LTCC based bioreactors for cell cultivation

H Bartsch, T Welker, K Welker, H Witte and J Müller
Institute for Micro- and Nanotechnologies IMN MacroNano®, Technische Universität Ilmenau, Gustav-Kirchhoff-Str. 7, 98693 Ilmenau, Germany
E-mail: heike.bartsch@tu-ilmenau.de

Abstract. LTCC multilayers offer a wide range of structural options and flexibility of connections not available in standard thin film technology. Therefore they are considered as material base for cell culture reactors. The integration of microfluidic handling systems and features for optical and electrical capturing of indicators for cell culture growth offers the platform for an open system concept. The present paper assesses different approaches for the creation of microfluidic channels in LTCC multilayers. Basic functions required for the fluid management in bioreactors include temperature and flow control. Both features can be realized with integrated heaters and temperature sensors in LTCC multilayers. Technological conditions for the integration of such elements into bioreactors are analysed. The temperature regulation for the system makes use of NTC thermistor sensors which serve as real value input for the control of the heater. It allows the adjustment of the fluid temperature with an accuracy of 0.2 K. The tempered fluid flows through the cell culture chamber. Inside of this chamber a thick film electrode array monitors the impedance as an indicator for the growth process of 3-dimensional cell cultures. At the system output a flow sensor is arranged to monitor the continual flow. For this purpose a calorimetric sensor is implemented, and its crucial design parameters are discussed. Thus, the work presented gives an overview on the current status of LTCC based fluid management for cell culture reactors, which provides a promising base for the automation of cell culture processes.

1. Introduction

The use of LTCC multilayer technology for the design of micro reactors provides the possibility of complex fluid handling in combination with electrical and electrochemical functions. These features of the technology brought forth a number of applications in the field of micro reactors [1], analytic devices [2-4] and PCR assays [5, 6].

First approaches in the field of LTCC based bio assays aim on the design of 2-dimensional electrode arrays [7]. The present work introduces a concept for the monitoring of 3D cell cultivation using an LTCC fluid system, which enables an optimized supply of the cell culture with oxygen and nutrients, guarantees stable temperature and flow conditions, and enables at the same time the monitoring of the cell growth by means of impedance analysis. The system consists of a temperature control unit, a cell culture chamber with impedance measurement system and a flow monitoring unit. At the current stage of development all components are realized as single modules linked via PEEK connectors [8]. This
work introduces these single components and their features and thus defines the basis for a monolithic integrated bioreactor based on LTCC technology. The integration of fluidic channels into LTCC multilayer requires an advanced process schedule to avoid deformation of the hollows during lamination and sintering. Therefore, proven methods for channel forming are summarized to give an overview of the applied methods.

2. Methods for channel manufacturing in LTCC reactors

2.1 Sequential lamination

One method to avoid sagging and to achieve geometrical exact fluid channels in the multilayer is the use of sequential lamination of the different reactor parts and their uniaxial joining. It makes use of dependency of the shrinkage rate from the densification of the ceramic green body. Figure 1 illustrates the process. A typical lamination pressure of 20 MPa is applied uniaxially to the channel part. In parallel, top and bottom parts are uniaxially joined at a reduced pressure of 2 MPa. All parts subsequently are laminated using the same reduced pressure of approximately one tenth part of the lamination pressure applied to the channel part. Hence the shrinkage of the top and the bottom part is

Figure 1. Process flow for the sequentially lamination method: (1) Uniaxial lamination of the channel parts at reduced pressure; (2) Force flow during uniaxial lamination of top and bottom part at reduced pressure; (3) Impact of the strain caused by different shrinkage rates during sintering.

Figure 2. Process flow for the isostatic lamination method with sacrificial inlays: (1) Assembly of uniaxially pre-laminated green body; (2) Isostatic lamination at familiar conditions; (3) Sublimation of the carbon inlay and homogeneous shrinkage during firing.

