Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 3, July - September 2013, p. 1205 - 1214

UNDERSTANDING THE CLEANING EFFECT WITH SODIUM HYPOCHLORITE OF E*NTEROCOCCUS FAECALIS* ENDODONTIC PATHOGEN USING ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY (EIS), ATOMIC FORCE MICROSCOPY (AFM) AND SURFACE PLASMON RESONANCE (SPR)

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Recurrent post-treatment endodontic infection is a frequent issue in today's dental practice. Our study presents a complex investigation on the formation of the acquired protein pellicle, a natural forming intraoral biostructure offering a novel approach in understanding this natural process. The dental pellicle can form on any structure of the oral cavity and in teeth with exposed root canal system to the oral environment. It enables subsequent interaction to a main endodontic pathogen *Enteroccus faecalis.* The present article investigates this interaction offering insight for dental clinicians. EIS observations together with AFM and SPR can offer a unique view of these complex processes and based on EIS fitting circuits we propose a model for the formation of this bio-layer and interaction with live cells. AFM images of the protein pellicle, adhesion of enterococci and effect of cleaning substances have been obtained in the main steps of our investigation confirming EIS data. Furthermore we follow the cleaning effect of sodium hypochlorite (NaOCl) on this structure with important clinical implications. SPR and AFM observations regarding action and optimal concentration were obtained by modeling a similar surface to the natural tooth dentine and simulating key conditions of the oral environment.

(Received July 4, 2013; Accepted September 7, 2013)

Keywords: ElS, SPR, *Enterococcus faecalis,* and sodium hypochlorite

1. Introduction

Many endodontic failures have as primary cause insufficient cleaning and disinfecting of the dental system[1]. One of the main microorganisms that reside in the dental endodontic system is *Enterococcus faecalis[2],* which has been frequently found in root canal-treated teeth from 30% to 90% of the cases [3]. This microorganism is reported to have a significant effect on the dentin complex allowing for growth and biofilm formation[4]. Root canal-treated teeth are more likely to harbor this microorganism than cases of primary infections reducing the positive potential of endodontic treatment[5].

Past investigations on the effect of sodium hypochlorite on the root canal wall have provided a starting point for the current investigation [1]. Based on previous published data we have successfully used EIS in dental material characterization expanding and explaining clinical implications[6]. The present study has in its focus monitoring of this cleaning substance on microbial interaction to the dental pellicle in teeth with open endodontic systems in the oral cavity[7]. The investigation uses complementary techniques EIS, AFM and SPR to better understand the clinical effect using live cells similar to oral bacteria. In our days biomedical

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engineering has introduced models [8] and physical chemical procedures and EIS has evolved from electrode process investigations and specific uses to bio-layer and medical applications[9].

2. Experimental

The present study has an interdisciplinary character being based on a twin approach; a clinical one related to the preparation of natural saliva from a clinical patient lot and a physical chemical one representing the use of EIS, SPR and AFM data.

Natural saliva was gathered from a clinical patient lot after their signing of the informed consent. Saliva has been prepared by centrifugation for 1 hour at 8000 rot/min with refrigeration and then sterile filtered with 0.2μm filters. It has been stored refrigerated at 4^0celsius.

Enterococcus Faecalis is a gram-positive bacterium frequently identified in the dental endodontic space, it is a commensal microorganism; in this study we have used bacteria strain ATCC 29212.

The bacterial strains were grown in Luria Bertani Agar (LBA) test tubes at 37 °C with medium composition: peptone, 10 g/L; yeast extract 5 g/L, NaCl 5 g/L and agar 20 g/L. The stock culture was maintained at 4 °C.

Table 1. Intra-canal electrical potential values obtained clinically

Patient	Electrical potential value (mV)
Patient 1	-70
Patient 2	-10
Patient 3	-52
Patient 4	-40
Patient 5	-25
Patient 6	-35
Patient 7	-15
Patient 8	-90
Patient 9	-20
Patient 10	-30

We resuspended the bacteria in sterile filtered saliva in order to recreate the original environment of growth favoring adherence specific surface proteins.

