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Abstract

Zr-Si organic–inorganic scaffolds fabricated by a two-photon polymerization technique were used for the primary culture of mouse embryonic neural cells. We observed that dissociated hippocampal cells adhere to the scaffolds, produce neurites, elongate and differentiate into adult neurons. Neuronal outgrowth and synaptogenesis were confirmed by immunohistochemical staining with antibodies against β III-Tubulin and synaptophysin. The formation of a functional neural network was assessed by the measurement of spontaneous activity using Ca²⁺ imaging of dissociated hippocampal cultures grown on Zr-Si scaffolds. The results of this study suggest that two-photon-induced polymerization of organic–inorganic hybrid biomaterials provides a robust model for 3D neuronal tissue engineering studies.

1. Introduction

The restoration of conductive pathway integrity in the spinal cord and cognitive function reparation following extensive craniocerebral injuries are considered as one of the main issues in modern regenerative medicine of the CNS (central nervous system). Such traumas are widespread and largely contribute to the development of socially-significant diseases. The current approaches to the therapeutic correction (regeneration) of such damage require an adequate test system *in vitro* simulating a neural network [1]. In this regard, 3D models allow us to investigate the mechanisms of CNS ontogenesis and neurodegenerative processes [2, 3]. Nowadays, scaffolds based on biocompatible polymers represent an appropriate platform for the growth of primary neural cultures [4] or stem cells capable of differentiation by a neuronal pathway [5], and might be used for the development of adequate 3D neuronal network models.

Primary cell cultures derived from the hippocampal tissue are characterized by particular cellular structure and genetically determined locality and are widely accepted as the most appropriate biological

model for studying the local network activity of the brain [6, 7]. Moreover, Ca²⁺ imaging, established in hippocampal neuron cell culture, allows us to not only study the formation and functioning of neural networks on a cellular level, but to evaluate the contribution of each neuron or astrocyte in the network activity [8]. For these reasons, primary hippocampal cultures represent the most appropriate tool for the 3D neural network model *in vitro*.

For the development of 3D model scaffolds, different materials have been tested [9, 10]. For the long-term *in vitro* culture, scaffolds should represent a stable non-degradable platform. On the other hand, they should be biocompatible to support the survival and functionality of the neuronal cells. The application of synthetic or combined natural and synthetic versus natural components for a 3D scaffold is supposed to be an appropriate technique for the analysis of neural network development under physiological and pathological conditions [2, 10].

Another critical property of 3D scaffolds for neuronal culture is the material porosity, required for cell survival and function. The scaffold should be permeable to low molecular weight molecules, gases, and

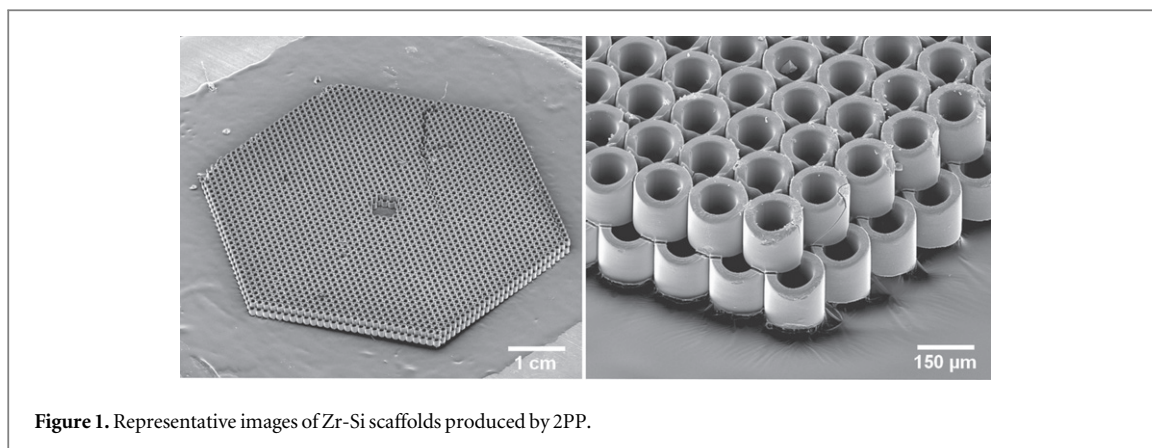


Figure 1. Representative images of Zr-Si scaffolds produced by 2PP.

nutrients, and avoid waste accumulation. Optimal pore size should permit cell–cell interaction, migration and neurite extension. At the same time, the surface area of the scaffold should be appropriate for cell attachment and allow physiological spreading. Therefore, an adequate platform for 3D neuronal cell culture assumes the application of scaffolds having a high fidelity and architectonics with specific dimensions at the elementary level above 20 microns according to the size of the pyramidal and interneuron cell bodies [11, 12].