Figure 3. Cross section of a fluid channel produced with the sequential lamination method.

Figure 4. Cross section through a wide fluid chamber produced using carbon inlays.
higher than that of the channel part, and the generated tensile stress spans the cantilever structure over the channel. Figure 3 shows a cross section of a channel produced using this method. It can be applied for channels with a width up to 1 mm. However, delimitation can occur due to inadequate contact of the single sheets after lamination.

Additional metallization layers influence the strain and consequently the geometry. The impact of metallization structures and layout on the channel sagging can be predicted by DoE methods [9]. Accordingly, the lamination pressure for the system is fixed. One disadvantage of the method thus consists in the fact, that the correction of shrinkage tolerances by adjustment of lamination pressure - a widespread technique in commercial LTCC fabrication - is not applicable. Therefore, tape charge specific and layout induced shrinkage deviations, which can reach an order of 2 %, cannot be corrected; this circumstance heightens the effort to meet narrow tolerances. However, for many fluid systems these tolerances are acceptable. Thus, the method is applicable for a wide range of systems.

2.2 Use of sacrificial materials

An alternative way to generate hollows in the green body is the use of volatile inlays. Starch and polymer based materials serve this purpose [10]. Additionally, sublimating carbon inlays can be used to sustain the hollows and chambers during isostatic lamination. Figure 2 depicts the process flow for this manufacturing method. Channel part, bottom and top are prepared in parallel. A carbon tape is laser cut, matching channel geometry and inserted into the prepared fluid channel. Then all parts are joint first uniaxially, and then isostatically laminated under familiar conditions. During firing the temperature is hold at 600°C for a certain time to sublimate the carbon. The holding time strongly depends on the amount of inserted carbon. The higher effort is justified by the benefit of a uniform and controllable shrinkage. Even large hollows as shown in Figure 4 can be produced by the method [11].

2.3 Microchannels

![Figure 5](image)

Figure 5. Micro channel produced by screen printing of fugitive carbon paste, isostatic lamination and subsequent firing. Typical dimensions are about 5-10 \( \mu \text{m} \) in height and more than 50 \( \mu \text{m} \) in width.

![Figure 6](image)

Figure 6. Embossed micro channels after uniaxial lamination at reduced pressure of 2 MPa (left) and after firing (right).
Fine channels are necessary to connect single scaffold tubes with the peripheral elements. Laser cut channels can be achieved with dimensions of 200 µm or larger. Channels with smaller dimensions can be manufactured using printed carbon layers or embossing. In the first case carbon paste is applied with stencils or standard screens. The channel height corresponds to the thickness of the printed paste. The tape is joined by means of standard isostatic lamination. The carbon sublimates during firing and leaves the channel behind. Figure 5 depicts the cross section of such a channel with typical dimensions. The method restricts the channel geometry to dimensions which have a height between 5 µm and 15 µm, a width larger than 50 µm and a characteristic shape resulting from screen printing.

An alternative method is the embossing of micro channels and subsequent uniaxial lamination at reduced pressure. Detailed descriptions of the embossing process are given in [12, 13]. The channels are moulded using silicon tools, for example. Exact geometries with rectangular cross section are achievable (see Figure 6). The finest achieved dimensions of fired channels are 35 µm x 35 µm. Larger geometries can be produced, free standing channels with a height of 50 µm and a width of 200 µm already were manufactured. The process requires an exact adjustment of the tool geometry and lamination process. Density gradients lead to shrinkage deviations, which are predictable but have to be adapted for new layouts. This effort is only justified for few applications with height requirements on the channel geometry.