The main investigation used was electrochemical impedance spectroscopy done in relation to the deposition process on a gold metallic surface. Gold was preferred as being extremely stable and non-influential to organic contents moreover the process investigated has been between protein layer and enterococci. The setup was placed in a small custom electrochemical cell comprised of a metallic gold surface representing the working electrode, Ag/AgCl as reference electrode and a platinum counter electrode. Electrochemical impedance spectroscopy (EIS) measurements were conducted with Autolab PGSTAT 302N in a logarithmic distribution range between 100 kHz – 10 mHz. The EIS fitting was performed using NOVA 1.8 software. The electrochemical cell had a working analyte volume of 60μl. Measurements were done at a slight negative dentine electric potential of -15mV imposed by the potentiostat [10-12].In order to further confirm the intra-canal dentine potential we measured the surface potential value on 10 patients. The requirement was that the endodontic system had to be exposed to the oral environment [Fig.1]. The measurement was done before any cleaning, just inserting the file into the infected canal to obtain optimal values [Table1]. A Headestroem file size 30 was inserted 4mm into the dental canal representing one measurement electrode and the second was a lip clip. The -15mV value was selected as being closest to physiological conditions and it was used as imposed surface value via the Autolab potentiostat.

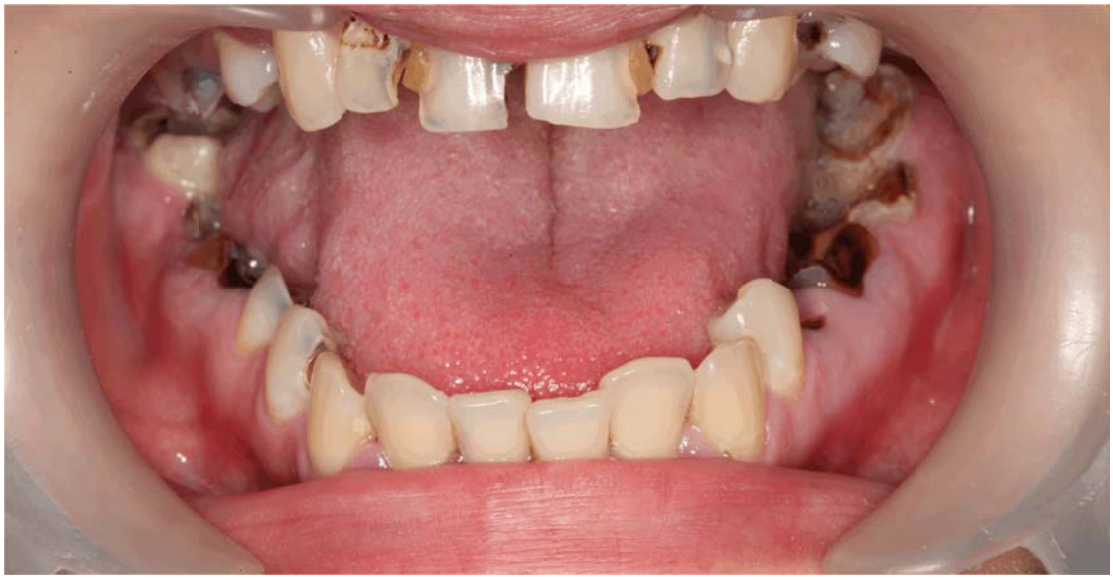


Fig.1 Example open endodontic systems in the oral environment

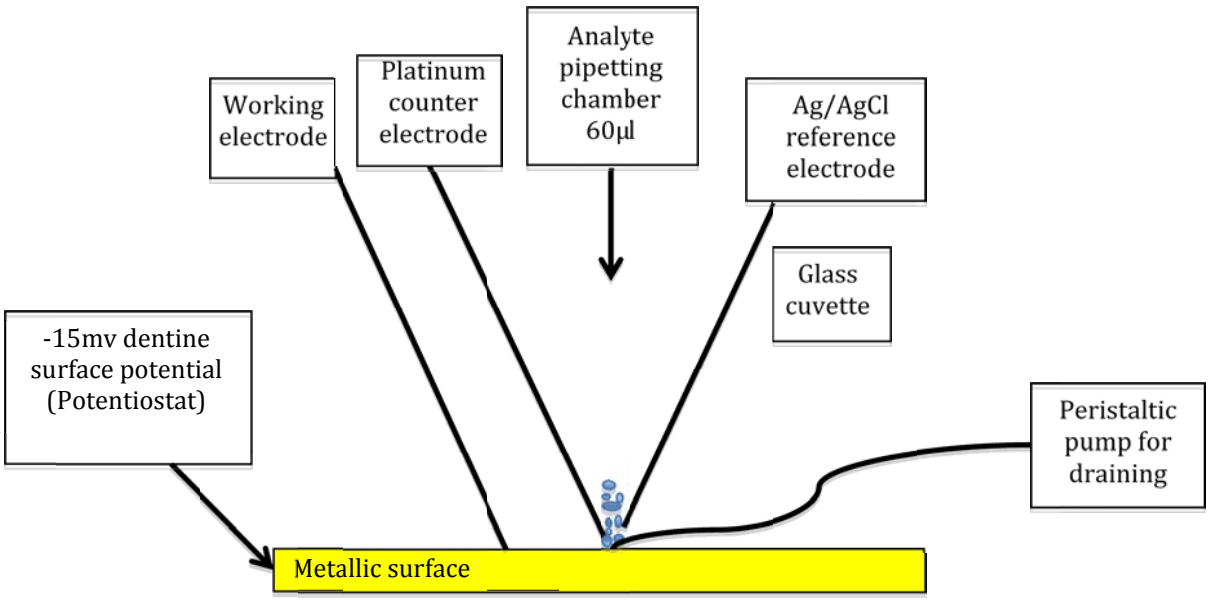


Fig.2 Diagrammatic representation of custom electrochemical cell used

In order to obtain exact SPR measurements a model was created that closely mimics the oral cavity on the gold chip. The SPR machine uses SPR gold chips. The SPR equipment is the Plasmonic® system which uses rectangular glass prisms with beveled margins coated on their upper surface with a 50µm gold layer. Each assembled chip has eight measuring channels. Each channel corresponds to a well that can sustain a volume of 20µl. The glass prisms coated with gold underwent a thorough cleaning protocol as follows: 10 minutes ultrasonication in an acetone solution and then 20 min in a 50-50% solution of KOH 0.1M and 30% hydrogen peroxide solution. Then the gold prisms were dried in vacuum at 50°C in a dry oven.

