Detection of the absorption of glucose molecules by living cells using atomic force microscopy

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Abstract A very small electrode (nanobiosensor) was constructed by immobilizing enzyme (glucose oxidase or hexokinase) on the surface of the cantilever of the atomic force microscope in order to detect the absorption of glucose molecules by living cells. If glucose is present, the nanobiosensor deflects, probably due to the reaction heat evolved in the process. Nanobiosensors built with inactivated enzyme or cantilevers without immobilized enzyme were not capable of producing this type of signal (deflection). This technique will be very useful in detecting the passage of specific molecules through a cell wall (or a cell membrane for other types of cells).

Key words: Baker's yeast; Atomic force microscopy; Absorption; Electrode; *Saccharomyces cerevisiae*

1. Introduction

Atomic force microscopy (AFM) is becoming a very important tool in the biological field. With this technique the cells are observed alive and do not require fixing methods commonly used in scanning electron microscopy [1,2].

AFM works as a profilometer works: by moving a commercial microfabricated tip across the sample. This tip is held at the end of a thin, flexible, gold cantilever (100 μm long, about the width of a hair) [3] (see Scheme 1B).

Recently, an interesting biodetector was developed by immobilizing enzymes on a gold surface (thermo-optical detector, Scheme 1A) [4–10]. This equipment is based on the heat evolved from an enzymatic reaction which provokes deflections of a laser beam and this result is registered as a graph [6–10]. Since AFM has almost the same parts as the thermo-optical detector (laser beam, lens and photodiode) (Scheme 1B), if an enzyme (glucose oxidase) was immobilized on a cantilever, it is hoped that the temperature gradient generated by reaction heat (between the enzyme and its substrate) could induce a deflection of the cantilever, transforming the AFM into an apparatus that could reveal the presence of specific biomolecules being absorbed by living cells, together with the image of the cells. These assumptions were demonstrated to be consistent with the experimental protocol and the goal of this paper is to describe a method of constructing nanobiosensors by immobilizing enzymes (glucose oxidase or hexokinase) onto the cantilever in order to detect biological molecules.

Saccharomyces cerevisiae was used for these experiments because of its importance in the food and pharmaceutical industries [11,12].

2. Materials and methods

2.1. Chemicals

Glucose oxidase was obtained from Sigma (diagnostic kit, item 510A) and hexokinase was obtained from Labtest Diagnóstica S.A. (Lagoa Santa, Brazil). The other reagents used were of analytical grade. Water was double-distilled and deionized. Glucose solution was obtained together with the glucose oxidase or hexokinase kits (100 mg/dl).

2.2. Biological materials

The industrial strain of *S. cerevisiae* was obtained from Itaiquara (Brazil). The stock suspensions were prepared by adding 1.0 g dry baker's yeast to 10 ml water (double-distilled and deionized) while stirring at room temperature. A drop of the suspension was placed on the surface of glass coverslips and allowed to dry for 15–20 min at room temperature to remove the excess water. Thus, the experiments were performed under a thin layer of water.

2.3. Nanobiosensors

1. Stock solution of glucose oxidase: 100 mg of glucose oxidase (or 10 mg of hexokinase) and 10 mg of bovine serum albumin (BSA) were dissolved in 1 ml double-distilled and deionized water. 5 μl of this glucose oxidase plus BSA solution was spread onto a 2 cm^2 area of a coverslip.
2. Building the nanobiosensor: a brand new cantilever was put in the cantilever holder of the AFM. An engage was given on the coverslips containing the solution of the glucose oxidase plus BSA. Then 1 μl of 25% glutaraldehyde (crosslinking agent) was added onto the area where the solution of glucose oxidase and BSA was spread. The resultant gel phase covering the cantilever was found to be stable in aqueous solution. After 30 s the cantilever was taken off the coverslip surface and dried for 3 h at room temperature. After drying, this nanobiosensor was put in a vacuum desiccator for conservation at 5°C. To test the effectiveness of this methodology, glucose solution was directly dropped onto the nanobiosensor while it was scanning a glass surface; the reaction catalyzed by the immobilized glucose oxidase was monitored by the deflections of the cantilever on the AFM monitor.