### 3. Temperature control

#### 3.1 NTC thermistors

The temperature control must maintain the temperature of the cell culture medium at 37 °C with an accuracy of 0.2 K. Temperature sensors must thus feature a high temperature coefficient, thus the material NTC-2114 (ESL ElectroScience) is chosen. Preliminary tests were carried out to determine sheet resistance and B parameter of the Steinhart–Hart equation (B parameter), and their respective variation as a function of the sensor geometry. A test structure with different length to width ratio is

| Length (mm) | Width (mm) | Resistance 30°C (kΩ) | SD (%) | Sheet resistance (kΩ/sq) | SD (%) | B parameter (%) | SD |
|-------------|------------|----------------------|--------|-------------------------|--------|----------------|-----|
| 1.3         | 1          | 14.7                 | 20     | 12.0                    | 20     | 2149           | 3   |
| 3.3         | 1          | 55.7                 | 10     | 18.1                    | 10     | 2225           | 3   |
| 1.3         | 0.65       | 25.0                 | 23     | 13.7                    | 23     | 2260           | 1   |
| 3.3         | 0.65       | 88.0                 | 7      | 18.2                    | 7      | 2256           | 2   |
| 1.3         | 0.325      | 67.4                 | 19     | 18.4                    | 19     | 2309           | 2   |
| 3.3         | 0.325      | 204.5                | 8      | 23.5                    | 8      | 2329           | 2   |
| 1.3         | 0.163      | 303.5                | 19     | 40.8                    | 19     | 2461           | 4   |
| 3.3         | 0.163      | 829.5                | 8      | 39.2                    | 8      | 2526           | 3   |

X : Mean value of the sample
SD: Standard deviation of the sample
used for this study. The paste is screen printed using a stainless steel fabric (325 mesh/inch and 0.024 wire thickness) with an emulsion thickness of 15 µm on DP 951 PX substrates (DuPont Nemours). Lamination and sintering is carried out under standard conditions. Two substrates with 40 test structures (5 of the respective geometry) are evaluated. The resistance of the test structures is measured by a multimeter (HP 34401) at 30 °C and 70 °C, respectively. The geometry data are captured by means of video measuring microscope (UHL VM Sergo, Walter UHL GmbH), and the respective sheet resistance is calculated using these data. The temperature was adjusted with a hotplate and referred by the use of an external PT-100 platinum resistance thermometer. The data determined are gathered in table 1. The sheet resistance is in good agreement with the datasheet value of 10 kΩ for 1.3 x 1 mm². Other dimensions cause strong deviations because of substrate-paste interactions and substrate-termination interactions as well. The sensor is fitted into the channel and has a width of 4.5 mm and a length of 0.8 mm. Two sensors are situated directly above the channel at the inlet and outlet, respectively. The calibration is carried out in a convection oven, the sensors were put inside for 1 hour at each temperature point to achieve a homogenous temperature distribution over the whole module, and the resistance values are determined using a multimeter (HP 34401). Sensor temperature and the correlated resistance value are recorded in the interval from 30 °C to 100 °C in steps of 10 K, whereby a PT-100 platinum resistance thermometer serves as reference. The actually achieved values for 30 °C and 40 °C are highlighted in table 2. Figure 7 depicts the resultant temperature characteristic. The deviation of the resistance values is lower than 7 %, this fact attests a stable process. The logged characteristics are the base for temperature control unit parameters.

### Table 2. Resistance R and B parameter of the sensors integrated into the fluid channel.

| R @ 30°C (kΩ) | R @ 40°C (kΩ) | B parameter (X, SD) |
|---------------|---------------|---------------------|
| 4.8 (6.68%)   | 3.9 (6.34%)   | 2052 (0.54%)        |

