Multilayer ceramics as integration platform for sensors in in vitro cell culture reactors

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Abstract

Monitoring systems that are capable to record neuronal activity in in vitro cell cultures are prerequisite to the comprehensive investigation of neuronal processes. Low temperature cofired ceramics are a suitable platform for rapid prototyping of biological reactors, entailing a wide assortment of integration-capable sensors. Neuronal spikes capture is fundamental for understanding of the signal propagation within the neuronal network. It requires reliable electrodes, which can be arranged 3-dimensionally in an in vitro cell culture. Thick film gold electrodes have been proven for such applications, however their characteristics especially at small dimensions stray strongly. This work investigates thin films separating small thick film gold electrodes and an electrolyte solution with regard to their influence on the charge transport processes in such systems. PEDOT:PSS layer and TiO_xN_y deposited on LTCC gold electrodes, including their impedance characteristics are discussed and compared. TiO_xN_y layers with serial resistance R_s of 32 kΩ and serial capacitance C_s of 4.1 pF measured at 1 kHz are proposed to be the used as sensing elements in 3-dimensional in vitro cell cultures. Copyright © 2018 VBRI Press.

Keywords: Bio-reactors, low temperature co-fired ceramics, LTCC, thick film technology, sensor integration

Introduction

Biological assays require constant conditions for an adequate management of fluid supply, guaranteeing uniform nutrient supply, constant temperature and reliable removal of metabolic products. Modern microfabrication offers a wide range of technologies enabling the design of bio-microreactors. Exemplarily, low temperature cofired ceramic technology (LTCC) provides a technology platform for rapid prototyping of microreactors with integrated electronics [1], [2]. **Fig. 1** depicts a reactor prototype manufactured using this technology.

The reactor contains a mixer structure [3]. Along the mixing path the fluid is tempered to 37°C [2]. The temperature control has an accuracy of 0.2 K for temperatures below 60°C. The current construction contains two independent heater elements, allowing an adequate temperature management. The flow sensor monitors the throughput. It has a linear characteristic up to maximum flow volume of 80 µl/min [4]. A light shaft provides the possibility to illuminate the assay chamber. The assay chamber can be equipped with different assays. Wire bonds connect sawed vias of mini-ceramic boards with the base plate. These are perpendicularly put through slots in the same. Epoxy seals the remaining gaps **(Fig. 1 b)**.

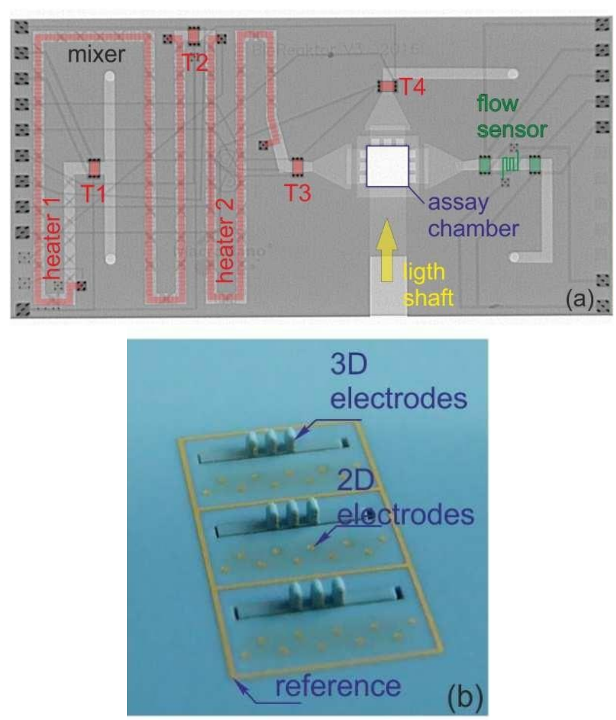

Fig. 1. Ceramic bio-reactor. (a) X-ray image, visualizing integrated mixer, temperature control and flow monitoring; (b) ceramic contact board, perpendicularly mounted through slots of the ceramic base plate.