At present, one of the promising approaches for the structuring of materials with the application of high-intensity radiation sources is the two-photon polymerization (2PP) technique [13]. The principle of this method is based on two-photon absorption of femtosecond laser pulses in a photosensitive material, resulting in its local polymerization. It has been demonstrated in a number of studies that 3D scaffold structures can be fabricated by the 2PP method using gelatin, hyaluronic acid, and fibrin [14–16]. It has also been extensively reported that hybrid organic–inorganic materials can be used for 3D scaffold fabrication by the 2PP technique [17–20]. Such scaffolds possess high affinity to stem cells, and can facilitate their osteogenic differentiation [17, 18]. Zr-Si organic–inorganic scaffolds were used for *in vitro* and *in vivo* studies for cartilage tissue engineering. It has been shown that 3D Zr-Si scaffolds are biocompatible for chondrocytes enabling them to recover the chondrogenic phenotype culture *in vivo* [19]. In our study, we focus on the investigation of characteristics of the morphological, structural and functional organization of neuronal networks on the 3D organic–inorganic Zr-Si scaffolds *in vitro*.

2. Materials and methods

2.1. Synthesis of Zr-Si-based organic–inorganic polymer ceramic composites

The Zr-Si hybrid material is a photosensitive sol–gel material that was prepared using a procedure similar to that previously described by [17, 20, 21]. The

material was synthesized from methacryloxypropyl trimethoxysilane (MAPTMS), methacrylic acid (MAA), and zirconium n-propoxide (ZPO). The photoinitiator 4,40-Bis(diethylamino) benzophenone (Sigma-Aldrich, St. Louis, MO) also known as ethyl Michler’s ketone was added to the solution at 1% w/w concentration and mixed for 6 h. The solution was filtered through a 0.5 μm syringe filter to remove undissolved particulates.

The Zr-Si hybrid material was drop-cast onto glass substrates. 1 ml of resin was deposited on 22 \times 32 mm glass coverslips, resulting in complete surface coverage. Substrates for scaffold fabrication were prepared by the drop-casting of 1 ml of liquid material onto 22 \times 32 mm glass substrates, followed by slow evaporation of the organic solvent over 24 h at room temperature and subsequent baking at 100 $^{\circ}\text{C}$ for 2 h. The Zr-Si films were baked for 2 h at 100 $^{\circ}\text{C}$ to form a hard gel for subsequent photopolymerization by 2PP.

2.2. Fabrication of scaffolds by two-photon polymerization (2PP)

A Ti:sapphire femtosecond laser system delivering 150 fs pulses at an 80 MHz repetition rate, was used for the 2PP fabrication of scaffolds (Chameleon, Coherent, Germany). The experimental setup is similar to one that was previously described [9]. An acousto-optical modulator was applied to trigger exposure of the sample. The beam is then passed through an EC Plan-Neofluar 20 \times objective (Zeiss, NA/0.5) and focused into the sample. To fabricate defined scaffold geometry, a custom-written computer code was used. The scaffolds consisted of two layers of hollow cylinder arrays (figure 1). The cylinders were located in a hexagonal arrangement within each layer. The inner diameter and the wall thickness of each cylinder were 100 and 30 μm , respectively. The 2PP-fabricated scaffolds were developed in 1-propanol for 4 h. The solvent was replaced twice during this duration to ensure complete removal of unpolymerized material. The scaffolds were washed and sterilized with isopropyl alcohol for 2 h in the dark. To remove alcohol

residues, the scaffolds were washed thrice with sterile deionized water for 10 min, thereafter, with a culture medium and finally the dissociated hippocampal neurons were plated onto the scaffolds.