The nanobiosensors should be refrigerated and kept under vacuum. Under these conditions they can be used up to 4 weeks after their preparation and the experiments using them are reliable and reproducible.

2.4. AFM

A NanoScope III AFM (Digital Instruments) operating in contact mode was used in the experiments with Nanotips (Digital Instruments), Si_3N_4 tips with 0.12 N/m spring constants were chosen. In some cases the images were low pass filtered to remove scan lines. All

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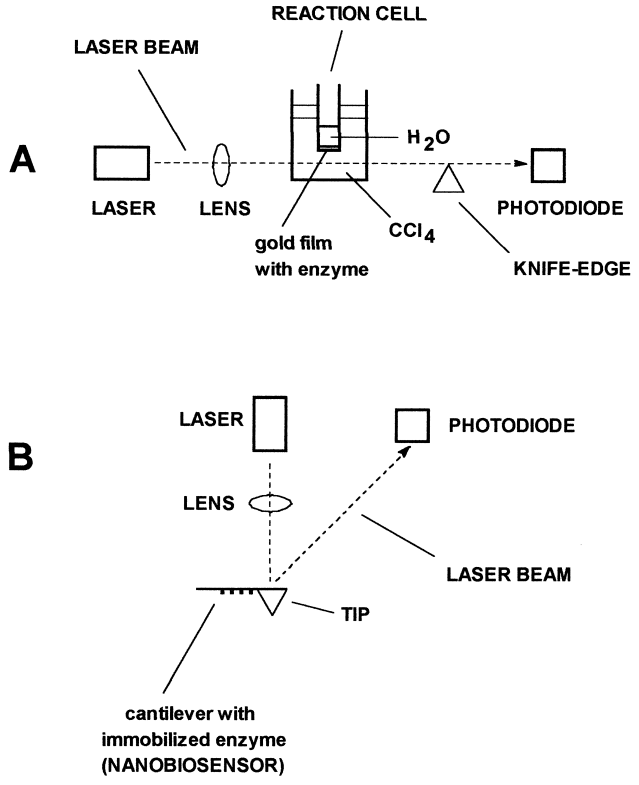

Scheme 1. Working principle of (A) thermo-optical detector [6–10] and (B) AFM [13].

images were collected on the AFM using a scan speed of 2.5 Hz and all imaging was done in air at room temperature.

The AFM works by moving a commercial microfabricated tip across a preparation while recording the *X*, *Y* and *Z* coordinates of the preparation being scanned. The *Z* dimension is calculated by reflecting a laser beam off the surface of the cantilever. The laser light reaches the surface of a split photodiode that records changes in the position with high precision. Deflections in the tip, which correspond to the surface topography, are sensed by a photodetector (Scheme 1B).

3. Results and discussion

3.1. Importance of the AFM

For a very long time, scanning electron microscopes have been used to obtain high resolution visualizations of the surfaces of biological samples. Normally, in order to scan the samples of *S. cerevisiae*, each preparation is coated with a film of evaporated gold (approximately 20 nm in thickness [14,15]). The application of this conductive coating to the surface of the sample masks important structures which exist on the cell wall [1,2] and kills cells in the preparation. With the advent of AFM, this fact changed and the cells are visualized uncoated and alive, at high resolution, producing excellent and reproducible images. Some years ago, my research group generated the world's first micrographs of different industrial strains of *S. cerevisiae* with AFM equipment [1] and we could observe structures, on the cell wall, never described before [1,2].

3.2. The concept of the nanobiosensor

Based on these advantages (to visualize cells uncoated and alive), a nanobiosensor was developed, as described in this paper, by immobilizing glucose oxidase (or hexokinase) on the surface of the cantilever of the AFM in order to detect the absorption of glucose molecules by living cells.

