more than the layer thickness obtained by free adsorption.

A reorganisation of the DNA self-assembled network at the surface was observed for the dsDNA-biosensor obtained at pH 7.0. The DNA film corresponds to a matrix with large uncovered areas of the HOPG electrode, the dark regions in the images, Fig. 1(D). The diameter of HOPG exposed areas varies in the range 100–500 nm. The dsDNA lattice was formed by coiled fibres, which condensed and twisted together in large and rather flexible loops and in end-to-end aggregations and stabilised on the surface. The thickness measured along the DNA network was not uniform; heights between 3.5 and 7.5 nm were observed. The values were much higher than the expected height of A-DNA, suggesting an increase of DNA condensation at this pH. Several layers of dsDNA molecules were involved in the condensation, by association and cross-over of sticky-ended linear molecules assuming less usual conforma-

tions and enhanced attractive lateral interaction between adjacent dsDNA helices caused by the applied potential.

An increase of superposition of the DNA molecules and a better coverage of the surface at pH 5.3 was noticed. Comparing the thickness and the electrode coverage of the two films obtained at different pHs on applying a potential of +300 mV it was concluded that the film obtained at pH 5.3 presented a self-assembled lattice that was more relaxed and extended on the surface. The results that were obtained by AFM corroborate previous observations that the best binding efficiency of dsDNA on hydrophobic surfaces occurs at approximately pH 5.5 [7]. For both pHs under study, the electrostatic interaction between the positively charged HOPG electrode and the negatively charged phosphate backbone increased the stability of the adsorbed dsDNA. The dsDNA film was robust enough to withstand application of high AFM forces without being removed from the surface.

3.2. Effect of pH on ssDNA films on HOPG

In ssDNA the bases are exposed to the solution, which facilitates the interaction of the hydrophobic aromatic rings of the purines and pyrimidines with the hydrophobic substrate. Consequently ssDNA interacts and adsorbs much more strongly to the HOPG surface, when compared with dsDNA for the same solution concentration. Fig. 2 shows the HOPG electrode modified by ssDNA films grown from 60 μg/ml DNA in pH 5.3 acetate buffer electrolyte and pH 7.0 phosphate buffer electrolyte, using free adsorption and adsorption under controlled potential, respectively. Images showed a dry uniform film covering the electrode almost completely with only a few defects. Insignificant differences were observed between the four immobilization procedures, Fig. 2, although all led to a surface showing similar holes. Therefore the study using ssDNA had to be carried out with smaller concentrations of ssDNA, in order to distinguish better the characteristics and particularities of each film.

3.2.1. Free adsorption of ssDNA

The surface of the ssDNA biosensor prepared on the HOPG electrode surface by free adsorption from 5 μg/ml ssDNA solutions as described in the experimental section was evaluated. The surface ssDNA-sensor obtained at pH 5.3 is distinguishable in Fig. 3(A). The large calf-thymus ssDNA molecules make intra- and intermolecular loops packing into large-scale two-dimensionally networks. The filaments form large networks over the HOPG with small circular pore arrangements that show uncovered electrode surface. The height of the ssDNA loops had a large distribution spectrum with values between 0.9 and 2.6 nm.

At pH 7.0 the two-dimensional lattices self-assembled on the HOPG surface were incomplete and larger areas of the electrode were uncovered by the ssDNA mole-

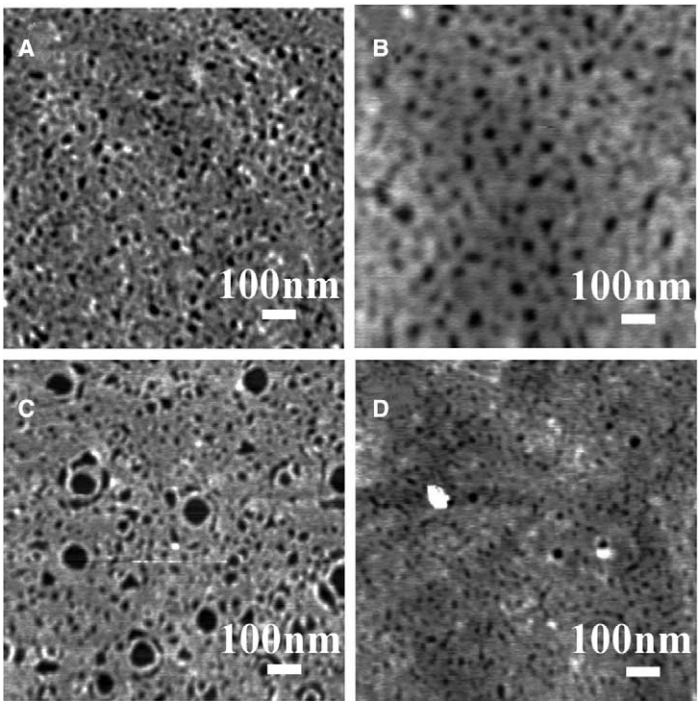

Fig. 2. MAC mode AFM topographical images in air of the DNA-biosensor surface prepared onto HOPG from a 60 μg/ml ssDNA after 3 min in 0.1 M electrolyte solution: (1) free adsorption: (A) pH 5.3 acetate buffer and (B) pH 7.0 phosphate buffer; (2) at a deposition potential of +300 mV (vs. Ag wire): (C) pH 5.3 acetate buffer and (D) pH 7.0 phosphate buffer.