2.3. Preparation of hippocampal neurons

The animal experiments were approved by the National Ministry of Public Health for the care and use of laboratory animals and by the Bioethics Committee of the Nizhny Novgorod State Medical Academy. Primary hippocampal cells were isolated according to the previously described protocol [22]. Briefly, hippocampi were isolated from embryonic C57BL/6J mice (E18) and mechanically cut under sterile conditions in Ca^{2+} - and Mg^{2+} -free PBS. The hippocampi were incubated with 0.25% trypsin (Invitrogen 25 200-056) for 25 min at 37 °C. The digested cells were carefully triturated and centrifuged at $1000 \times g$ for 3 min. The cell pellet was resuspended in Neurobasal medium (Invitrogen 21 103-049) containing 2% B27 Supplement (Invitrogen, 17 504-044, USA), 0.5 mM L-glutamine (Invitrogen, 25 030-024, USA), 5% fetal calf serum (PanEco K055, Russia) and plated onto scaffolds with a density of 9×10^3 cells mm^{-2} . The control cultures were grown on 18 mm coverslips coated with the positively charged hydrophilic substance—polyethyleneimine (Sigma P3143) as a widely accepted substrate for neuronal culture [23]. Both cultures, plated on scaffolds or coverslips, were maintained under standard cell culture conditions. Half of the medium was changed every 2 d.

2.4. Immunocytochemical staining

Neuronal cells on Zr-Si scaffolds were stained by a two-step indirect method. First, the scaffolds were fixed in 4% paraformaldehyde. Then, the cells were permeabilized by methanol pre-cooled at 20 °C for 3 min. A 2% bovine serum albumin (BSA/PBS) solution was used to block unspecific antibody binding, thereafter, primary antibodies (in 2% BSA/PBS solution) were applied overnight at 4 °C. This step was followed by incubation with antibodies against β III-tubulin (1:1000, mouse monoclonal IgG2a, clone 2G10, Sigma-Aldrich) or synaptophysin (1:50, rabbit polyclonal H-93, Santa Cruz, Heidelberg, Germany). After several washing steps, horseradish peroxidase-conjugated goat anti-rabbit/goat anti-mouse IgG (H + L) secondary antibody (1:100, Dianova, Hamburg, Germany) was added to the scaffolds for 1 h. The staining with peroxidase was visualized by incubation with 3-amino-9-ethyl-carbazole substrate in sodium acetate buffer (0.1 mol L^{-1} , pH 5.2) containing hydrogen peroxide.

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) of scaffolds populated with dissociated hippocampal neurons was performed after fixation of the cells in the daytime

in vitro 14 (DIV14). The procedure for the preparation of samples for SEM analysis is described elsewhere [17].

2.6. Ca^{2+} imaging

Ca^{2+} imaging was performed according to the protocol described previously [11, 24]. Hippocampal cultures grown on scaffolds and coverslips were treated with Oregon Green 488 BAPTA-1 AM (OGB-1) ($0.4 \mu\text{M}$; Invitrogen O-6807) pre-diluted in dimethylsulfoxide (DMSO) and 4% pluronic acid. Then the samples were washed by dye-free medium for 15 min. A confocal laser scanning microscope (Zeiss LSM 510, Germany) with a W Plan-Apochromat $20 \times /1.0$ objective and 488 nm line of argon laser radiation and emission detection with a 500–530 nm filter was used for the recording of spontaneous Ca^{2+} activity in neurons and astrocytes by registration of OGB-1 fluorescence changes. A time series of images (256×256 -pixel) with a duration of 10 min and $420 \times 420 \mu\text{m}$ field of view were recorded at 4 Hz. To obtain an axial optical slice resolution of $1.6 \mu\text{m}$ a confocal pinhole of 1 airy unit was applied. The evaluation of Ca^{2+} fluctuations was performed by the automatic counting of Ca^{2+} events in manually selected regions of fluorescent images in custom-made software C++ Builder. The Ca^{2+} fluorescence in selected cells was evaluated by the calculation of the average fluorescence intensity (F, relative units from 0 to 255) of the pixels within the defined region.