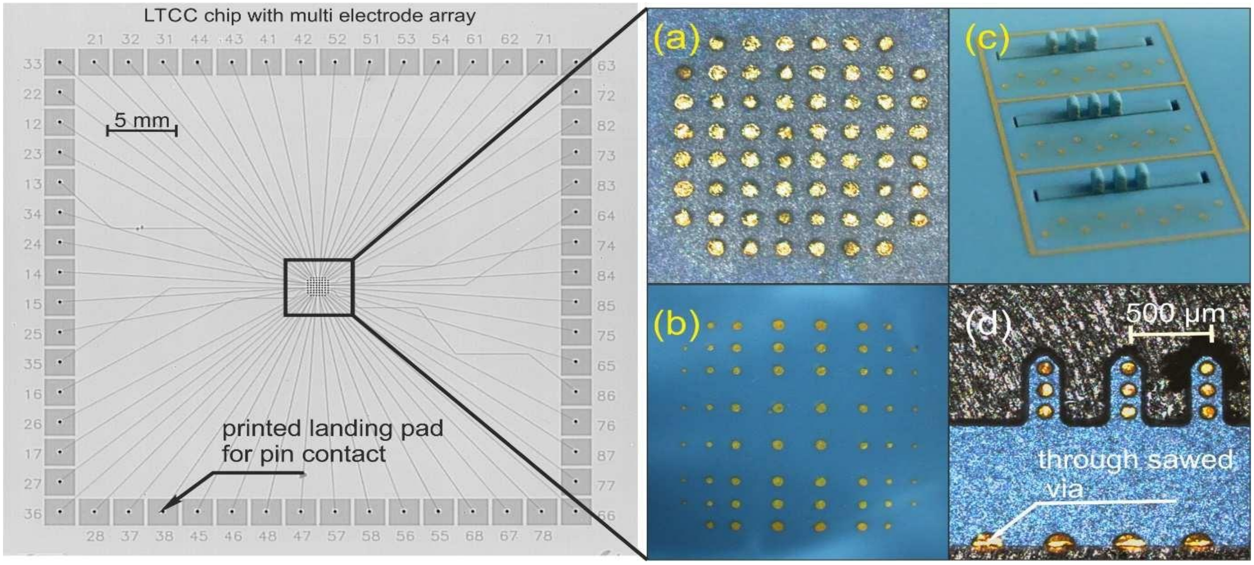

Fig. 2. X-ray image of the LTCC chip with 2D electrode layout; the respective electrode area is varied in three types:

a) Type a: Array of 60 test electrodes with 86 µm diameter; the chip type was designed for the assessment of functionalization layers on small electrodes.
b) Type b: Array of test electrodes with a diameter of 86, 129,172 and 215 µm; it is designed for the study of electrode size effects.
c) Type c: 3D test chip with 27 electrodes assembled perpendicular (diameter 86 µm) and 30 electrodes on the base plate; this chip type allows 3-dimensional signal capturing in cell tissue.
d) Miniaturized ceramic contact board with 86 µm electrodes, arranged with a pitch of 150 µm on electrode fingers.

Materials and methods

LTCC multielectrode arrays can be used for the monitoring of cell cultures [5]. The suitability of LTCC chips with 3-dimensional thick film gold electrodes for capturing of neuronal signals was proven using primary rat hippocampal and cortical cell cultures prepared from E 17 over a period of more than six months [6]. Nevertheless, there is a need of improvement for the thick film gold electrodes. Surface adsorbates at gold electrodes effect the transition characteristic of the sensors. Plasma treatment [7] and wet chemical cleaning [8] change the behavior for the better. In this work, different functionalization on thick film gold electrodes are assed concerning an improved impedance characteristic.

Test chip types

The investigations in this work use LTCC test chips containing different multi electrode arrays (MEA). The test chip types are depicted in **Fig. 2**. Printed contacts provide an interface to the impedance measurement adapter. Ceramic chips are made of 4 layers Green Tape™ DP 951 PX (DuPont Nemours). The electrodes are screen printed using the gold metallization DP 5740A (DuPont Nemours). Buried wirings and landing pads are screen printed using the same paste. For 3D MEAs, a miniaturized ceramic contact board is prepared using 4 layers of Green Tape™ DP 951 PT (DuPont Nemours). Electrodes and wiring are screen printed with the gold paste DP 5740A. The contour is laser cut in the green state. After firing it has a thickness of 400 µm. On 3D chips, three of these elements are assembled through slots, which are laser cut into a 2D LTCC base plate **(see Fig. 2 c and d)**. The electrical contact is accomplished over wire bonds at the backside of the ceramic chip, pulled from the cut surface through the via elements to the screen-printed landing pads on the backside of the base plate. The remaining gaps between the elements and base plate are sealed using the epoxy resin (HIE, IKTZ GmbH Jena) from the backside. A silica ring with an inner diameter of 11 mm and a height of 7 mm is glued around the multi-electrode area using PDMS (Silguard 184) in order to provide a container for the cell culture medium.

