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2013 J. Phys.: Conf. Ser. 434 012072

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## In vivo assessment of corneal barrier function through non-invasive impedance measurements using a flexible probe.

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**Abstract.** The cornea is a transparent structure composed of three layers: the epithelium, the stroma and the endothelium. To maintain its transparency the stroma remains in a constant state of dehydration. Consequently, any ion flow disorder through the covering layers can compromise the barrier function and, therefore the corneal homeostasis. Since ionic permeability has a fundamental impact on the passive electrical properties of living tissues, in this work it is proposed and demonstrated a diagnosis method based on tetrapolar impedance measurements performed by electrodes placed on the corneal surface. The contribution of each cornea layer to the total measured impedance has been analysed over a frequency range. Following the obtained guidelines, a flexible probe with integrated electrodes has been developed and manufactured using SU-8 photoresin. The feasibility of the proposed method has been evaluated *in vivo* by monitoring corneal epithelium wound healing. Obtained impedance measurements have been compared with measurements of permeability to sodium fluorescein from different excised corneas. Successful results demonstrate the feasibility of this novel flexible sensor and its capability to quantify corneal permeability *in vivo* in a non-invasive way.

### 1. Introduction

The corneal epithelium is the outer surface of the cornea and serves as a barrier to prevent chemical, biological, and physical insults from the environment from entering into the eye. This barrier function is critical for corneal homeostasis, including maintenance of corneal transparency [1]. Because of this, quantitative measurements of the epithelium permeability are of special clinical interest. In animal models, the most commonly used technique is based on the measurement of the corneal permeability to carboxy fluorescein. However, this technique requires the excision of the corneas from euthanized animals. As an alternative, approaches based on measurement of passive electrical properties for *in-vivo* monitoring of corneal tissues were envisioned in the past [2]. In fact, such strategy was employed for assessing the corneal barrier function *in-vitro* [3–5]. Recently, Trans-layer Electrical Resistance (TER) measurements, which have consistently been used in *in vitro* studies, have been modified in

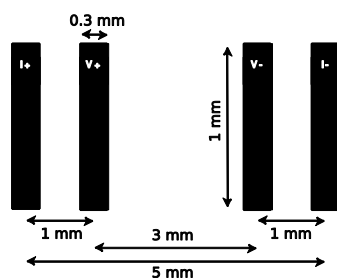
order to be implemented in living animals [6,7]. Nevertheless, invasiveness of these last approaches makes them inconvenient for continuous monitoring of corneas and absolutely banned for clinical use.

To overcome the invasiveness of the above referred techniques some numerical studies have been carried out [8,9]. Here, a novel development based on the use of a flexible substrate integrating the methodology presented in a previous work from the authors is disclosed [10]. With this new flexible probe, no pressure to ensure the electrical contact of the electrodes with the corneal surface is needed. Hence, the maximum separation between the electrodes is not limited as it was when using a rigid substrate, which increases its sensitivity. Moreover, the non-invasiveness of the presented method allows monitoring the corneal barrier function evolution in the same individual. This fact, apart from reducing the quantity of animals required to perform corneal barrier function studies, lays the first stone for its further clinical use.

## 2. Impedance sensor

### 2.1. Sensor design

The measurement method is based on tetrapolar impedance measurements in order to minimize the parasitic effects of the electrode-electrolyte interface impedances. In a tetrapolar system, the electrical properties of each sub-volume of the tissue have a different contribution to the measured impedance [11]. Consequently, the correct choice of each electrode size and the position relationship between them is important for focusing the measurement on the region of interest. In parallel, in previous studies, it was concluded that the most important parameter of the sensor geometry is the sensor width due to its relation with the spatial resolution of the measurement [9,12]. Furthermore, the contribution of each corneal layer to total measured impedance depends on the frequency range, being within the low frequency range (Freq. < 50 kHz) where the corneal epithelium has the largest contribution. However, at this frequency range the contribution of the tear film is also important, constituting a limitation in the resolution of the proposed method since little variations in the tear film thickness could produce variations on the performed measurements[10]. For that reason, among the 4 different electrode configurations available in the fabricated sensing device, the one with the biggest distance between the pair of electrodes was chosen for this study (geometry scheme shown in figure 1).



**Figure 1.** Schematic representation of the used geometrical relationship between electrodes.



**Figure 2.** Image of the sensing probe applied to rabbit's eye during the *in vivo* experiments.

### 2.2. Sensor fabrication

The sensor fabrication is based on the construction of SU-8 free standing structures over a SiO<sub>2</sub> sacrificial layer grown on a 4 inch Si wafer. The fabrication process consists of three standard photolithographic steps. The first stage defines the device structure on a 25 μm of SU-8 layer. Secondly, electrodes and metal connections were patterned on a previously evaporated 20/200 nm Ti/Au layer. Finally, a thin layer of 1 μm of SU-8 was patterned to insulate the metal tracks and define the electrodes and connections. The structures are finally obtained by etching the SiO<sub>2</sub> sacrificial layer in a HF-based chemical bath. Moreover, electrodes have been electrochemically coated with black platinum to minimize the parasitic effects produced by the electrode-electrolyte impedance [10].