X : Mean value of the sample
SD: Standard deviation of the sample

Figure 7. Temperature characteristic of the NTC sensors

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**3.2 Heater control**

The analogue voltage drop over the NTC sensors is transformed into a digital value with a resolution of 24 bit (AD7794, Analog Devices). The reference voltage amounts to 5 V. The resulting voltage resolution of 298 nV and corresponds to a resistance resolution of 1.42 mΩ. This value is interpreted by the microcontroller (Atmel ATmega32 8-bit AVR RISC), which here constitutes a PI controller. The control sets the electrical power of the heater element, which is arranged directly underneath the fluid channel. The silver composition DP6142D (DuPont Nemours) is used for the heater, the screen printing is carried out with a stainless steel fabric (325 mesh/inch and 0.024 wire thickness) with an emulsion thickness of 15 µm. Sensors and heater are covered with one sheet DP 951 C2. Figure 8 depicts an X-ray tomogram and a schematic cross section through the module. The channels have a cross section of 1 mm x 1 mm. The fabrication follows the method described in section 0. The resistance of the heater amounts to 12.5 Ω at 35 °C and to 15 Ω at 50 °C. The power consumption in
this temperature range is less than 1 W. The time constant is determined analyzing the step response recorded at a temperature of 90 °C. It amounts to 24 s.

Three modules as depicted in Figure 8 are characterized in terms of their control accuracy. The actual temperature values at the inlet and the outlet are determined from the filed temperature characteristic of the respective NTC sensor and plotted in Figure 9. The temperature at the inlet varies between 26 °C and 30 °C. A warming up of approximately 2 K above ambient temperature is observed when the preset temperature is set to 90 °C. At the outlet the preset temperature is adjusted with high accuracy: the maximum deviation observed amounts to 0.2 K for preset temperatures up to 60 °C and to 2 K for higher preset temperatures.

4. Assay chamber
The core of the system is the cell culture chamber. The open cavity is formed with high accuracy by the use of silicon inlays during isostatic lamination [14]. The complex 3D fluid supply is embedded in a chamber module, which is schematically depicted in Figure 10. Inside of the chamber a scaffold with Schwarz P polymer structure serves as 3-dimensional framework for the cell growth. In the current case, a urethane dimethacrylate (UDMA) scaffold produced via two-photon polymerization [15, 16] is

Figure 8. X-ray tomogram of the temperature conditioning module (above) and schematic cross section through the functional elements.

Figure 9. Temperature measured at the inlet and outlet of 3 modules.

Figure 10. Schematic view of the cell culture chamber with fluid supply, Schwarz P scaffold and impedance sensors.
inserted into the LTCC reaction chamber as shown in Figure 11. The micro channels at the interface must match the scaffolds’ pitch of 440 µm; the clear span of the scaffold channels amounts to 150 µm. In the first development stage the micro channels are laser cut in DP 951 PX, and the chamber body is laminated using carbon tape as sacrificial material (see section 0). The channel width amounts to 200 µm. The fluid concept intends to supply each second channel of the Schwarz P scaffold with nutrient solution in diagonal sequence and to use the other channels for the removal of metabolic products. The refinement of the system will apply embossing (see 0) for the channel forming to achieve finer dimensions and to enable addressing single channel for precise drug delivery in a defined region of the cell culture. Thus, e.g. the influence of pharmaceuticals on the differentiation of stem cells to osteoblasts and their mature to bone tissue can be investigated.

One method to gather information about the state of a cell culture is the impedance spectroscopy. It monitors the dielectric properties of biological cells and tissues. Conventionally, small AC voltages are applied, and the response of the system is measured [17]. The impedance sensors are electrodes, which are situated at the bottom of the assay chamber. A simple design, which is easily feasible in LTCC technology, is a round electrode. In the current design those electrodes have a diameter of 130 µm and are printed with the paste DP 5740A on gold vias (DP 5738). The impedance of these electrodes was measured in phosphate-buffered saline (PBS) as electrolyte at 1 kHz using an impedance measurement adapter described in [18]. The median impedance of the electrodes amounts to 24 kΩ.

5. Flow monitoring

5.1 Principle
The scaffold in the chamber is perfused permanently by the cell culture medium with a flow rate of 200 µl/min. The flow monitoring controls the steady transport of metabolic products. Flow control in LTCC fluid systems can be done optically [19] or thermally [20]. In the current application case a calorimetric principle with four temperature sensors allocated around a heater is applied. Two sensors are situated upstream and two downstream, as the X-ray tomogram of the module depicts in Figure 12. Figure 13 illustrates the temperature distribution over the sensor elements for a blocked channel (flow = 0) and a faultless one (flow > 0) schematically. The flow controller interprets the temperature deviation of upstream and downstream components based on the resistance change and detects a blockage.

