EFECTUL POTENȚIALULUI ELECTRIC INTRAORAL ASUPRA *Candida albicans*
THE EFFECT OF INTRAORAL ELECTRICAL POTENTIAL ON *Candida albicans*

VIRGIL PENTA, CAMELIA UNGUREANU, ANDREI BOGDAN STOIAN, CRISTIAN PIRVU*
University Politehnica of Bucharest, Department of General Chemistry, 1-7 Gheorghe Polizu Str., 011061, Sector 1, Bucharest, Romania

Candida albicans is the most frequently isolated pathogen yeast and is present in the oral cavity of almost half of the population. Since there have been several seemingly contradictory studies on the effect of electricity on C. albicans behavior, we propose a new method of measurement: Electrochemical Impedance Spectroscopy (EIS) coupled with Surface Plasmon Resonance (SPR). Along with other electrochemical methods and high-resolution Atomic Force Microscopy (AFM) imagery we investigated the effect of electric potentials measured between metallic intraoral restorations materials on C. albicans.

Candida albicans este cel mai frecvent izolată drojdie patogenă și este prezentă în cavitatea bucală a aproape jumătate din populație. Deoarece au existat mai multe studii aparent contradictorii cu privire la efectul electricității asupra comportamentului Candida albicans, propunem o nouă metodă de măsurare: Spectroscopia de impedanță electrochimică (EIS) cuplată cu Rezonanța de Suprafață a Plasmonilor (SPR). Împreună cu alte metode electrochimice și cu imagini de înaltă rezoluție cu ajutorul Microscopiei de Forță Atomică (AFM) s-a investigat efectul potențialelor electrice măsurate între materialele metalice intraorale asupra Candida albicans.

Keywords: *Candida albicans*, *biofilm, Surface Plasmon Resonance, Electrochemical Impedance Spectroscopy, Atomic Force Microscopy*

1.Introduction

Candida albicans (C. albicans) is the most commonly isolated pathogen yeast and is present in the oral cavity of almost half of the population [1]. *C. albicans* may form biofilms on the surface of implantable medical devices because these serve as biofilm substrates [2, 3]. The problems of this yeast reinfection from full denture prosthetics represent a major issue in the actual clinical environment [4]. The biofilms of this yeast are composed of a mixture of cell types, including yeast cells (blastospores), pseudo hyphal, and hyphal cells, and include an extracellular matrix (polysaccharide and protein). Polysaccharides contain different monosaccharide units but may contain also non-carbohydrate groups. It can be affirmed that the most polysaccharides are structurally complex, and they may be attached to protein molecules or to other polysaccharides. Polysaccharides can be depolymerized by acids and heat, some enzymes, and high pH following oxidation. Structural modification makes the polysaccharides molecules even more complex and perhaps, polydisperse [5, 6].

Hyphal development strongly contributes to *C. albicans* success as a pathogen [7]. Yeast to hypha conversion occurs through an intermediate germ tube stage. In the case of the yeast, a germ tube can be induced from the yeast cell and developed into the mycelia form. This morphological transition can be induced by changes in a variety of environmental factors like temperature, ambient pH (a pH value in the range 6-8 is critical for germ-tube formation) and growth medium. The mechanism whereby these factors induce germ tube formation in *C. albicans* is virtually unknown [8]. Growing hyphal cells normally generate an electrical current so that positive charge enters the hyphal termination and exits from the rear [9-11].

There have been several seemingly contradictory studies on the effect of electricity on *C. albicans* behavior from which we selected a few. An applied electrical field exert a dramatic influence on the polarity of cell growth, causing disruption of normal biosynthetic and metabolic processes of the cell [12]. The switch from yeast cells to hyphal cells growth depict a transition to a more polarized form of growth since budding occurs predominantly by isotropic wall expansion while germ tube growth is restrict to the apex [13].

Electrical fields could inhibit germ tube extension and caused a delay in germ tube formation. However the growth still occurred even at the highest field strengths. A part of these yeast cells grows or move near the cathode, some to the anode and others to both anode and cathode according to the strength of the field or the medium conditions. The nucleus and septum positioning in *C. albicans*

* Autor corespondent/*Corresponding author*,
E-mail: cristian.pirvu@upb.ro

are not affected by electrical fields. The general shape of the yeast and hyphal forms are also not affected [14].

At the same time, the *in vitro* effects of a low intensity constant direct current of 0.2 to 1 mA, applied for 2 to 18 hours on *C. albicans* yeast inhibit the yeast growth [15]. The inhibitory action was proportional to the magnitude and application time of the electric current.

An increasing number of patients nowadays present very different metallic intraoral restorations of questionable compatible compositions. We stress the fact that oral galvanism (the currents being conducted by saliva, food, and nerves) and symptoms associated with this issue pose important health risks and should not be treated lightly. The surfaces of implantable medical devices generate oral galvanism which means electrical currents and voltages in the mouth [16]. In the resulting complex oral environment with many heterogenic elements such as polymeric denture materials, cast metal restorations and even metallic or polymeric restorations it is vital to view and understand interactions and possible risks.

In this direction we propose the current research regarding *C. albicans* and the implications of different surface potential values on the adhesion and interaction of this microorganism to the acquired protein pellicle [17]. Interaction of this microorganism to the oral plaque formation is complex and the exact moment of interaction difficult to quantify in the multitude of 400-600 bacterial species of the oral environment. As such we wish to continue our previous work [18] in understanding cell interaction to oral protein pellicle [19] and quantify the intimate interaction of the said microorganism to a model of the human acquired pellicle, a protein formation that naturally coats all types of materials in the oral cavity.

2. Materials and Methods

2.1. Materials

With their *consent,* the human saliva was collected from healthy declared non-smoker volunteers. The conditions for saliva quality were strictly followed: no food or drink for 8 hours before experiment (in the morning), negative for HIV and no drug intake for 14 days prior experiment. The saliva has been processed by centrifugation 1h at 8000 rpm, sterile filtered with 0.2 µm filters and stored at 4 °C.

The strain *Candida albicans* ATCC 10231 was grown on Malt Extract Agar (abbreviated „MEA") (Merck) with medium composition: malt extract 17 g/L, and agar 20 g/L.

The yeast ($5 \cdot 10^6$ yeast cells) was resuspended in saliva to stimulate like oral environment protein expression. The cultures grown in saliva for about three days have been used for our experiments. Natural saliva promotes *C. albicans* growth and specific gene expression like human intraoral environment [20].

2.2. Methods

2.2.1. Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy measurements were carried out using Autolab PGSTAT 302N in a logarithmic distribution range between 10 KHz and 10 mHz. The EIS fitting was performed using NOVA 1.10 software. All measurements were performed in a custom-built electrochemical cell with three electrodes: a working electrode (Au disk thin-film electrodes with an exposed surface of 0.03 cm^2), a platinum counter-electrode and an Ag/AgCl reference electrode.