We then deposited a hydroxyapatite thin layer similar to the natural root canal. For this we used a hydroxyapatite suspension in dried ethanol and the setup was as described in a well-known

protocol [13]. After obtaining the inorganic layer to mimic the natural root we deposited filtered natural saliva on this structure to observe the formation of the acquired dental pellicle again simulating the intraoral environment. The cleaning experiment conducted used different concentrations of NaOCl: 0.5%,2%,3%, 4% respectively.

The experiment was comprised of three phases, first deposition of prepared saliva on the working electrode at the given potential, recording impedance values in a custom electrochemical cell [Fig.2,]. The second was concerned with enterococcus adherence to this protein pellicle and intimal interaction promoting specific adherence. The third phase investigated the effect of NaOCl 3% and other concentrations on intra-canal protein pellicle structures AFM images were obtained in all the key steps of our study to confirm EIS findings.

The deposition of saliva was done by pipetting a quantity of 60 micro liters in the glass cuvette canal and waiting for 30 minutes. Then we washed two times with 500µl in order to remove unbound constituents. We obtained EIS measurements at T0 initial pipetting time, T1 after 10min and after T2 20min, each measurement had a duration of 10min. We confirmed the structure of the acquired pellicle via AFM.

We then pipetted 60µl of bacteria suspended in natural saliva and waited for aprox. 40min observing interaction via EIS. After the experimental time we washed with 2x500µl of distilled water to remove unbound constituents. We then viewed the remaining bacteria and dental pellicle via AFM.

In order to understand the effect of NaOCl on adhered intra-canal bacteria we investigated the effect of a 3% sodium hypochlorite solution viewing surface interaction after application with the AFM.

AFM in contact mode was used to determine the surface changes of the after coating with oral proteins. The same procedure was applied to observe changes to the protein pellicle after enterococci adherence and modifications after sodium hypochlorite use.