5.2 Realization
All elements, heater and sensors, are situated on a free standing bridge, which is spanned in the middle of a 1 mm high fluid channel. The bridge consists of one layer DP 951 PX, heater and sensors are screen printed using a stainless steel fabric (325 mesh/inch and 0.024 wire thickness) with an emulsion thickness of 15 µm and covered with one layer DP 951 C2. The resulting thickness of the fired bridge amounts to 255 µm. The heater consists of the platinum thick film paste DP 9896. It is designed with a line width of 100 µm and a pitch of 200 µm and covers an area over the channel of 0.8 x 0.3 mm. The thermistor paste DP 5093 D is used for the temperature sensors. They are arranged symmetrically around the heater upstream and downstream. The respective distance amounts to 0.95 mm and 0.45 mm from the heater outline. They have a design length of 850 µm and thus span the whole fluid channel. The width was varied to secure higher resistance values upstream despite of manufacturing tolerances of the buried elements. The design values amount to 1060 Ω and 710 Ω for sensor 1 and 2 (upstream) and 530 Ω and 850 Ω for sensor 3 and 4 (downstream). The fabricated resistances feature a deviation between 10 % and 20 % from the design values. The bridge is laminated with a reduced pressure of 2 MPa and the fluid structure is laser cut subsequently. The fluid channels are punched with a 1 mm round tool and laminated at 20 MPa. The whole module is sequentially laminated applying a pressure of 2 MPa (see section 0).
5.3 Characteristics

The temperature characteristic of the particular sensors is recorded with a PT-100 platinum resistance thermometer as reference. The mean of the temperature coefficient amounts to 2.1 $\Omega/K$. However, the temperature characteristics of the paste show very strong deviation up to 40 %. Based on the particular data for each sensor, the real temperature distribution inside the system as a function of the flow rate is logged. For this purpose a constant flow is driven with high accuracy into the module by means of a High Pressure Liquid Chromatography (HPLC) analysis pump (LC-20AD, Shimadzu). The respective sensor temperature is determined from the actual resistance values measured with a multimeter while the heater is driven with a constant power of 0.45 W. Figure 14 depicts the plotted temperature data for the particular sensors and the heater element as well. The over temperature amounts to 40 °C at the heater element for the nominal flow rate of 200 $\mu l/min$. The resistance difference between upstream and downstream sensor amounts to 30 $\Omega$ at the outer sensors and to 15 $\Omega$ at the inner sensors. Figure 15 depicts the resistance difference as a function of the flow rate for the outer and inner sensor pair.
Each system must be attuned to compensate manufacturing tolerances of the sensors. For this purpose the heater is driven with a constant power and the resistance difference of upstream and downstream sensor is compensated with a potentiometer in series with the downstream sensor. A second potentiometer in the feedback path serves for the adjustment of the desired minimum flow rate. Then the flow monitor module is ready for use.

6. Summary
The current work describes three modules of a fluid handling system for cell cultures realized in LTCC multilayer technology. A temperature control based on buried NTC temperature sensors is described, which guarantees the comfort temperature for cell growth of 37°C with high accuracy of 0.2 K. The tempered cell culture medium enters subsequently into the cell culture chamber, which is equipped with a Schwarz P scaffold as support frame for the 3-dimensional cell growth. At the bottom of the chamber impedance sensors examine the cell growth. The steady perfuse of cell culture medium necessary for nutrient supply is monitored by means of a calorimetric sensor system, which gives an alert if the flow rate falls below a critical value. These modules build the basis for a cell monitoring system which allows the automated cell cultivation and analysis in bioreactors easy to use in bio labs.

7. References
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