The experiments were conducted by registering EIS measurement over 30 minutes to record salivary pellicle formation. To observe microorganism interaction EIS measurements were recorded every 20 minutes for duration of 60 minutes after their insemination in the cuvette.

2.2.2. Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance Autolab Esprit was synchronized with EIS for measurements, offering an advantage to real time observation of microorganism interaction. We used this determination to monitor the deposition of the salivary pellicle and subsequent *C. albicans* interaction. The experiments had the following protocol, which was completely automatized by the Autolab Esprit to reduce error:

1. Baseline acquisition with ultrapure water 50 µL;
2. Drain 40 µL - pipetting 40 µm natural filtered saliva on the gold chip;
3. Wait time 30 minutes;
4. Washing step: 2×500 µL ultrapure water to eliminate unbound constituents from surface;
5. Pipetting of 50 µL ultrapure water;
6. Wait time to view new baseline and compare to initial value;
7. Draining 40 µL of cuvette content and pipetting of 40 µL *C. albicans* grown in natural saliva;
8. Wait time 60 minutes;
9. Final washing step: 2×500 µL ultrapure water;
10. Pipetting of 50 µL ultrapure water to acquire baseline;
11. Final wait time.

All the experiments had a fixed temperature to 37 °C to reproduce the natural environment and promote genuine phenomena occurring.

Table 1

Potential values (mV) measured in Afnor artificial saliva for different metallic materials
Valorile potențialului (mV) măsurate in saliva artificială Afnor pentru diverse materiale metalice

Alloy	Fe-Cr-Ni	Cr-Ni	Cu-Al	Co-Cr
Fe-Cr-Ni	-	282	64	145
Cr-Ni	282	-	194	152
Cu-Al	64	194	-	11
Co-Cr	145	152	11	-

2.2.3. Atomic Force Microscopy (AFM)

Atomic Force Microscopy scans were performed using an A.P.E. Research A100-SGS atomic force microscope. The saliva films and *C. albicans* interaction were investigated for each disk after step 4 and step 11 respectively.

3. Results and discussion

3.1. Electrical Potential measurements

The differences of electrical potential between crowns of different metals (Table 1) were measured in Afnor artificial saliva (NaCl – 0.7 g/L, KCl – 1.2 g/L, Na_2HPO_4 H_2O – 0.26 g/L, $NaHCO_3$ – 1.5 g/L, KSCN – 0.33 g/L, urea – 1.35 g/L, pH = 7.6). This is the starting step of our experiments, since we found a wide electric potential range between different materials.

3.2. Cyclic Voltammetry (CV)

To assess the viability of *C. albicans* and to have an initial overview of the electrical processes CV tests were performed on *C. albicans* suspended in natural saliva over a wide potential range between -0.5 V and 1 V vs. Ag/AgCl using a small scan rate of 1 mV/s, allowing the microorganisms to adapt to the potential change. The cyclic voltammogram (Figure1) shows that there are no major processes taking place at low potentials. The peak around 600 mV is attributed to living microorganisms [21] and is caused by a charge transfer. The peaks around 1 V represent the electrolysis of water. Also marked on the figure are the potentials on which the SPR and EIS analysis were made which are close to the values measured between the metallic materials.

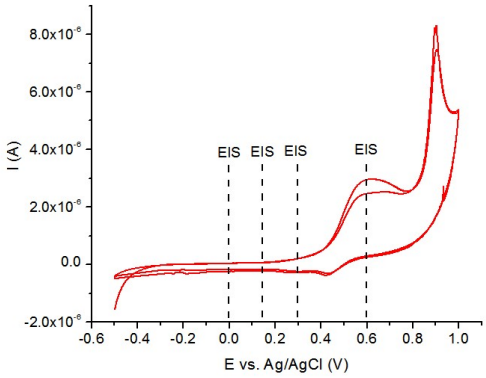


Fig. 1 - Cyclic voltammogram for *C. albicans* suspended in natural saliva crossed *over the upper part of the scale becoming unreadable/ Voltamograma ciclică pentru C. albicans suspendată în salivă artificială*

3.3. SPR analysis

Salivary pellicle formation was monitored for 30 minutes using EIS coupled with SPR and were carried out at open circuit potential (OCP), 150 mV, 300 mV and 600 mV vs. Ag/AgCl. Subsequent fungal interaction to the acquired pellicle layer was observed in the same manner for 60 min. Figure 2 presents the SPR diagrams corresponding to gold surface interaction with salivary proteins and *C. albicans* cells. The graphic shows that the association curves corresponding to the acquired protein layer formation and *C. albicans* interaction are influenced by the increase of the electric potential.

The graphic shows strong protein layer formation and *C. albicans* interaction on the gold chip despite two washing steps even at OCP.

The second experimental surface potential was 150 mV. SPR data showed increased signal in the initial association curve corresponding to acquired pellicle formation with higher remnant signal after the washing steps. Fungal interaction viewed through SPR showed an increased association curve corresponding to increased surface-microorganism interaction. The final signal after washing steps shows important surface modifications.

The third potential investigated was that of 300 mV. SPR data shows accelerated protein build-up with high residual signal after the washing steps. The high remaining signal after the washing steps points out that a larger mass of proteins and *C. albicans* interacted with the surface.

We underline the fact that it is highly unlikely that variations of 600 mV can be present with reduced clinical symptomatology, and indeed, our electrical potential measurements did not reach this value. However, we considered that the presence of the 600 mV peak in the CV should be investigated further. SPR data shows a very steep adhesion curve corresponding to a high angle modification. The remaining signal after the washing steps was much higher than previously observed in any of the anterior experiments. The interaction of this protein pellicle with subsequent fungi was impossible to view by SPR. The values of the acquired signal crossed over the upper part of the scale becoming unreadable.

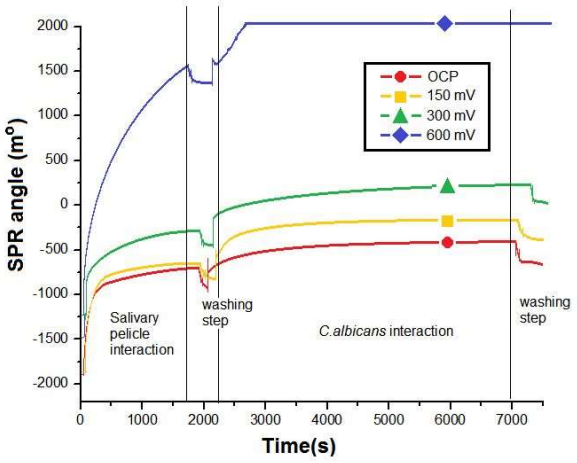


Fig. 2 - Acquired pellicle formation and *C. albicans* interaction / *Grafic SPR înregistrat pentru formarea peliculei de C. albicans*

3.4. EIS investigation

3.4.1. Interaction at OCP

EIS data at OCP (Figure 3) revealed that the saliva pellicle acts as a complex two-phase element. The first phase represents the light salivary components that form complex protein constructs of the dental pellicle passively. The second phase represents the high weight salivary components that subsequently interact with the gold surface. The constant phase element, CPE 1, represents the layer vacancies that are becoming filled with proteins and CPE 2 represents the heterogenous phases present in the film. The fitted data values show that the salivary film is stable in time and that there is no important contribution from the saliva to the electrical properties of the film. For *C. albicans* interaction we retain a two-phase system for the equivalent circuit where Phase 1 represents *C. albicans* in the system and Phase 2 represents the salivary pellicle.