3. Results

3.1 EIS and AFM in understanding acquired pellicle deposition

Initial application of prepared saliva on the metallic surface promoted the formation of the acquired protein pellicle in a similar fashion to the intraoral construct. Nyquist, Bode modulus and phase plots show the rapid formation of a resistive layer on the surface.(Fig.3)

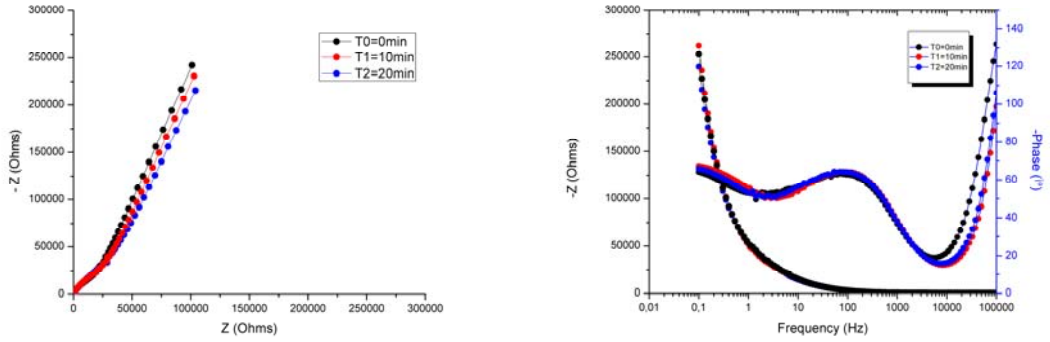
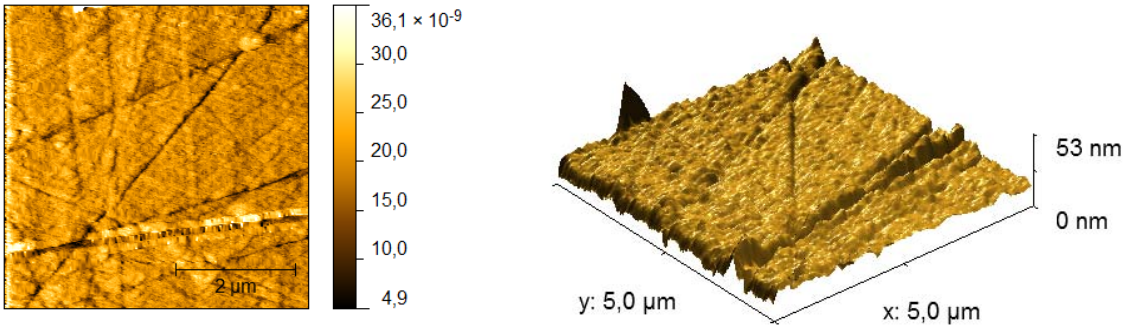


Fig. 3 Nyquist and Bode diagrams showing the formation of the salivary protein pellicle.



Ra= 2.28nm

Fig. 4 AFM image of protein layer formation after 30 minutes.

After 30 min the protein construct has been investigated by AFM surface investigation to confirm EIS findings. The image showed a reduction in surface roughness when compared to plain gold of 0.76nm (Fig.4). An initial rough surface was preferred to grossly mimic the uneven topography of root dentine.

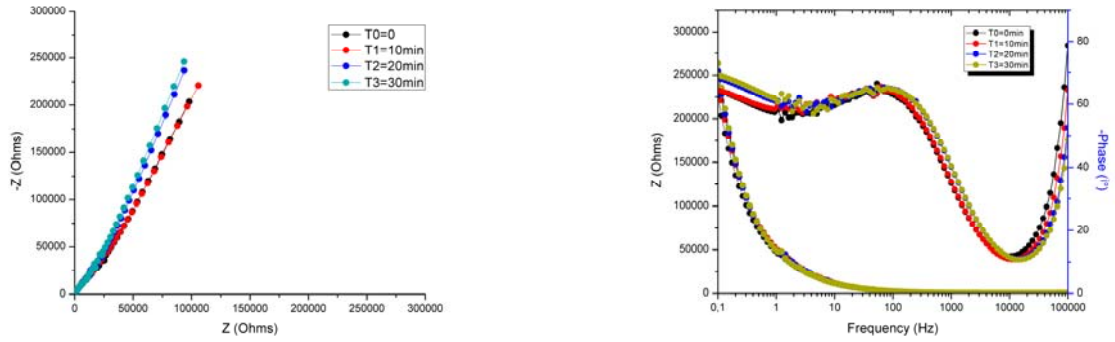


Fig.5 Nyquist vs Bode modulus and phase corresponding to Enterococcus Faecalis adhesion