2.7. Evaluation of cell viability

To study the cytocompatibility of Zr-Si scaffolds with cultured cells we used histochemical live/dead cell viability assay. The viability of dissociated hippocampal cells cultured on scaffolds was evaluated by the calculation of the percentage ratio between the number of dead cells stained by propidium iodide (Sigma-Aldrich) and the total number of cells stained by bisBensimide (Invitrogen, H3570) in the cultures on the DIV7 and DIV14 [24].

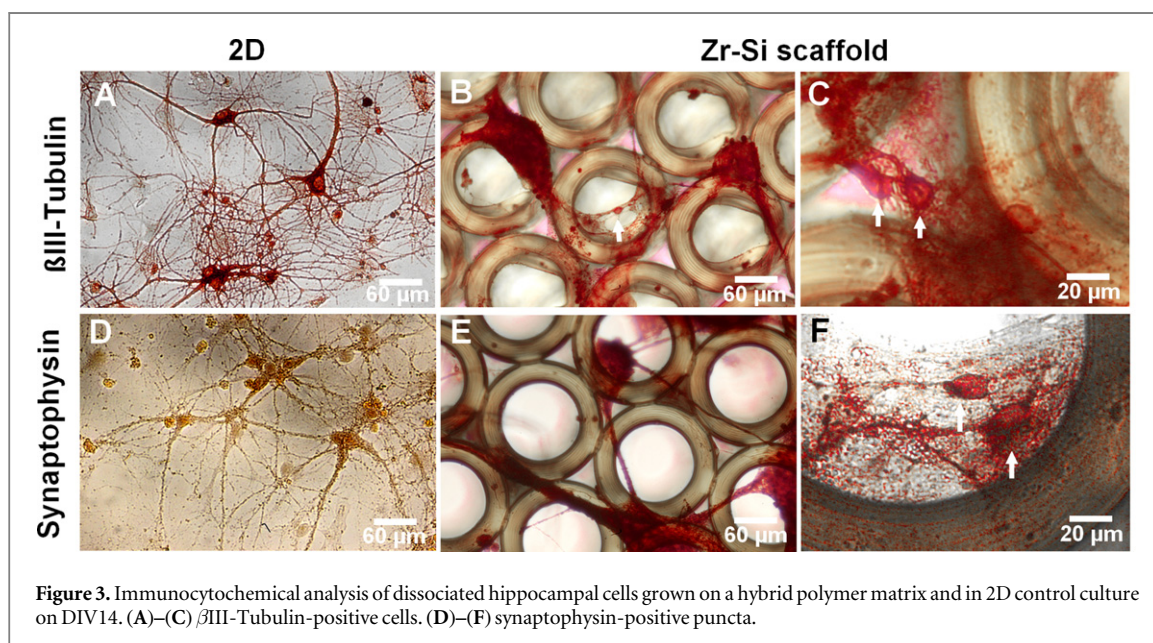
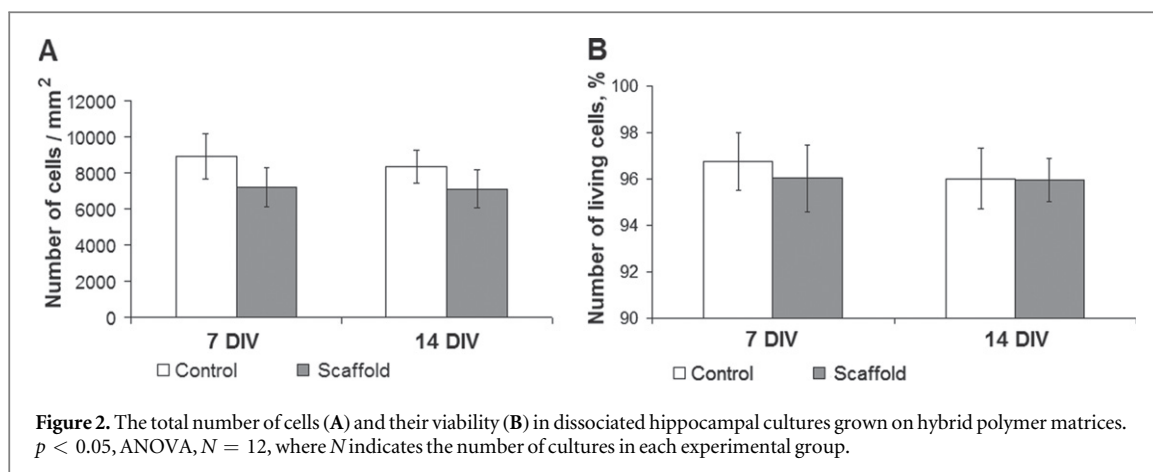
2.8. Statistical analysis