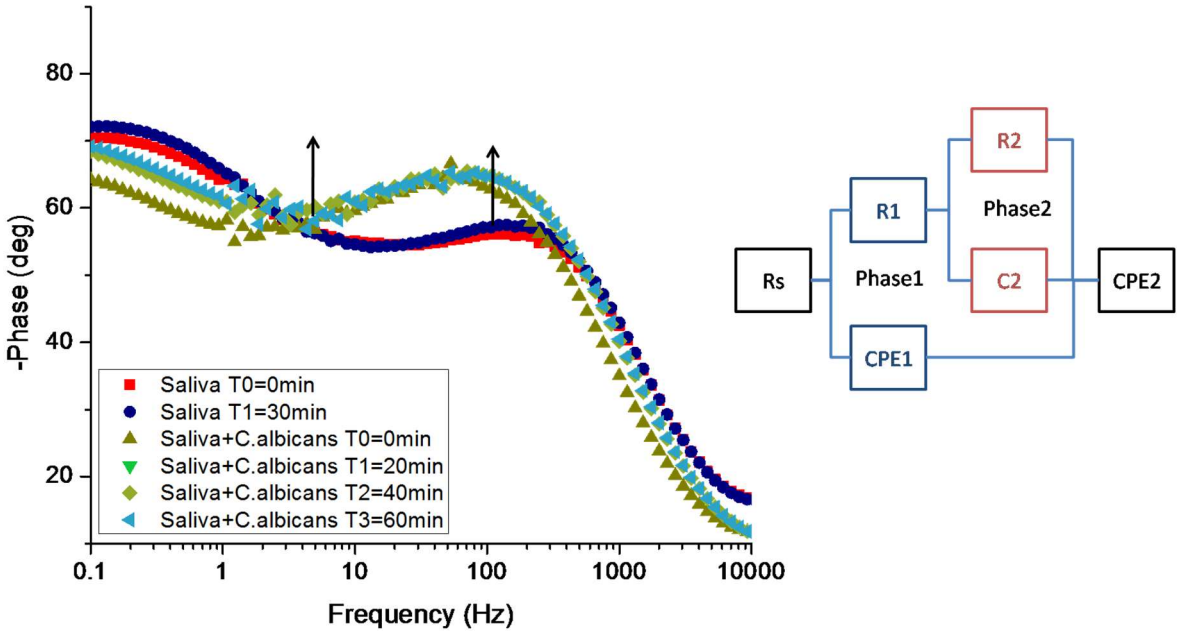
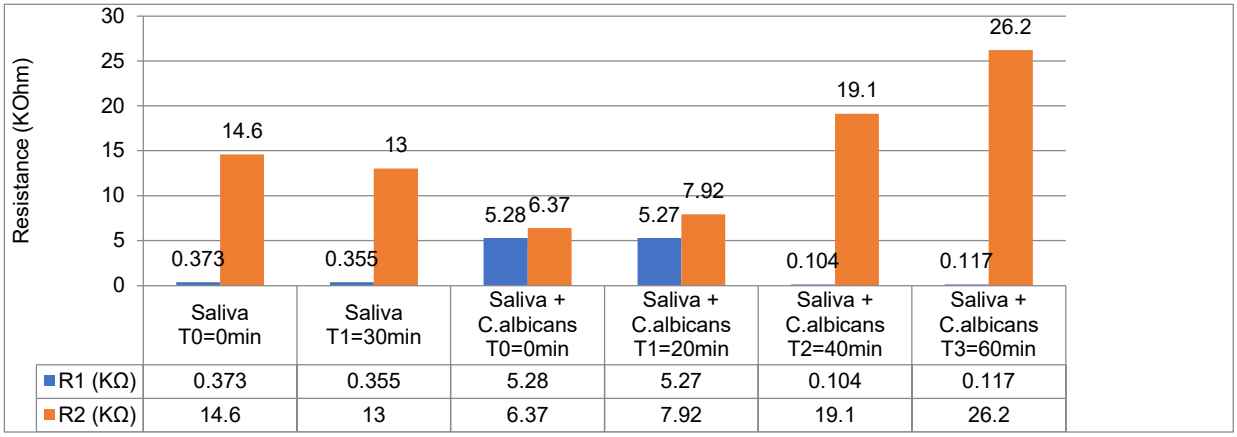


Fig. 3 - Phase Bode plot for salivary pellicle formation and *C. albicans* interaction at OCP with inserted equivalent circuits / *Grafic Bode de fază înregistrat pentru formarea peliculei salivare și interacțiunea cu C. albicans la OCP (potențialul circuitului deschis). Inserat circuitul echivalent.*



	Saliva T0=0min	Saliva T1=30min	Saliva + C.albicans T0=0min	Saliva + C.albicans T1=20min	Saliva + C.albicans T2=40min	Saliva + C.albicans T3=60min
R1 (KΩ)	0.373	0.355	5.28	5.27	0.104	0.117
R2 (KΩ)	14.6	13	6.37	7.92	19.1	26.2

Fig. 4 - Fitted EIS resistance variation data for salivary pellicle and C. albicans film at OCP

Rezultatele rezistențelor calculate din fitarea datelor EIS pentru pelicula salivară și pentru interacțiunea cu C. albicans la OCP