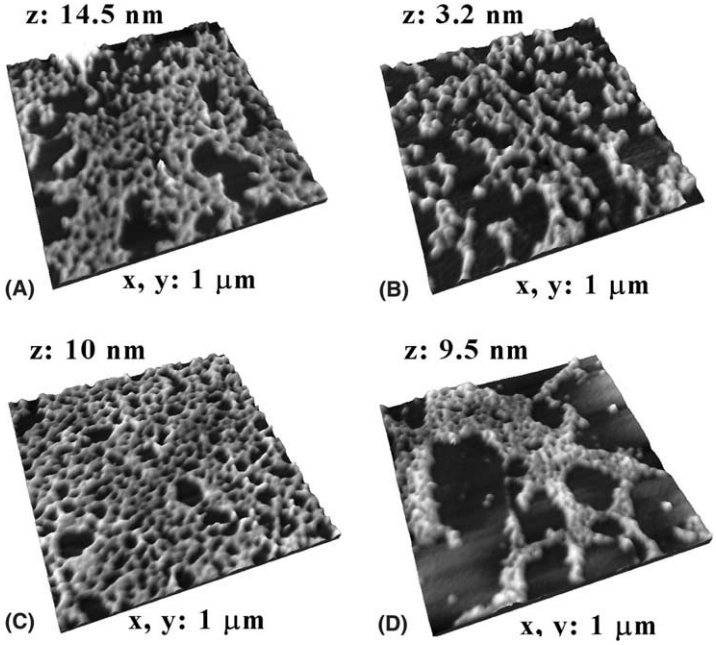

Fig. 3. MAC mode AFM topographical images in air of the DNA-biosensor surface prepared onto HOPG from a 5 μg/ml ssDNA after 3 min in 0.1 M electrolyte solution: (1) free adsorption: (A) pH 5.3 acetate buffer and (B) pH 7.0 phosphate buffer; (2) at a deposition potential of +300 mV (vs. Ag wire): (C) pH 5.3 acetate buffer and (D) pH 7.0 phosphate buffer.

cules, Fig. 3(B). The thickness of the film measured by section analysis had values between 0.8 and 1.7 nm.

At both pHs portions of ssDNA coexisted with double-stranded parts, due to the capacity to associate with arbitrary complementary portions of other ssDNA molecules by means of a base pairing mechanism.

3.2.2. ssDNA adsorbed under applied potential

The pH of the buffer electrolyte of a ssDNA solution drastically affects DNA condensation on the HOPG electrode at +300 mV (vs. Ag wire). At pH 5.3, packing of ssDNA molecules into a filamentary matrix which spreads all over the surface with equal size pores through the film that make the electrode surface visible, Fig. 3(C), was noticed. Dimensions of 1.2 to 3.5 nm were determined by section analysis inside the images.

The positive potential of 300 mV applied during the adsorption changed the properties of the ssDNA layer obtained at pH 7.0 markedly. Images of ssDNA on HOPG surface showed an even lower coverage of the electrode surface, Fig. 3(D), as compared to that obtained at pH 5.3. Large sheets covered big areas of the substrate and larger zones of the HOPG were not covered at all. The measured thickness of the film was 2.37 ± 0.4 nm.

At both pH 5.3 and pH 7.0 more than one monolayer of ssDNA molecules condensed together as a result of the influence of the interaction between neighbouring ssDNA strands but less condensation then in the case of dsDNA for the same pH occurred.

For ssDNA and for all immobilisation procedures used the density of the exposed surface pores was very high but the dimensions of the pores were rather small, due to multiple adsorption contacts between the ssDNA and the HOPG and higher DNA–DNA interactions. As in the case of dsDNA, better, more compact films were formed in pH 5.3 acetate buffer than in pH 7.0 phosphate buffer. The applied potential increased the robustness of the ssDNA adsorbates.

4. Conclusions

The DNA lattices are held together at the HOPG surface by non-covalent interactions such as hydrogen bonding, base stacking, electrostatic, Van der Waals and hydrophobic interactions. As shown the existence of pores in the DNA layer leaving HOPG uncovered areas can cause misleading results when using a DNA-electrochemical biosensor for detection of hybridization or DNA–drug interactions, due to non-specific adsorption of oligonucleotides or drugs on the uncovered surface. DNA-biosensors with a low degree of non-specific binding require high concentrations of DNA solutions, which induces the formation of more than one monolayer of DNA. Films grown in pH 5.3 acetate buffer always presented enhanced DNA surface density due to overlaying and superposition of the DNA molecules, with respect to films formed in pH 7.0 phosphate buffer.

The DNA immobilisation procedure must be a compromise between the degree of electrode coverage and the strong adsorption offered by the applied potential. The DNA–electrode surface interaction is stronger and more stable when a potential is applied during adsorption. Knowledge of the morphology of adsorbed DNA and the coverage of the electrode surface, controlled by the appropriate choice of DNA solution concentration, pH and applied potential, will help in the improvement and development of DNA electrochemical biosensors.

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