This nanobiosensor works based on heat released when an enzyme reacts with its substrate. The concept of this small electrode was based on an interesting methodology developed recently by analytical chemists: the thermo-optical detector, which uses an enzyme (catalase) immobilized on a gold film (Scheme 1A) [6–10]. The very small amount of heat evolved from the enzymatic reaction was sufficient to produce a deflection of a laser beam [6–10]. As the AFM has almost the same parts as this thermo-optical detector (laser beam, lens and photodiode), it was assumed that an enzyme immobilized on the surface of the cantilever could produce the same effect (deflection of the cantilever and, consequently, the laser beam). This assumption proved to be correct.

3.3. Difference between nanobiosensor and thermo-optical detector

The nanobiosensor generates images from the sample with qualitative analyses while the thermo-optical detector produces a graph with quantitative analyses.

3.4. The use of a nanobiosensor in detecting absorption of glucose molecules by living cells

At the beginning of the experiment, glucose molecules (500 μl of glucose solution to 10 ml of medium) were added to the medium with *S. cerevisiae* cells. A drop of this suspension was placed on a coverslip and dried at 25°C during 15–20 min to remove excess water. Fig. 1A shows the scanning of this sample (glucose molecules+*S. cerevisiae* cells) by the nanobiosensor (glucose oxidase or hexokinase immobilized on the cantilever surface). When the surface is scanned by this nanobiosensor, a reaction between the glucose oxidase (from the cantilever) and glucose molecules (from the medium reaction) takes place and heat is evolved from the process. This small amount of heat provokes a deflection of the nanobiosensor which is observed on the monitor of the AFM. In the case of Fig. 1A, successive deflections occurred and it was impossible to visualize the image of the cells. Fig. 1B shows that after 3 h of incubation it is already possible to observe the cells image partially, due to the absorption of glucose molecules by *S. cerevisiae* cells. In some regions of this scanned area, there are glucose molecules and this fact is observed by deflections of the nanobiosensor. Fig. 1C shows that the process of glucose absorption is continuous and Fig. 1D,E shows that nearly all glucose molecules were absorbed by these living cells. As observed before, each industrial strain of *S. cerevisiae* has a different biochemical behavior [11,16] and the time of absorption of glucose may vary from strain to strain.

To prove that the experiments made with the nanobiosensor were not an artifact, the same sample was scanned with a common cantilever (without immobilized enzyme) (Fig. 2A–E), under the same conditions and the same period of time as the experiment shown in Fig. 1. No deflections were observed, proving that the presence of the enzyme is necessary to produce them.

Enzyme inactivated by heat and afterwards immobilized on the surface of a cantilever was also incapable of producing deflections.