Interaction of *enterococcus faecalis* with the acquired protein pellicle determined the formation of a complex layer. The construct has a paradoxical capacitive effect when viewed on the Nyquist diagram determining a capacitive not resistive trend (Fig.5). The explanation is in the intimate adhesion process of living bacteria to the protein surface. AFM confirmation of enterococci adherence was obtained. (Fig.6)

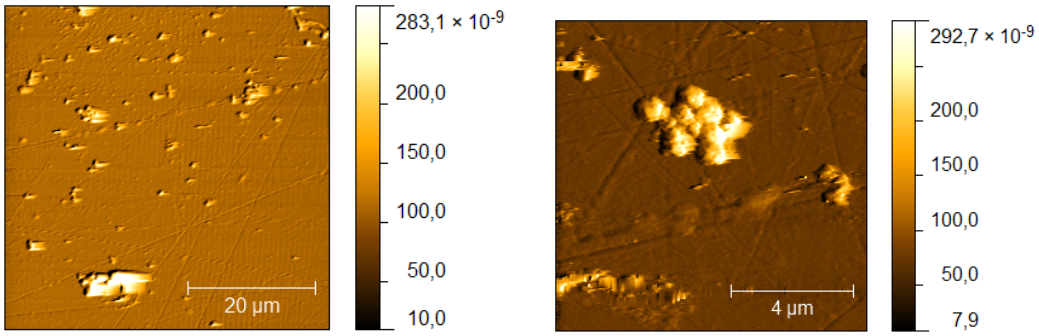
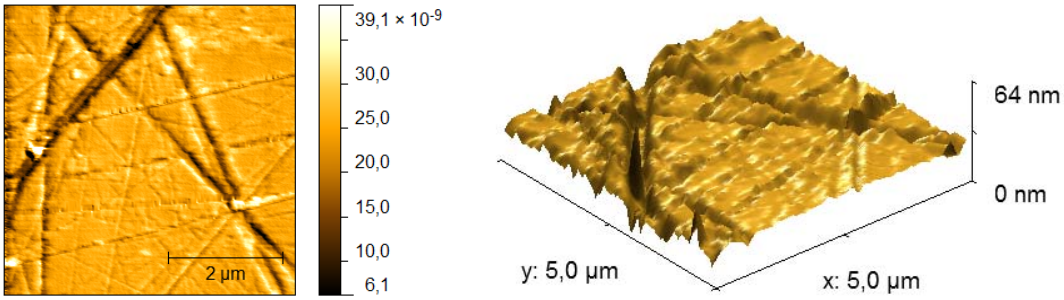


Fig.6 AFM image of remaining adhered enterococci on acquired protein pellicle formation after washing step.

The next experimental step was to wash with 3%NaOCl and observe the effect. After washing with this solution we observed a complete removal of the previously deposited layer. AFM confirmation was obtained showing a clean layer of gold with no protein or bacteria present (Fig.7).

Based on the potent effect of NaOCl we propose following the effect of different concentrations on the protein layer viewed by SPR.



Ra: 3,05 nm

Fig. 7 AFM image of clean gold after NaOCl application for 60s showing complete removal of the initial bio-layer

3.2 SPR results

Fig. 8 shows formation of the protein pellicle, washing with sodium hypochlorite and removal of adhered pellicle proteins regardless of concentration used

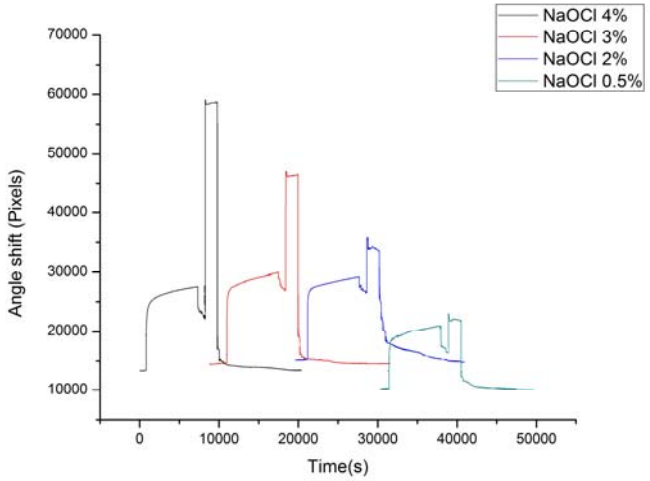


Fig.8 SPR sensogram for deposition and washing with sodium hypochlorite of various concentrations