Test procedure

Cleaning

All samples were cleaned for 20 minutes in acetone, 20 minutes in isopropanol and finally rinsed 5 minutes in distilled water. Drying was carried out under nitrogen flow using an air blow gun. Subsequently, they were treated in oxygen plasma for 5 minutes at 200 W.

Laminin coating

Laminin is an extracellular matrix protein and it is often used to provide a suitable environment for in vitro cell cultures. It has a positive influence on cell adhesion and is thus often used to cover the inorganic surface of cell culture reactors. The conducted test aimed to evaluate the influence of this coating on signal transmission through this coating. One chip type c with 30 2D electrodes and 27 3D electrodes was used to perform the study in order to simulate multiple use of this type, which contains glue joints prone to leakage. Beside of the information about the influence of the coating, changes caused by repeated steps including coating and cleaning on the electrode impedance and chip assemblage are tested. All preparation steps were performed under sterile conditions in a laminar flow hood. The MEA preparation protocol includes cleaning for 15 minutes with 70 % ethanol, rinsing with distilled water, air-dry under sterile conditions, coating with poly(ethyleneimine) (PEI, 0.05% w/v, Sigma Aldrich, Taufkirchen, Germany in aq. dest. filled into the MEA, stored for 1 h dwell time at 20°C and removed), four times rinsing of the MEA with aqua dest. and drying. The prepared MEAs are laminin coated: 100 ml laminin (Sigma Aldrich) : 5 ml sterile buffered saline solution (PBS) are filled in the MEA, stored for 1 h and then replaced by Dulbecco's Modified Eagle's Medium. 10 % fetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin are added. This cell culture medium is named 10 % DM in the following.

A solution of 1g Tergazym (ALCONOX/Z273287-1EA, Sigma Alsrich) dissolved in 100 ml aqua dest. Is used for recycling. This tergazym cleaning removes residues from cell culture growth. Multiple chip use involves thus numerous cycles of coating and tergazym cleaning. Chips are stored in the cleaning agent overnight at room temperature. After rinsing and drying they can be handled again.

PEDOT: PSS functionalization

Thick film gold electrodes are coated with a doctor knife with Clevios^{TM} PH 1000 (Heraeus). The wetting agent Triton^{TM} X-100 (Sigma Aldrich) with a concentration of 0.01 % was added in two experiments. Further parameters are drying temperature and blade gap (see **Table 1**). Curing is carried out at 200°C for 30 minutes with a ramp of 30 minutes in a lab furnace.

Table 1. Parameter variation for PEDOT:PSS coating.

Chip N°	Blade gap [µm]	Wetting agent [%]	Drying temp. [°C]	Mg Imp @ 1 kHz [kΩ]	Phase angle @ 1 kHz [°]	R_s @ 1kHz MW [kΩ]	C_s [pF]
22	150	no	65°	81	54.5	37	2.5
23	300	0.01	65°	281	67	55	1.0
24	300	0.01	75°	333	70	53	0.9

The dispersion wets the electrodes well independently form wetting agent content. Impedance measurements at 1 kHz are executed. The blade gap has been found to be the strongest influence on the layer thickness. It causes an evident effect on the electrical characteristic. Fig. 6 depicts the results measured on sample 22 in three independent cycles and Fig. 7 the respective Nyquist plot of median values.

TiO_xN_y functionalization

The impedance change caused by sputter deposition of TiO_xN_y layers is assessed using three chips type a. 25 nm thick layers are sputtered on thick film gold electrodes in a RF plasma with a power of 200 W applied for 2 min at a total process pressure of 1.3 mbar (gas flow 80 Sccm N_2 : 0.5 Sccm O_2) The thickness of the films is 25 nm. Serial resistance and serial capacitance box plots are depicted in Fig. 6 a and b. Fig. 7 shows Nyquist plot of the median values obtained from all measurements.