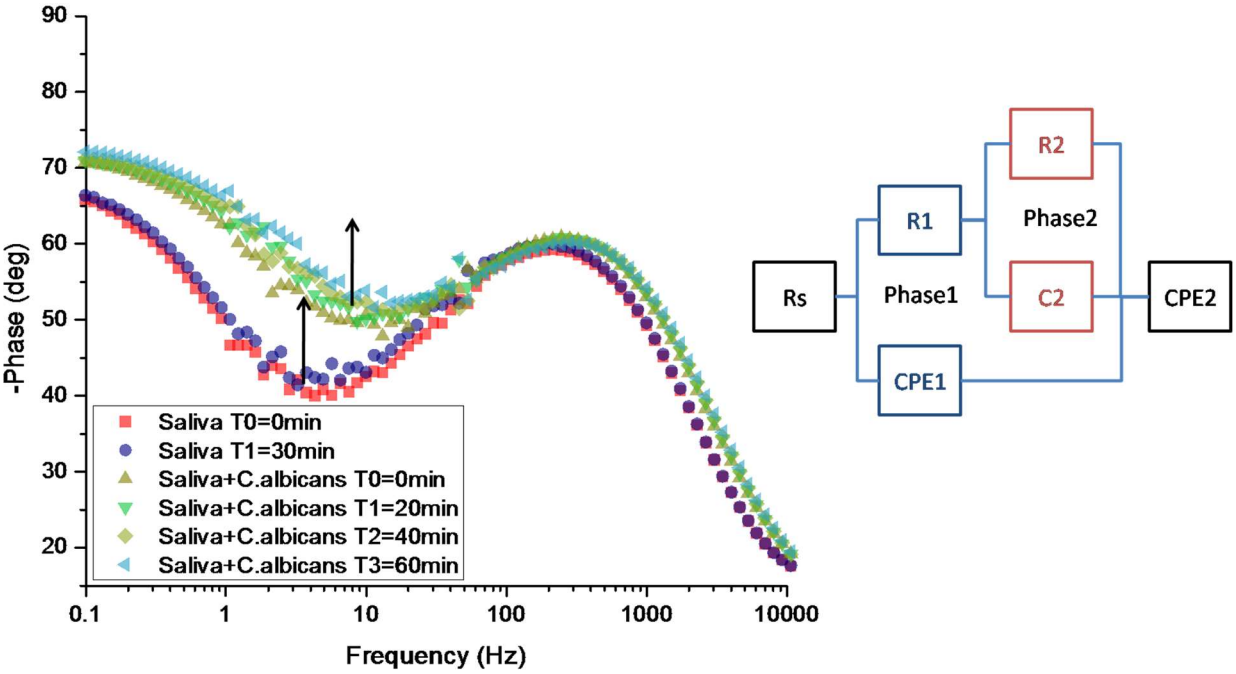
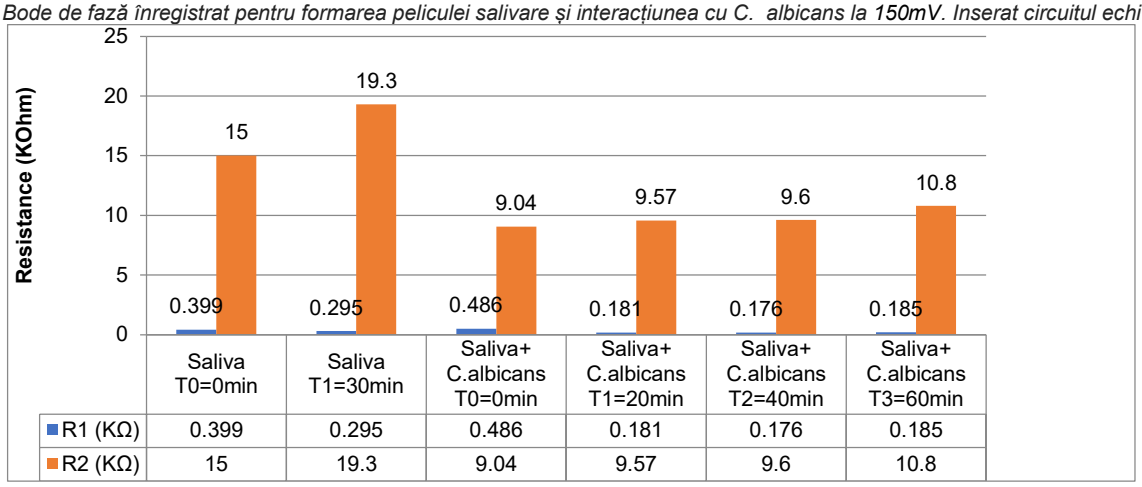


Fig. 5 - Phase Bode plot for salivary pellicle formation and *C. albicans* interaction at 150mV with inserted equivalent circuit
Grafic Bode de fază înregistrat pentru formarea peliculei salivare şi interacţiunea cu C. albicans la 150mV. Inserat circuitul echivalent.



	Saliva T0=0min	Saliva T1=30min	Saliva+ C.albicans T0=0min	Saliva+ C.albicans T1=20min	Saliva+ C.albicans T2=40min	Saliva+ C.albicans T3=60min
R1 (KΩ)	0.399	0.295	0.486	0.181	0.176	0.185
R2 (KΩ)	15	19.3	9.04	9.57	9.6	10.8

Fig. 6 - Fitted EIS resistance variation data for salivary pellicle and *C. albicans* film at 150 mV
Rezultatele rezistenţelor calculate din fitarea datelor EIS pentru pelicula salivară şi pentru interacţiunea cu C. albicans la 150 mV

Immediately after the first washing step (T0 – Saliva + *C. albicans)*, there is a decrease in resistance for the salivary film (R2) compared to the resistance value calculated for the salivary pellicle (T1 - saliva) attributed by the partial removal of the conductive species form the surface of the gold disk and the remaining film has a pseudo capacitive behavior.

In the first 20 minutes the system is composed by two separate layers: *C. albicans* suspended in saliva and an adhered salivary pellicle that are not interacting, hence the high resistance values for R1. After 40 minutes the system changes and *C. albicans* adheres to the salivary pellicle and displaces it. R2 becomes the resistance element attributed to *C. albicans* combined with the salivary pellicle. R1 represents the film formed by light salivary components dislocated from the initial film (see Figure 4).

3.4.2. Interaction at 150mV

At 150 mV the EIS data (Figure 5) for the formation of the salivary pellicle shows some differences to the ones registered at OCP. The proteic film remains composed of the two distinct phases given by the different weights of the salivary components. The formation of the salivary pellicle is accelerated producing some modifications in the film. There is an increase of the values of the element R2 (Figure 6) which gives a hint to a more compact layer on the surface of the disk.