All data quantification is presented as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using a two-way analysis of variance (ANOVA) implemented in the SigmaPlot 11.0 program (Systat Software Inc.). Student–Newman–Keuls (SNK) was used as a post hoc ANOVA test. The difference between the groups was considered significant if the *p* value was less than 0.05.

3. Results

3.1. Zr-Si scaffolds represent a favorable environment for 3D cell cultivation

By using the 2PP technique we have fabricated scaffolds with a 3D structure, which are composed of



two layers of cylinders (height 100 μ m, inside diameter 100 μ m, outside diameter 160 μ m) and arranged in squares with 2 mm size (figure 1). Due to the shifting of the upper layer by 80 μ m with respect to the lower one, a porous 3D structure was formed that provided cultivated cells with an effective transport of nutrients and internal ledges for the additional support of cell growth and the formation of cellular extensions.

3.2. Zr-Si scaffolds are highly compatible with dissociated cells from hippocampus

To investigate the capacity of the Zr-Si scaffolds to support neuronal growth and survival, we used primary cultures of hippocampal neurons, which allowed for the visualization of the morphological changes and for functional analysis. In this experiment, cells were plated onto scaffolds without polyethyleneimine pre-treating. For the analysis, we selected a time window DIV14, when well-defined synapses are already formed [25].

Histochemical live/dead cell viability assay has shown that the scaffold material has a high affinity to the cells of nerve tissue, neurons and astrocytes. No significant difference in the total number of cells was found between the scaffolds and control group, neither on DIV7 nor DIV14 (figure 2(A)). Importantly, the viability of cells grown on the scaffolds was comparable with the viability of cells cultured in control conditions (figure 2(B)). These results indicate a non-cytotoxic character of Zr-Si scaffolds related to primary hippocampal cells.

3.3. Zr-Si scaffolds induce the formation of neuronal networks in 3D cell culture

The morphological analysis of the dissociated hippocampal cells at DIV14 has shown that the development of the cultures on the Zr-Si scaffolds corresponded to the main development traits typical for the normal formation of the neuronal networks in primary culture. At DIV1 viable cells were attached to the scaffolds. The formation of neuronal networks was verified by the detection of neuronal cells with