The results were complete removal of bound proteins indifferent of cleaning solution concentration. (Fig.8)

4.Discussion

Investigations conducted with EIS revealed the two-step protein pellicle formation as viewed through the Nyquist fitting circuits with specific time constants (Fig.3). The first corresponding to low molecular weight proteins, proline rich acidic proteins and second large weight molecular proteins representing the salivary glycoproteins MG1 and MG2 and other smaller constituents[14, 15].

Table 2 Fitting circuit values for T1 and T2 salivary pellicle formation

Element	Rs - solution	R1	R2	CPE1	CPE2	C1
T0=0min	10 Ω	395 Ω	-	Y0=5.24μMho N=0.683	-	12nF
T1=10min	10Ω	430 Ω	34.1kΩ	Y0=4.55μMho N=0.733	Y0=1.40μMho N=0.86	6.85nF
T2=20min	10Ω	430 Ω	35kΩ	Y0=4.30μMho N=0.784	Y0=1.41μMho N=0.858	5.80nF

MG1 is a family of glycoproteins with mass higher than 10^6Da and MG2 is the second family of glycoproteins with lower mass values of around 1.2-1.5x10^5Da. MG1 mucin core is a large oligomeric protein composed of di-sulfide subunits; it is secreted by all major salivary glands except the parotid. It is extremely heterogenic and presents many glycoforms. Bacteria and viruses can bind to this form [16].

MG2 is a small nonmultimerizing protein found in all salivary products and has a main role of interaction to microorganisms [17]. Both of these glycoprotein families are actively involved in dental pellicle formation [18].

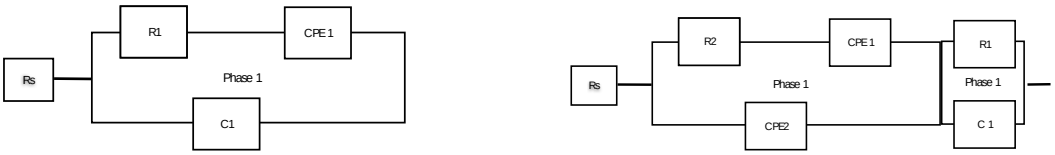


Fig.9 Presenting left T0 fitting circuit and right T1 and T2 fitting circuits

The initial fitting circuit for the T0 measurement presents just one time constant (Fig9). It represents a complex protein layer in formation. The resistance R1 has a low value of just 395 ohms showing the adsorption of low molecular weight acidic proteins to the surface. The surface potential similar to the human dentine potential promotes an acquired protein pellicle similar to the intraoral one. The CPE1 describes a diffusive electrical layer based on the n value of 0.6. Based on electrical charge and molecular weight, proteins in prepared saliva adhere to the metallic surface creating a complex construct, first step in biofilm formation. The C1 capacity describes a general character of this formation similar to a capacitive film-like layer (Table 2).

Further investigation on pellicle formation at T1 and T2 moments after 10 and 20 min show a maturation of the acquired pellicle layer with close interdependence to multi-layer formation as shown by the fitting circuits (Fig.9). The first time constant represents the low molecular weight initial adsorption to the metallic surface and constitution of the first protein layer. The second time constant represents the formation of the acquired pellicle bulk layers of protein allowing for maturation and growth as shown by R2 with increasing values from 34.1kΩ to 35kΩ. The general character of this growth is given by CPE2 showing a general diffusive character close to a capacitive trend with n values of 0.85-0.86, meaning a formation process with gaps in the structure at measurement time (Table 2). CPE 1 represents individual layer formation and based on a diffusive character given by n values of 0.7 and 0.78 hints to a dynamic protein adherence process.