In this case *C. albicans* adheres faster on the surface displacing the salivary pellicle. Phase 1 represents the adhered microorganisms on the salivary pellicle and Phase 2 represents the total salivary pellicle. There are no great variations in the resistance elements. R1 represents the light salivary components and R2 represents a mixture

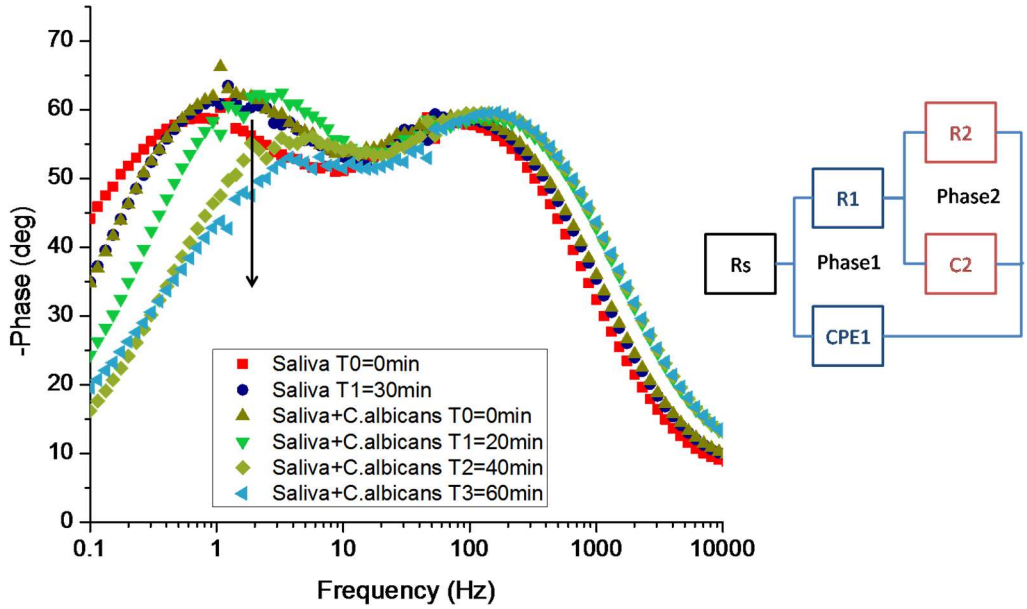
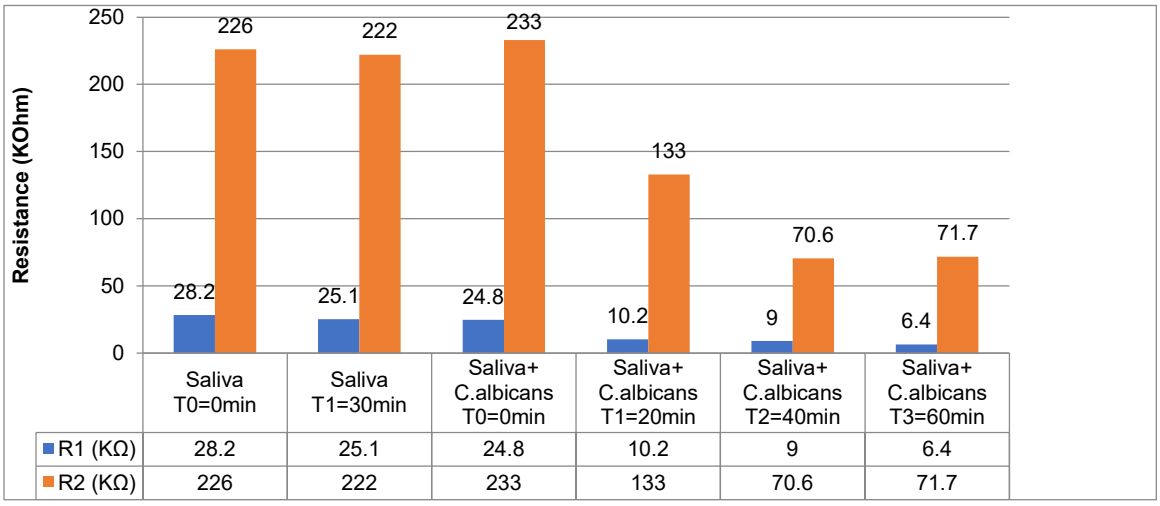


Fig. 7 - Phase Bode plot for salivary pellicle formation and *C. albicans* interaction at 300 mV with inserted equivalent circuits
Grafic Bode de fază înregistrat pentru formarea peliculei salivare și interacțiunea cu C. albicans la 300mV. Inserat circuitul echivalent.



	Saliva T0=0min	Saliva T1=30min	Saliva+ C.albicans T0=0min	Saliva+ C.albicans T1=20min	Saliva+ C.albicans T2=40min	Saliva+ C.albicans T3=60min
R1 (KΩ)	28.2	25.1	24.8	10.2	9	6.4
R2 (KΩ)	226	222	233	133	70.6	71.7

Fig. 8 - Fitted EIS resistance variation data for salivary pellicle and *C. albicans* film at 300 mV
Rezultatele rezistențelor calculate din fitarea datelor EIS pentru pelicula salivară și pentru interacțiunea cu C. albicans la 300 mV

of *C. albicans* and saliva. Both elements show a semi stabile behavior.

3.4.3. Interaction at 300 mV

At 300 mV the saliva is strongly attracted on the surface of the gold disk. The elimination of the final CPE element is due to the well-ordered structure of the film in which the phases of the salivary pellicle are well separated and exhibit a capacitive behavior (Figure 7). The resistance of the film is highly increased, a phenomenon attributed to the forming of a more compact layer. Once the *C. albicans* is introduced in the system, it is immediately attracted to the surface.

The overall resistance of the film decreases abruptly and constantly (Figure 8), but this process cannot be attributed to the degradation of the film. Instead, based on the CV curves, we can assess that there is a charge transfer process that is taking place in the film attributed to the activation of intracellular mechanisms of *C. albicans*. We believe that is the result of a chain reaction initiated by the oxidation of the Nicotinamide Adenine Dinucleotide (NADH) as a response to the oxidative stress which takes place according to the relation

$$NADH + H^+ \rightarrow NAD^+ + 2H^+ + 2e^-$$

triggered by the oxidative stress beginning around 300 mV.

3.4.4. Interaction at 600 mV

At 600 mV SPR data show the fast formation of a compact layer of proteins on the surface of the gold chip which exhibits a high capacitive behavior which remains stable