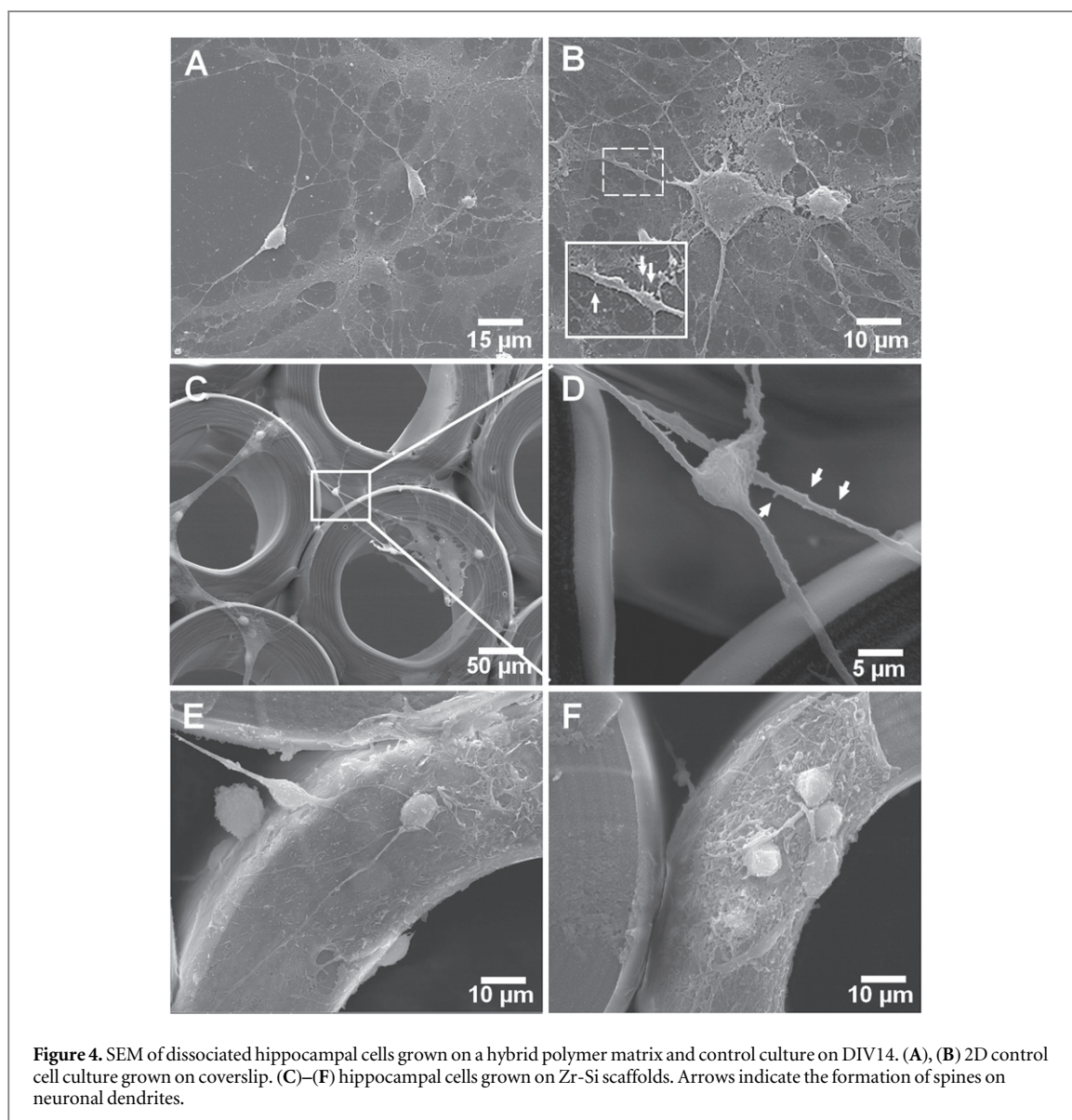


Figure 4. SEM of dissociated hippocampal cells grown on a hybrid polymer matrix and control culture on DIV14. (A), (B) 2D control cell culture grown on coverslip. (C)–(F) hippocampal cells grown on Zr-Si scaffolds. Arrows indicate the formation of spines on neuronal dendrites.

antibodies against β III-Tubulin (neuronal marker) and synaptophysin, which indicates the development of differentiated neurons capable of synaptic contact formation. As demonstrated in figure 3, cells cultured on Zr-Si scaffolds for 14 days express both specific markers similar to neurons in 2D culture.

SEM analysis of the dissociated hippocampal cultures grown on scaffolds revealed that the morphological structure of the neuronal cells corresponded to that in neurons grown on polyethyleneimine-coated coverslips. A well-developed dendritic tree with typical spine morphology was observed on the surfaces of the rings of scaffolds, as well as inbetween single scaffold segments, confirming that scaffold architecture promotes neuronal growth and spine formation in 3D cell culture (figure 4).