Enterococcus faecalis interaction to the protein pellicle

The process of adhesion is comprised of four generic steps. (Fig.10)[18]

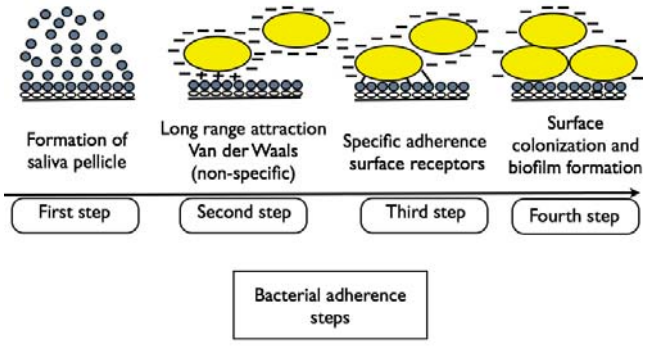


Fig.10 Diagrammatic representation of bacterial adhesion

These steps describe the process of biofilm formation. In order to understand the mechanism of bacterial adhesion and to estimate bacteria-surface interactions, the AFM has proven to be a valuable technique and roughness, as parameter, has a key role [19, 20].

Although bacteria have negatively charged surfaces and the dental structure is also negatively charged the process of adherence does occur. It has been proposed that at the micro and nano level, microorganism and protein size, there is a charge delocalization determining local positive charge on a globally negative structure. (Fig.10)[21, 22]

Furthermore adhesion is determined by two generic factors, surface roughness and surface energy[21]. Surface roughness is essential to the adherence process of bacteria in a dynamic fluidic flowing environment because at the interface between fluid and material fluid flow is reduced in relation to roughness. In our experiment we used a static environment because we believe that inside the dental root canal the situation is very similar to static one. Also the surface potential of our gold metallic surface can be set close to the dentinal one.

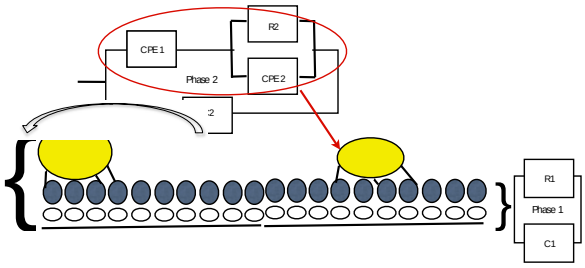


Fig.11 Diagrammatic representation of corresponance of Nyqust fitting circuit to acquired pellicle layers

When viewed with the AFM we observed tightly adhered bacteria on the protein pellicle layer proving specific adherence to this organic substrate [Fig.8]. This way we can show that there is specific interaction between the dental pellicle construct and *Enterococcus faecalis* in the dental root canal structure leading to invasion of dentin canaliculi and further surface colonization and biofilm formation[23].

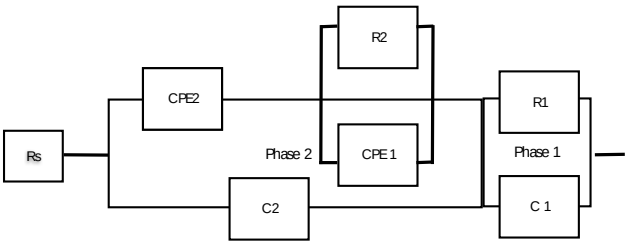


Fig.12 Fitting circuit for Nyquist diagram

The EIS fitting circuits are comprised of two time constants (Fig.11, 12). The first step describes the surface adsorption of acidic proline rich proteins that have a low molecular weight. The second complex phase describes the bulk salivary layers in formation, together with the adhesion of *Enterococcus faecalis*. The process described by this phase is extremely complex because there is a multitude of interactions occurring in a single time frame, interaction between metallic surface and salivary proteins, surface salivary adhered proteins and enterococci, inter-enterococci interaction and salivary proteins suspended in saliva and un-adhered enterococci.