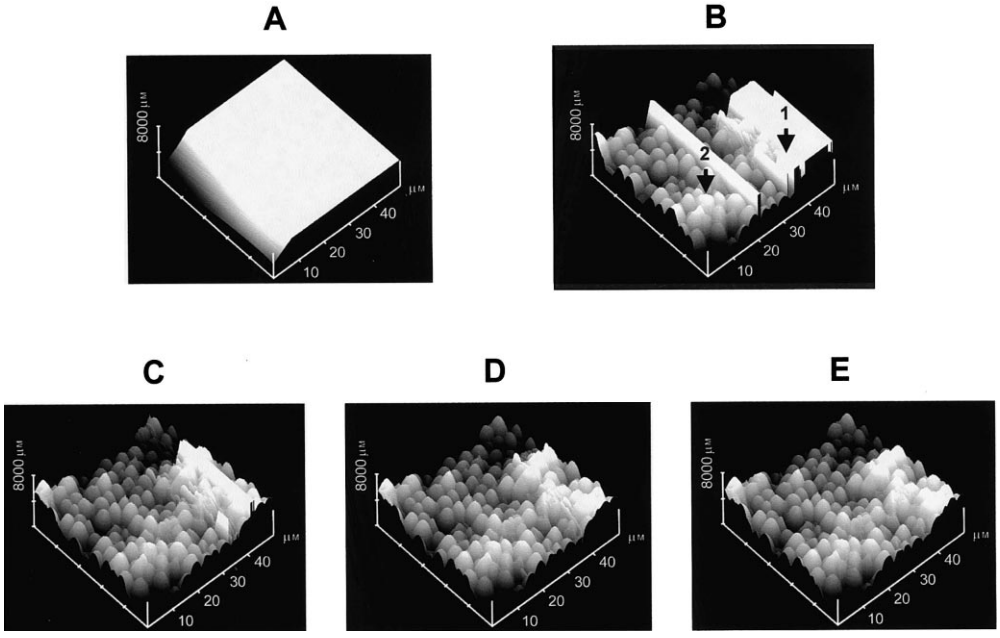

Fig. 1. Scanning of *S. cerevisiae* cell surface by the nanobiosensor (glucose oxidase (or hexokinase) immobilized on cantilever surface). A: The presence of high concentrations of glucose molecules induces a great number of deflections impeding the visualization of the cells. B: After 3 h, part of the glucose molecules were absorbed by the cells and since the number of deflections is lower, it becomes possible to partially visualize the cells image. Arrow 1 indicates the deflections of the cantilever due to the presence of glucose molecules and arrow 2 indicates the *S. cerevisiae* cell. C: 4 h after the first scanning (A) more glucose molecules were absorbed and the image of the cells is almost totally visible. D,E: 5 h after the first scanning (A) it is possible to observe the entire cell image, indicating that nearly all glucose molecules were absorbed by *S. cerevisiae* cells.

3.5. Other types of nanobiosensors

When this experiment was repeated using another enzyme (hexokinase, an enzyme also used to identify glucose molecules in clinical analysis) immobilized on the surface of cantilever, the same pattern of deflections and the absorption of glucose molecules were observed (data not shown). Other types of nanobiosensors were made with superoxide dismutase or catalase in order to study reactive oxygen species (manuscript in preparation). These are important facts indicating the possibility of using other enzymes to build nanobiosensors specific for other molecules in the future, since heat is evolved from the reaction catalyzed by the chosen enzymes.

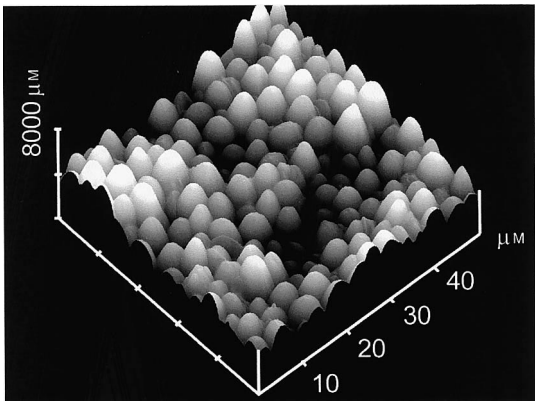

Fig. 2. Scanning of *S. cerevisiae* cell surface by a cantilever without immobilized enzyme. No deflection was detected, indicating that the presence of the enzyme is necessary to produce deflections. During 5 h of scanning the same image was obtained.

3.6. Other potential applications of this technique

This type of technique has potential applications in several areas: fermentation science (alcohol ethyl production) [17], organic chemistry and medicinal chemistry (chiral alcohol production, by *S. cerevisiae*, to synthesize important chiral drugs) [12,18], biotechnology, nutrition and food engineering (purification of waste waters using *S. cerevisiae*, production of vitamins by microorganisms, bread and brewing) [19–21], pharmacology (test of drugs) [22], pathology and clinical analysis (increased levels of glucose associated with diabetes mellitus and conditions interfering with glucose absorption) [23], analytical chemistry (new types of electrodes and bioelectrodes) [4], physiology and biophysics (transport of molecules through cell walls or biological membranes) [24].

4. Final remark

This is a reproducible, reliable and simple method which works even when the cantilever is put in contact with enzyme solution (without using the immobilizing method described in this paper) and allowed to dry.

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