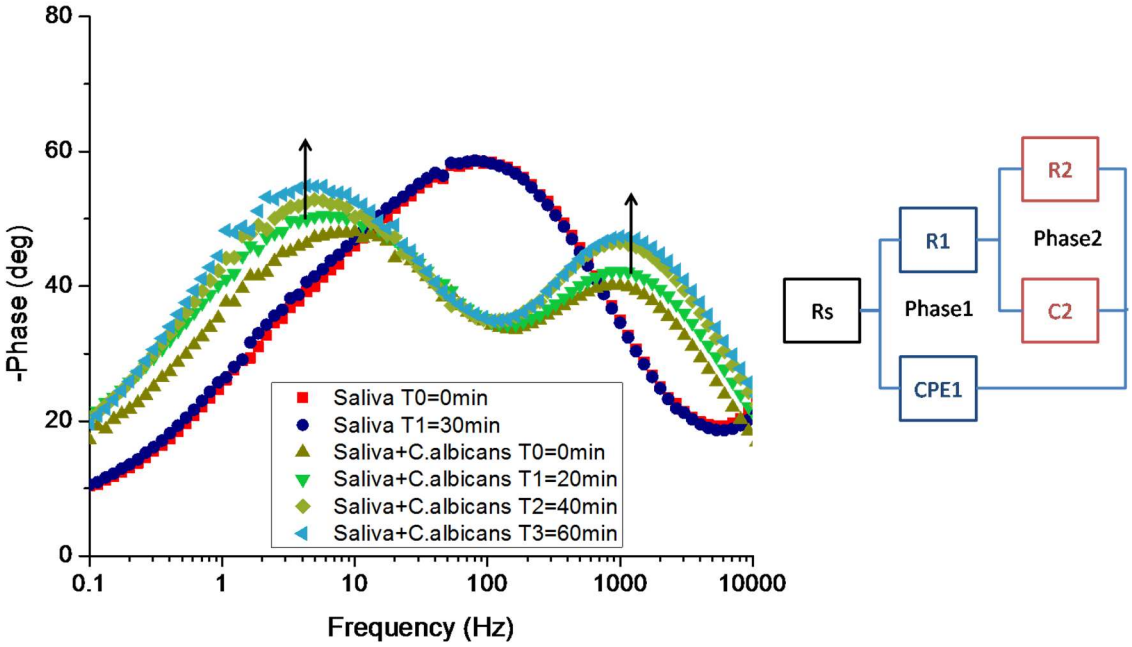


Fig. 9 - Bode plot for salivary pellicle formation and C. albicans interaction at 600 mV with inserted equivalent circuits
Grafic Bode de fază înregistrat pentru formarea peliculei salivare și interacțiunea cu C. albicans la 600 mV. Inserat circuitul echivalent.

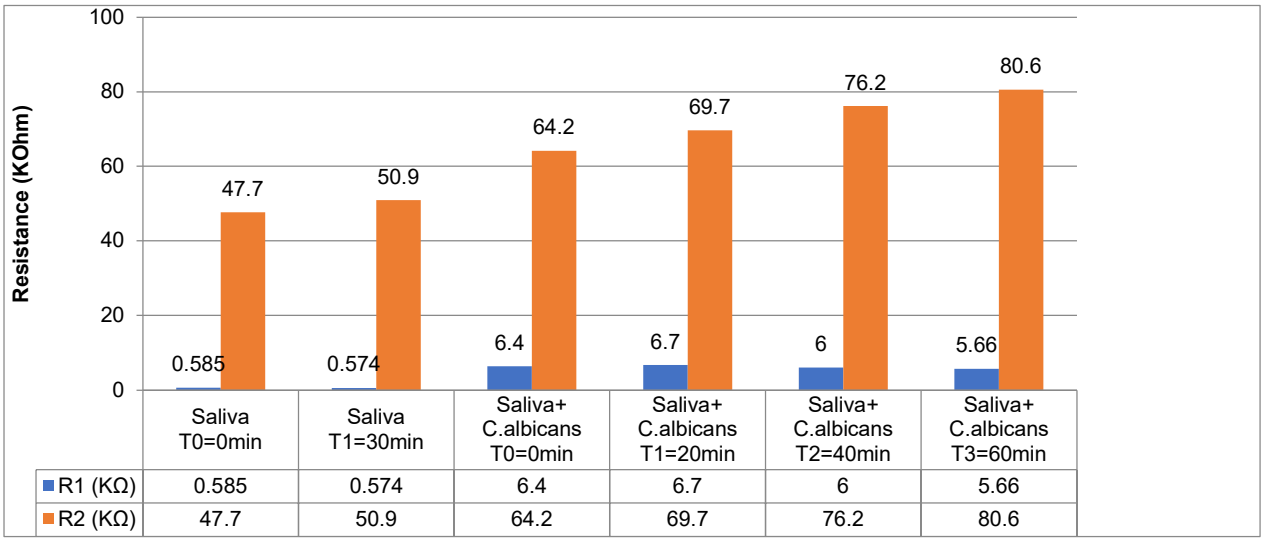


Fig. 10 - Fitted EIS resistance variation data for salivary pellicle and *C. albicans* film at 600 mV
Rezultatele rezistențelor calculate din fitarea datelor EIS pentru pelicula salivară și pentru interacțiunea cu C. albicans la 600 mV

throughout the experiment. The *C. albicans* film exhibits a constant increase in resistance. This increase of resistance that is found only in Phase 2 (Figure 10) of the equivalent circuit, attributed in the fitting circuit with the *C. albicans* pellicle and correlated with the decreasing CV signal (Figure 9), indicates the degradation of the microorganisms. As the *C. albicans* begin to die, the overall charge transfer reactions attributed to counteracting the oxidative stress begin to shut down, leading to a decrease in the CV signal and the increase of resistance observed in the EIS experiments. The remains of the microorganisms behave like a compact and resistive film.

3.5. AFM microscopy

AFM images taken for the salivary pellicle at OCP (Figure 11a) after the first washing step shows that the whole investigated surface is covered with a layer that does not exhibit any structural features and has a low roughness. This layer represents the natural saliva deposition since no electric potential was applied. AFM images taken after the 60 minutes of *C. albicans* interaction and final washing steps (Figure 11b) show the presence of *C. albicans* on the salivary pellicle. The microorganism has been found scattered on the surface of the gold chip. The dimensions of the parent yeast cells range between 200 and 400 nm, but some clusters of about 2.4 μm across have been found.

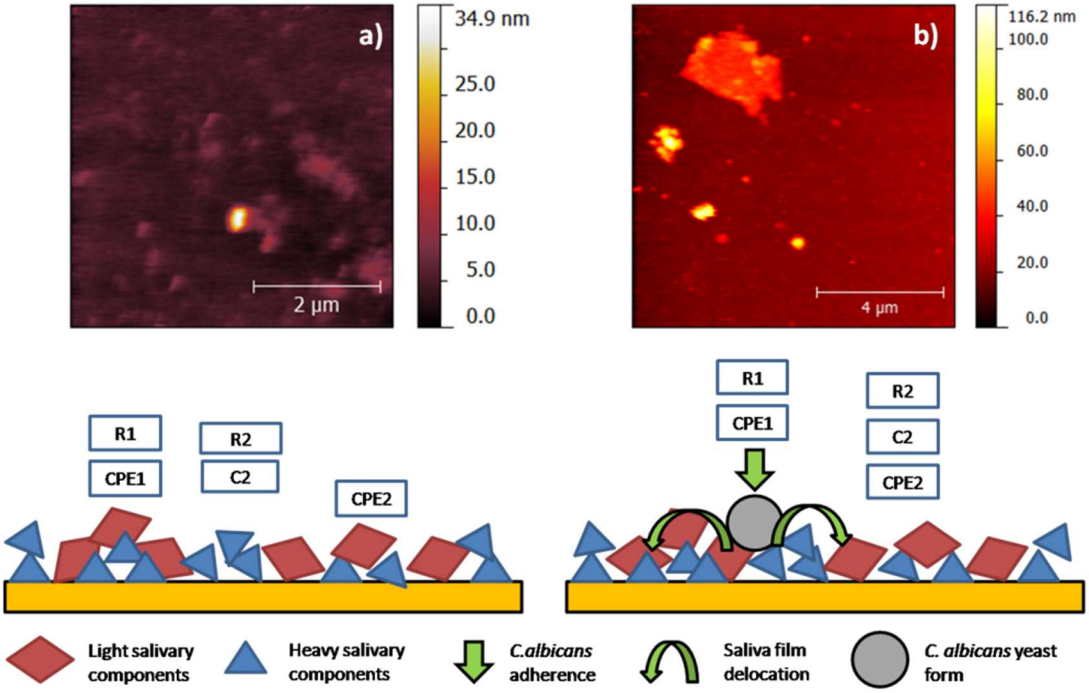


Fig. 11 - AFM topography images for saliva pellicle (a) and *C. albicans* (b) at OCP and modeled systems
Imagini topografice AFM pentru pelicula salivară (a), C. albicans (b) la OCP și sistemele modelate