Impedance measurement

The measurement adapter bases on the integrated circuit (IC) RHD213 (Intan Technologies LLC). It is designed for high-impedance monitoring of the biopotential. These chips encompass a complete acquisition system for electrophysiological signals consisting in: (i) low-noise amplifier array with analogue front-end; (ii) 16-bit analogue-to-digital converter (ADC, multiplexed); (iii) flexible electrode impedance measurement module; (iv) digital serial bus interface. Two IC are connected over 60 spring contacts with the landing pads of the LTCC chip. An aluminium frame guarantees mechanical stability and electromagnetic shielding. An USB/FPGA interface board (Opal Kelly XEM6010) connects the measurement setup with the control PC and the software RHD2000 (Intan Technologies LLC) controls the measurement.

PBS serves as electrolyte for the all measurements. A two-electrode setup is used with thick film gold as working electrode and platinum wire as counter/reference electrode. The frequency was swept between 20 – 5,000 Hz. Derived from the signal characteristic of neuronal spikes, 1 kHz is considered as nominal value for impedance measurements. Statistical analyses were realized via the software MiniTab17.

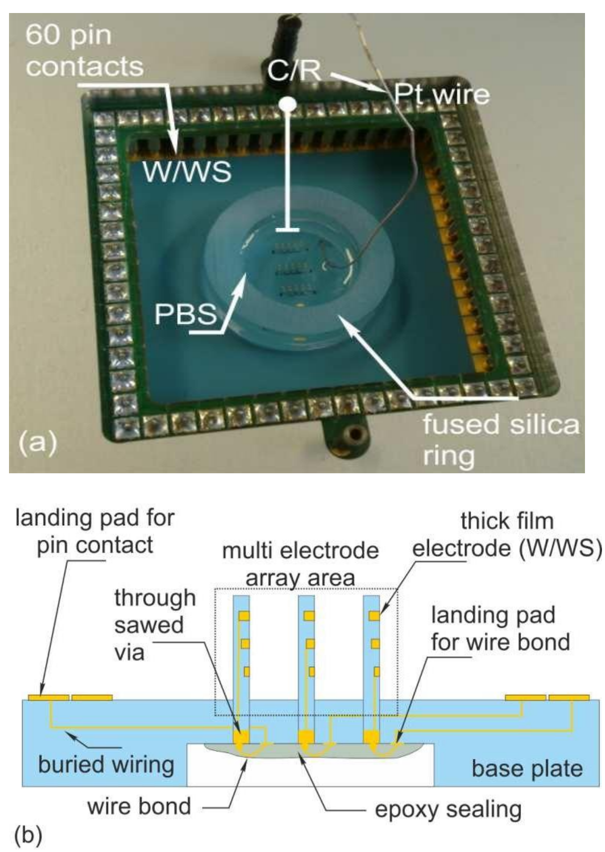

Fig. 3. LTCC chip mounted in the impedance measurement adapter (example of type c chip).

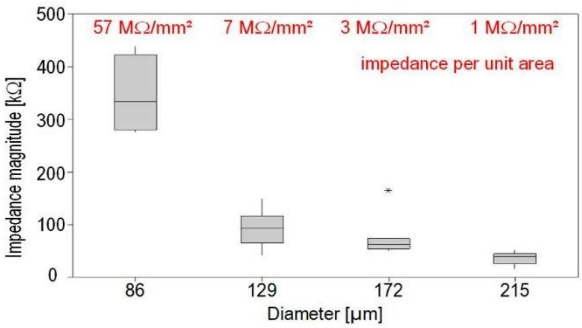

Fig. 4. Impedance magnitude dependency on electrode diameter (thick film gold electrodes, nominal frequency 1 kHz, chip type b).

Results and discussion

Electrode size influence

MEA type b allows the evaluation of electrode size effects. The layout contains thick film gold electrodes with electrode diameter from 86 µm to 215 µm, allocated in rows. Impedance measurement on uncoated chips are performed. **Fig. 4** presents the impedance magnitude box plot at nominal frequency 1 kHz as a function of the electrode diameter.