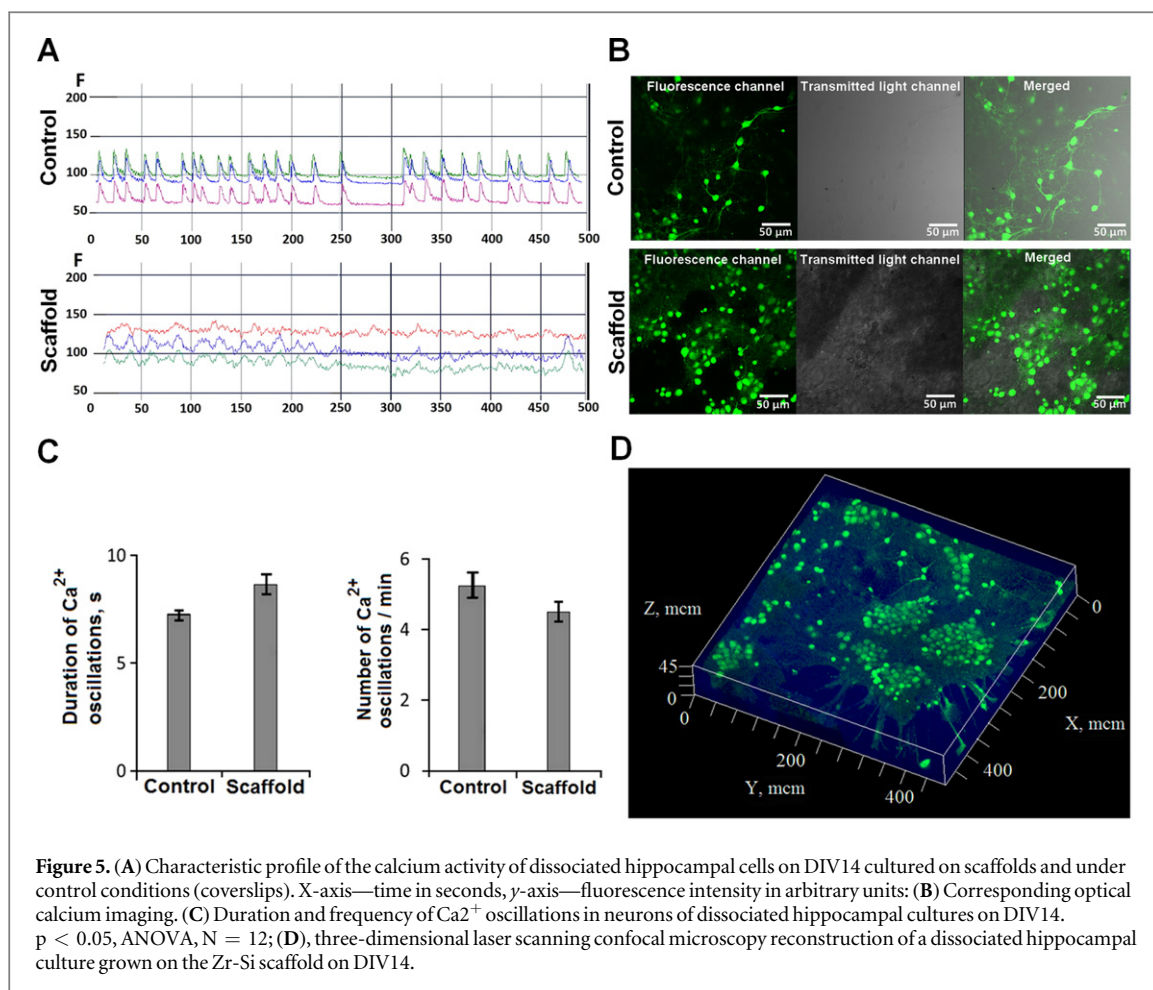
3.4. Dissociated hippocampal cultures grown on Zr-Si scaffolds demonstrate functional calcium activity

The mature chemical synapses become predominant on DIV14 and can be used for the functional analysis

of the neural network formation *in vitro* [26]. Therefore, we have chosen this time point for the Ca^{2+} imaging analysis. In our experiments, we observed the functional calcium activity of dissociated hippocampal cultures grown on scaffolds on DIV14, suggesting that neural networks exhibiting functional calcium activity were formed (figure 5).

Our previous studies revealed that spontaneous Ca^{2+} oscillations appear in dissociated hippocampal cultures beginning with DIV7. On the DIV14, a large number of neurons have similar patterns of Ca^{2+} oscillations with an oscillation duration of 6 s. This is explained by the fact that hippocampal neurons are genetically determined on the formation of networks that generate spontaneous burst [7].

The duration of calcium oscillations on the scaffolds ranged between 5 and 12 s, their frequency being equal to 0.9–2.0 oscillations per minute (figures 5(C), (D)). The occurrence of spontaneous variations of the intracellular calcium concentration in the dissociated hippocampal cultures pointed to their normal *in vitro*



development and corresponded to the functional ontogenesis typical for this development stage (10–14 d) [26].