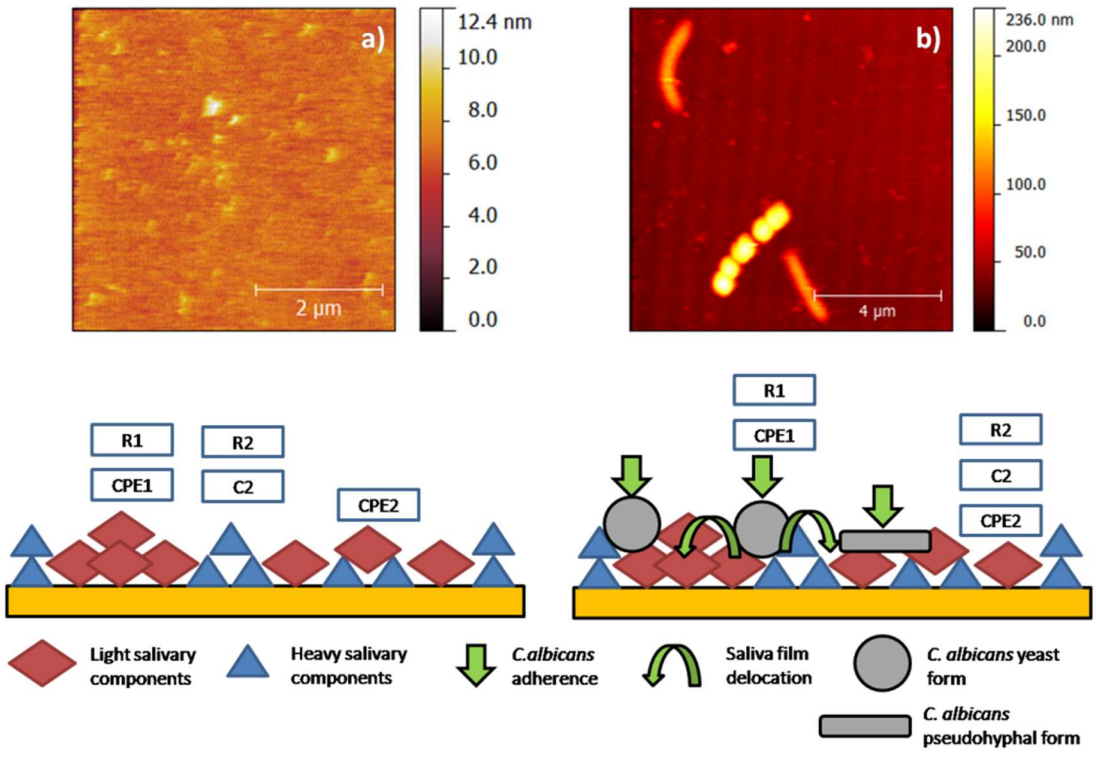


Fig. 12- AFM topography images for saliva pellicle (a) and *C. albicans* (b) at 150 mV and modeled systems
Imagini topografice AFM pentru pelicula salivară (a), C. albicans (b) la 150 mV și sistemele modelate

The AFM imagery taken for the salivary pellicle at 150 mV (Figure 12a) shows that the surface of the pellicle is more homogenous than at OCP. AFM data confirms the formation of a different type of pellicle structure based on overall aspect at this surface potential.

AFM images taken after final washing steps to confirm cell interaction to the protein pellicle reveal increased cell presence on the protein layer compared to anterior potential values. The yeast and pseudohyphae forms were found on the surface (Fig. 12.b). Yeast individuals had diameters

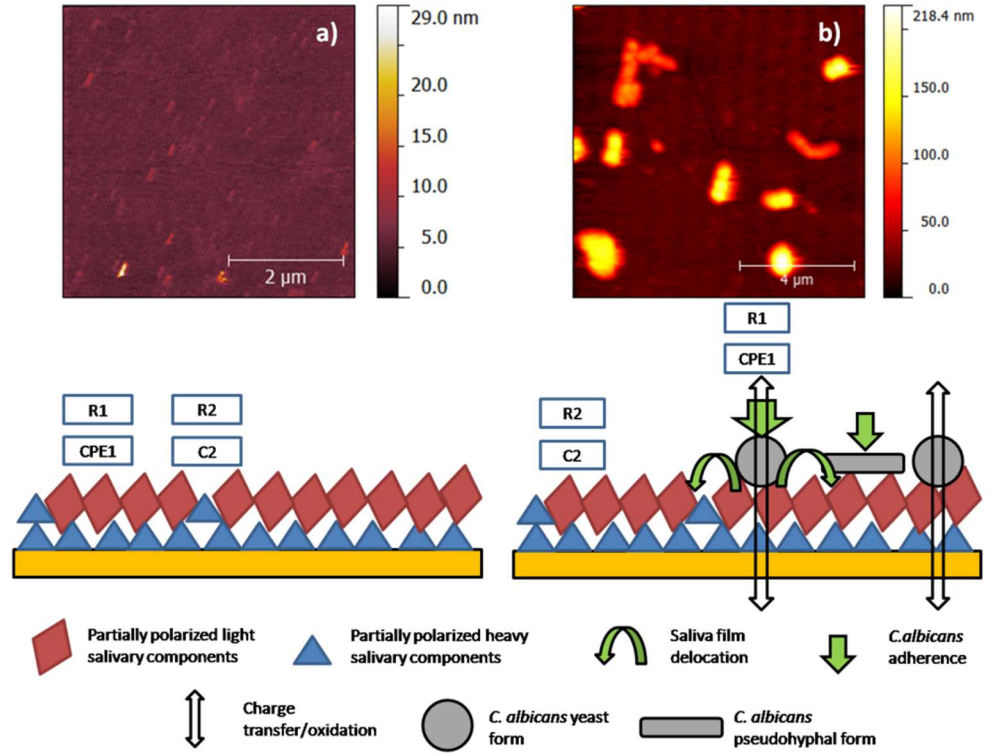


Fig. 13 - AFM topography images for saliva pellicle (a) and *C. albicans* (b) at 300 mV and modeled systems
Imagini topografice AFM pentru pelicula salivară (a), C. albicans (b) la 300 mV și sistemele modelate