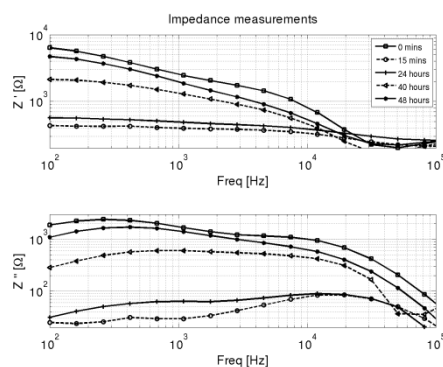
### 3. Experimental procedures

#### 3.1. Corneal epithelial wound

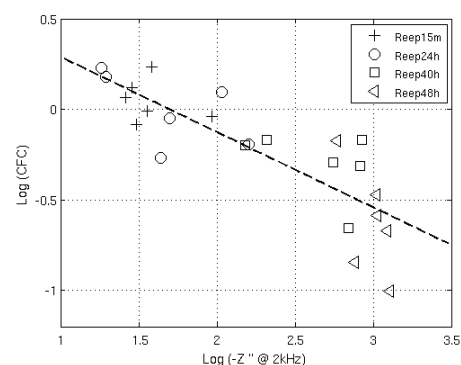
To study the wound healing of the corneal epithelium, a total of 30 New Zealand white rabbits weighing between 2.5 and 3 kg were used. The rabbits were deeply anaesthetized with an intramuscular injection (50 mg/kg of ketamine plus 7 mg/kg of Xilacine). Then, both eyes were kept open by a blepharostat and a circular wound was performed in the central corneal epithelium by applying a 6 mm diameter paper disc soaked with 10  $\mu$ l of n-heptanol for 30 seconds. After removing the paper disk, the corneas were rinsed with 10 ml of sterile saline solution. It has been reported that this protocol generates an epithelial wound with little or even no damage to the underlying stroma [13,14].

#### 3.2. Epithelial permeability to sodium fluorescein measurements

Corneal epithelial permeability to fluorescein was evaluated as an indicator of corneal epithelial integrity. After sodium fluorescein instillation (50  $\mu$ l, 1 mg/ml) to the ocular surface, animals were euthanized with intravenous pentobarbital at 200 mg/kg and corneas were excised. Homogenization of each cornea was performed in 300  $\mu$ l of PBS and samples were centrifuged at 14000 rpm and 4°C during 10 minutes. Fluorescence in supernatants and in a fluorescein standard was measured using a Victor II plate reader (Perkin Elmer) by duplicate.



**Figure 3.** Representation of the impedance measurements for the following-up of the corneal epithelial wound healing process. Data is presented as the mean value of each group (n=6).



**Figure 4.** Representation of the Corneal Fluorescein Content (CFC) in function of the impedance imaginary part at 2 kHz. Dash line shows a linear regression of the logarithmic values of both magnitudes.

#### 3.3. Impedance measurements

A custom made tetrapolar impedance analysis system was used to perform impedance spectroscopy measurements as it was described in a previous work [10]. To evaluate the wound healing, both impedance and permeability to sodium fluorescein measurements were carried out before and after (at 15 min, 24 h, 40 h and 48 h) damage. Accordingly, each group of measurements is composed by 6 eyes.

### 4. Results and discussion

Fig. 3 shows the impedance measurements performed to monitor the wound healing process of the corneal epithelium. These results are presented by plotting the mean of all the measurements for each group. As expected, the measured impedance increases with the time after wounding can be observed. It is interesting to note that the last measurement do not recover the basal value, which denotes that the healing process has not finished yet. The imaginary part of the impedance measured at 2 kHz has been proposed as an indicator of the corneal epithelium permeability. This choice is based on previously

simulation results [9], where is shown that, within this frequency range, there is an optimum compromise between the corneal epithelium and the tear film contributions. Thus, the measurement is minimally affected by the tear film. Moreover, the tear film should not contribute to the imaginary part as it is mainly composed by a saline solution. It can be observed that its value after wounding is almost null, which denotes that it is mainly affected by the corneal epithelium.

Table 1 show the data from both the fluorescein measurements and the proposed indicator mentioned above, which were performed in the same individuals and at the same time. As far as both measurements correspond to the same phenomena, a similar behaviour is expected. To show this relation both logarithmic values are depicted in Fig. 4, where a trend can be perceived although the variability of the data in both measurements methods. In particular, the lack of sensitivity of fluorescein quantification in corneas at 40 and 48 hours post wounding, which had very low fluorescein content. Moreover and to better show this trend, a linear regression of the logarithmic values of both magnitudes is represented (dash line). These results indicates that the non-invasive tetrapolar impedance measurements described in this work can be used for *in-vivo* experiments, paving the way for further clinical use of this technique.

**Table 1.** Summary of the experimental data. Data presented as mean  $\pm$  Std from 6 eyes per group.

Time	Corneal fluorescein (CFC) [mg]		Impedance [k $\Omega$ ] at 2kHz	
	Mean $\pm$ Std	$\Delta$ (%)	Mean $\pm$ Std	$\Delta$ (%)
Pre-wounding	0,11 $\pm$ 0,05	0	1,22 $\pm$ 0,23	0 %
15 minutes	1,12 $\pm$ 0,33	918 %	0,04 $\pm$ 0,03	-96,7 %
Post-wounding	1,00 $\pm$ 0,47	809 %	0,07 $\pm$ 0,06	-94,3 %
24 hours	0,50 $\pm$ 0,17	354 %	0,55 $\pm$ 0,30	-54,9 %
40 hours	0,24 $\pm$ 0,21	118 %	0,99 $\pm$ 0,27	-18,9 %
48 hours				

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