Unsurprisingly, the impedance magnitude rises when diameter becomes smaller. Considering 86-µm-electrodes and 129-µm-electrodes, the impedance per unit area increases by a factor of 8. The respective surface area ratio corresponds to 2.25. A significant increase of the variation for smaller electrodes is evident. Field distortion at the electrode circumference cause this non-linearity. The consequential high signal attenuation on small electrodes causes high measurement uncertainty.

Influence of the laminin coating and repetitive applications

Laminin coated type c chips are filled with 10 % DM and impedance was measurement at 1 kHz after 24 h storage at 37°C. Impedance measurement were carried out after 7 days dwell time. Measured chips were treated with tergazyme following the above-mentioned protocol. Iterating the process four times (cycle 1-4), the data depicted in **Fig. 5** are obtained. The test reveals that coating significantly decreases impedance magnitude. It reduces to one half compared with uncoated ones. A significant deviation appeared in cycle 2. The effect is attributed to different layer thickness caused by manual coating. Nevertheless, the tests have shown that repetitive use is not harmful for thick film electrode performance. Impedances measured in cycle 4 fall below of these of the first trial. Scaling effects reduce significantly: although impedance per unit area of 86-µm-electrodes was higher, the factor diminish to less than three.

Functionalization influence

Coatings improve the impedance characteristic significantly: the serial resistance of 86-µm-electrodes reduces when functionalization layers are applied on the thick film surface.

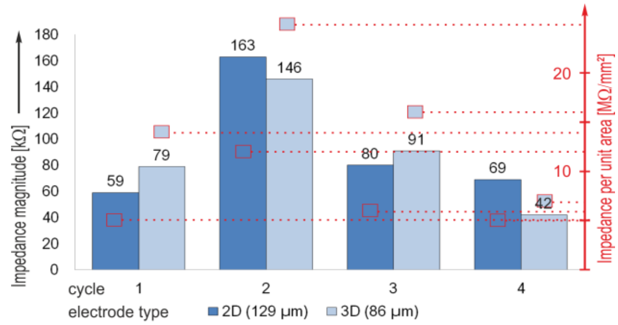

Fig. 5. Median of the electrode impedance magnitude and impedance per unit area, measured at 1 kHz for the type c MEA, thick film gold. One cycle encompasses the laminin coating after the described protocol, after each cycle the MEAs are cleaned with tergazym. After repeated coating the measurement was repeated.

Fig. 6 reveals that thick film gold electrodes show low Cs-values (0.35 pF @ 1 kHz) and high R_s-values (117 kΩ@ 1 kHz). **Fig. 7** compares Nyquist plots of uncoated and coated electrodes. Thick film gold electrodes have a circular characteristic, at low frequencies capacitive behaviour dominates. A simple serial equivalent circuit (SEC) can approximate the electrode. It consists of a serial resistance R_s and serial capacitance Cs. This characteristic involves high signal attenuation at low frequency. The characteristic of the coated electrodes is more complex because charge transfer and diffusion are present. Zeta potential and charge transfer mechanism differs significantly. **Fig. 8** proposes an equivalent circuit, which considers additional components.

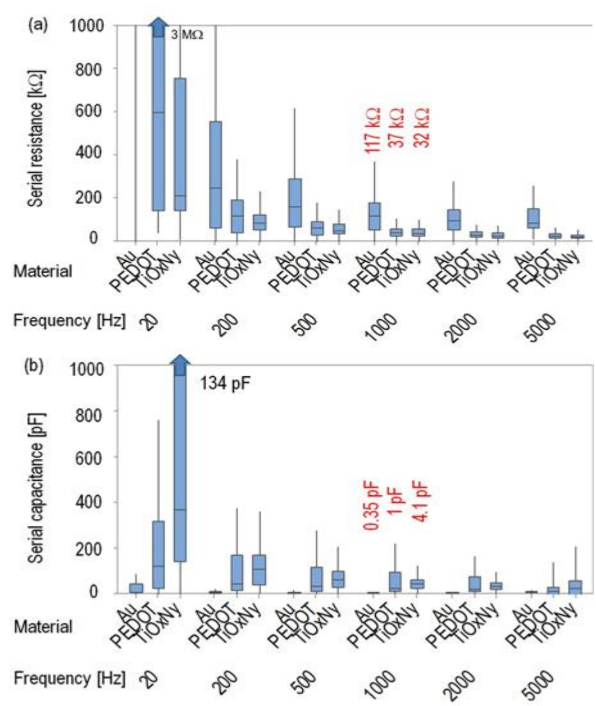

Fig. 6. SEC elements plotted over frequency, comparison of measurements on 86 µm electrodes; data base: Au = pure thick film gold – type c; PEDOT = PEDOT:PSS coated thick film gold – type a and TiO_xN_y = sputter coated thick film gold – type a; a) serial resistance; b) serial capacitance.