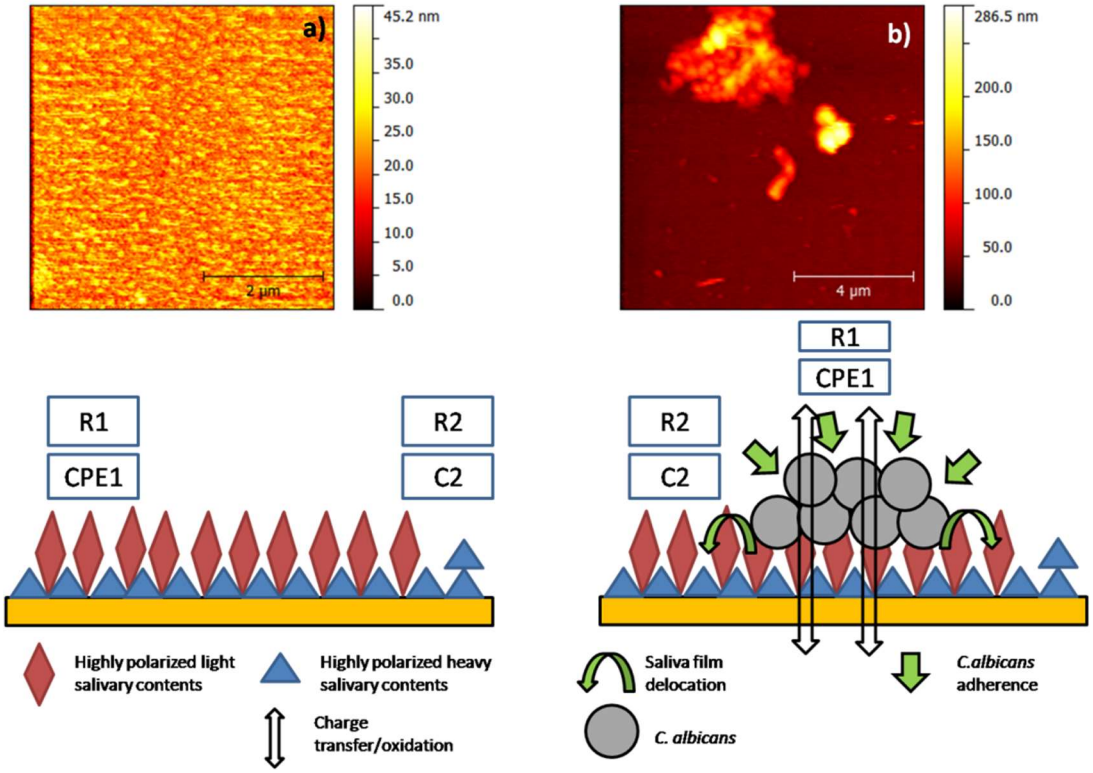


Fig. 14 - AFM topography images for saliva pellicle (a) and *C. albicans* (b) at 600 mV and modeled systems
Imagini topografice AFM pentru pelicula salivară (a), C. albicans (b) la 600 mV și sistemele modelate

of about 400 nm and the pseudohyphae were large with lengths of about 2.5 µm and widths of about 0.5 µm.

Another interesting aspect is the shape of fungal cells. Usually, globular cells are present in cultured *C. albicans* but in our case because we

cultured *C. albicans* in natural filtered saliva and reproduced the natural environment we were able to view the pseudohyphae form. We believe this to be of great importance in relation to surface specific interaction drawing as close as possible to natural interaction in the oral environment. The increased number of individuals present on the surface can be attributed to a polarization of the yeast membranes. Yeasts usually have membranes that are negatively charged at approx. -150mV [22] and such, applying a positive potential on the gold disk surface, more microorganisms are forcedly attract on the surface by depolarizing the negative part of the membrane and hyperpolarizing the positive part. The depolarization may activate the ionic channels of the membrane. It has been reported that the electrically induced activation of the Ca^{2+} ionic channels is linked to the phenomenon of galvanotropism, which activates the formation of germ tubes and pseudohyphae [22].

AFM images taken on the surface protein construct revealed a change in surface aspect happening at 300 mV (Figure 13a). It appears that the protein components of saliva were partially polarized and arranged in the same direction at this high potential.

The microorganisms have been found in both yeast and pseudohyphal forms and with an increased volume. The average diameter of the yeast form is 1.6 μm and the length of the pseudohyphae averages 3 μm (Figure 13b).

To counter the oxidative stress microorganisms have defence mecanisms that include enzimes such as NADH oxidase, glutatione reductase, catalase etc. Furtehemore, in *C. albicans* the response to oxidative stress is linked with the activation of the glucose mecanism [23]. However due to the complex metabolic reactions that involve electron transfer chains and other enzimes, coenzimes and cofactors we are not able to explain the fullness of the processes. An explanation, sustained by the AFM scans (Fig.9b) which shows the majority of the microorganisms aligned in the same direction, is that the candida cells exhibit galvanotropism at high potential. This seems to be triggered at 150 mV, but is more active in this case. Galvanotropism is closely connected whith the activation of the Ca^{2+} ionic channels which triggers the unilateral growth of pseudohyphae and the orientation of the microorganisms.

The ability to form hyphae has been proposed as a virulence factor [24], as these structures are often observed invading tissue, and strains that are unable to form hyphae are defective in causing infection. Pseudohyphae share many similarities with yeast cells [7] but their role during candidosis remains unknown; germ tube formation and hyphal elongation can be the result of polarized growth [25].

AFM imagery performed at 600mV shows a totally different protein construct that has a. distinctive aspect compared to the other potential variations. AFM images reveal a unique aspect with high surface roughness and a distinctive appearance (Fig.14a) hinting the hyperpolarization of the proteic compounds. AFM images of fungal presence (Fig.14b) are showing atypical cell association on the protein layer. These large flat structures, more than 5 μm across and with a height of about 170 nm were composed or irregular individuals. The cells were intimately grouped and presented a smaller diameter than previously seen in the other experiments although the cells used were from the same culture as in anterior experiments.

4. Conclusion

The electrical potential influence on oral bio-layer forming processes is extremely important. Based on our observations high potential variation determines the formation of distinctive microlayers. Although high potential variation has been found in dental patients, we believe that toward the upper part of the variation phenomena become markedly disturbing of physiological conditions. We have observed an increase in surface interaction of fungi with potential. Overall, the increased oral surface potential does affect adhesion in the sense of an increase. This fact is extremely important clinically because we can better understand plaque build-up on metallic surface apparently not prone for these phenomena.

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