Table 3 Circuit fitting values for the bacterial adherence process

Element	Rs solution	R1	R2	C1	CPE1	CPE2	C2
T0=0min	340Ω	450Ω	204kΩ	1.22μF	Y0=23.4μMho N=0.212	Y0=6.32μMho N=0.749	888nF
T1=10min	340Ω	450Ω	210kΩ	1.25μF	Y0=17.8μMho N=0.256	Y0=5.92μMho N=0.764	852nF
T2=20min	340Ω	450Ω	228kΩ	2.04μF	Y0=18.0μMho N=0.279	Y0=5.44μMho N=0.825	830nF
T3=30min	340Ω	450Ω	232kΩ	2.13μF	Y0=16.9μMho N=0.302	Y0=5.26μMho N=0.838	814nF

The C1 in the first phase describes a capacitive layer that forms almost instantaneously on a surface when exposed to low molecular weight salivary proteins (Table3). Furthermore the low resistance R1 describes this layer accordingly. The second phase complex R2, CPE2 represents bacterial layer maturation the diffusive character described by the n values of CPE2 has a growing trend towards a capacitive character again showing dynamic layer assembly (Fig.12, Table 3). R2 has an ascending trend showing that this layer increases in electrical resistance simultaneously with the process of bacterial adherence also increasing in proportion. CPE1 in conjunction with the R2, CPE2 complex enforces the description of a non-uniform layer with variable surface geometry.

As a general indicator of layer formation and evolution of growth C2 describes maturation in structure with a capacitive character reflecting complex organic layers that are in the process of assembly. In order to view the effect of 3% sodium hypochlorite endodontic cleaning solution we applied it on the bio-layer in the glass cuvette after observing the adhered enterococci for aprox.40 mins and waited for 60s.

It can be concluded that sodium hypochlorite is efficient in removal of bound intra-canal bacteria by removal of the acquired pellicle formation as viewed by AFM. Furthermore we believe that a lower concentration of sodium hypochlorite could be used clinically rendering the same powerful effect but reducing the occurrence of clinical side effects. For this purpose we use an SPR determination (Fig.8).

The experiment results conducted using different concentrations of NaOCl are presented in the sensogram in fig.8 expressing the fact that the SPR effect is sensitive to binding of analyte due to the associated increase in thickness causing a proportional increase in refractive index, observed as a shift in the resonance angle. The experiment had three important events. The first event corresponds to building the acquired protein pellicle on the artificial dental surface. The second corresponds to washing step with sodium hypochlorite and the third corresponds to the dissociation curve meaning the removal of bound protein from the hydroxyapatite surface. The results showed the strong influence of this substance in the removal of surface proteins. These proteins represent a mass of glycoproteins that attach to the surface and form a layer that is both protective to the tooth surface and also a linking interface for oral bacteria in building the oral plaque.

The cleaning effect was very powerful regardless of concentration. Even the 0.5% NaOCl removed all the adhered proteins of the acquired protein pellicle. This practically means that based on the in vitro experiments the clinical working concentration of the solution could be lowered becoming less invasive to the oral tissue while retaining its cleaning and disinfecting ability. The SPR sensogram shows a complete removal of the acquired pellicle glycoproteins rendering a surface prone to promote sealer adhesion.

5. Conclusion

EIS can be a useful procedure to monitor the formation of the acquired protein layer and can offer precious insight on intimate internal protein construct. This method offered the possibility to view low molecular weight proline rich protein adsorption and subsequent bio-layer formation. Furthermore investigations in regard to *Enterococcus faecalis* adherence to this layer as found in the oral environment in teeth with open endodontic systems showed specific interaction to the dental pellicle at dentine surface potential. This interaction and the effect of 3% NaOCl on the bio-layer have great importance on the long-term outcome of endodontic treatment. It is apparent that a 3% sodium hypochlorite solution is potent enough to completely remove the acquired protein pellicle and subsequent adhered bacteria. Also based on SPR observation we can conclude that a clinical reduction of concentration can provide optimal cleaning decreasing risk of harmful side effects.

The visualization of these complex organic layers via EIS represents an important application of the technique helping dental clinicians understand eclectic processes and bacterial interactions in the oral environment. AFM and SPR techniques support understanding of the cleaning effect with sodium hypochlorite of endodontic pathogen

Acknowledgement

Authors recognize financial support from POSDRU/107/1.5/S/76909, and would like to thank Camelia Ungureanu for live bacterial cells management and Bogdan Stoian for obtaining AFM images.

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