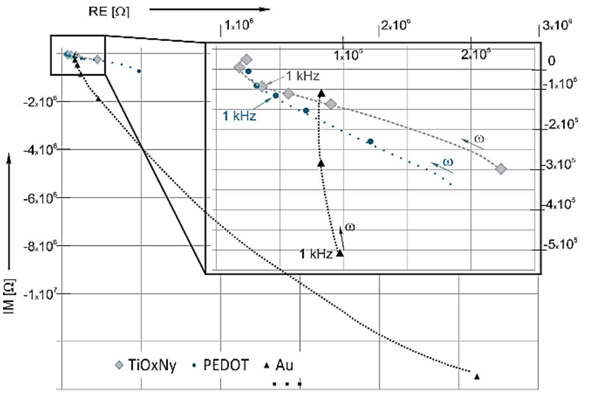

Fig. 7. Nyquist plot of median values for thick film gold electrodes, pure and coated with PEDOT:PSS and TiO_xN_y. The data points for PEDOT:PSS and TiO_xN_y represent the medians of all measurements on chip of type a. The data points for Au represent the medians of all measurements on 86 µm electrodes on chip of type c.

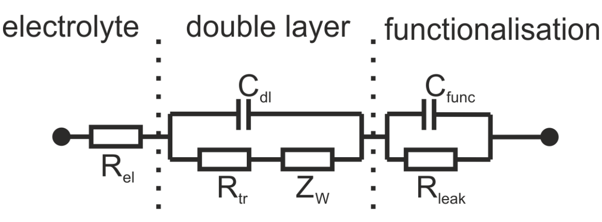

Fig. 8. Equivalent circuit of a functionalized electrode. R_{el}-resistance of the electrolyte; C_{dl}-double layer capacitance; R_{tr}- charge transfer resistance; Z_W – Warburg impedance; C_{func} – capacitance of the functional layer; R_{leak}- leak resistance of the functional layer.

TiO_xN_y functionalization changes the transmission characteristic; the equivalent circuit must therefore consider diffusion and charge-transfer. Between 200 Hz and 1 kHz, diffusion dominates the behavior. The typical Warburg characteristic with a slope of approximately 45° is present in the Nyquist plot. Towards frequencies, serial capacitance and serial resistance decrease. R_s amounts to 20 kΩ at 5 kHz. Since the layer thickness of the TiO_xN_y coating is only 25 nm and the permittivity is high, it should cause a high capacitance of the functionalization itself. PEDOT:PSS functionalization effects a mixed characteristic. R_s is higher in comparison with TiO_xN_y functionalization. As a consequence PEDOT:PSS thickness, which amounts to a few hundred nanometer, C_s is significantly lower.

Conclusions

The high impedance magnitude of thick film gold electrodes is assigned to the intense influence of the double layer capacitance on the signal transmission. Variance of the impedance per unit area increases strongly with decreasing electrode diameter. Therefore, measurement uncertainty on small electrodes is high and limits thus the application of thick film gold electrodes for the monitoring of neuronal cell cultures. Coating with laminin reduces this influence. Manual application of the agents leads to significant changes in the resulting performance. A recycling test over 4 cycles revealed that electrodes are robust against repeated coatings.

Functionalization decreases the high double layer barrier of thick film gold electrodes. Doctor blade application of PEDOT:PSS layers and sputtering of TiO_xN_y cause mixed kinetic and charge-transfer electrode characteristic. Small electrodes with 86 µm diameter functionalised with TiO_xN_y have an increased serial capacitance 4.1 pF and low serial resistance of 32 kΩ at a frequency of 1 kHz. This promises best signal transition of all electrodes investigated in this study. These electrodes should therefore be appropriate for signal capturing in neuronal in vitro cell cultures.

Acknowledgements

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