4. Discussion

Scaffolds made of Zr-Si hybrid polymer are known to support the growth of several cell lines and mesenchymal stem cells [17–19]. However, primary neuronal culture is associated with certain difficulties. Usually, coated tissue culture plates are a prerequisite for seeding. The most commonly used coating reagents are positively charged polymers such as poly-L-lysine, polyethyleneimine or biologically purified adhesive molecules, such as collagen. In this study, we have shown that Zr-Si scaffolds fabricated by the 2PP technique, exhibit a favorable platform for the 3D growth of primary neurons, without any additional coating. For designing the structure of a 3D matrix, the formation of a surface micro-relief suitable for the development of nerve cells is important. Surface micro-relief is known to play a key role in the formation of intercellular contacts and the morpho-functional structure of neuron-glia networks [27]. When selecting the pore diameter, as well as the special scaffold architectonics, we followed the body size of the nerve cells and specific properties of their

outgrowths, such as adhesion to certain substrates. The displacement of the subjacent layers in the scaffold constructs makes it possible to create multilevel functional cellular conglomerates characteristic to the native nervous systems, providing bioavailability of the cellular medium components. Moreover, the porosity of the scaffold structure allows cellular outgrowth to penetrate it and makes it possible for the formation of transition-conducting pathways directly through the polymer structure.

Our investigations revealed that the properties of the Zr-Si hybrid polymer 3D scaffolds provide a proper adherent material for the nervous system cells and facilitate the formation of functional neural networks. Thus, the neuronal growth and synaptogenesis were confirmed with specific antibodies against neuronal marker β III-Tubulin and presynaptic marker synaptophysin. The studies of the cell viability demonstrated that the scaffold material was not toxic for cells. The total number of cells and their viability in the dissociated hippocampal cultures grown on 3D scaffolds did not change for a long time, indicating that the scaffold material remained non-toxic during 14 d *in vitro*. Our data demonstrate the functional calcium activity of the neural networks developed in the dissociated hippocampal cultures grown on the 3D scaffolds. Earlier studies have shown that the increased postsynaptic

Ca^{2+} entry was associated with reduced filopodial motility and with stabilization of spine morphology, suggesting that the Zr-Si hybrid polymer 3D scaffolds might induce the spine formation and development of a functional neural network [28, 29]. Moreover, being a secondary messenger, calcium ions play a special role in the intracellular signaling system, so that changes in their concentration serve as ‘trigger stimuli’ for the implementation of various biochemical signaling mechanisms of the cells. For that reason, changes in the intracellular concentration of calcium ions are a reliable indicator of the functional activity of neuronal networks.

A particular characteristic of hippocampal neurons is that during ontogenesis pyramidal neurons form connections between themselves and interneurons forming a closed neural network, which precipitate spontaneous Ca^{2+} activity [30, 31]. The neurons which belong to the neural networks of the hippocampus interact with glial cells, thereby the major manifestation of this functional activity is also Ca^{2+} oscillations. Thus, the presence of spontaneous oscillations in hippocampal cell cultures is a reliable indicator of the formation of a high-grade functional neuron-glia network. According to our previous studies, primary cultures of hippocampus on day 14 in culture are characterized by the presence of synchronous oscillations of a concentration of cytoplasmic calcium. Such calcium oscillations have a pronounced leading-edge characteristic of neural networks with a predominance of mature axon-dendrite chemical synapses [26]. Synchronism calcium events in primary cultures of hippocampal cells are bound to form a functional neuro-glia network with numerous synaptic contacts. Reducing the number of active synapses can significantly affect the network settings of calcium oscillation and provide a basis for changing the functional characteristics of a particular neuron-glia network. In primary hippocampal cultures on the scaffold, as revealed, spontaneous calcium oscillations are generated partly synchronized. The duration and frequency of the oscillations recorded in the neural networks, cultivated on the scaffold, match the calcium oscillations in control primary hippocampal cultures recorded for the same period of ontogenesis.

Our results prove that hybrid Zr-Si-based composite scaffolds, structured by the 2PP technique can be applied, as biocompatible material for the development of 3D neural network models *in vitro*. Physiologically relevant models of neural tissue require prioritization among complexity, control and reproducibility. Completely biological materials (e.g. collagen, fibrin) for the fabrication of neuronal scaffolds can direct the growth of tissues and can be important in forming architecturally functional tissue such as aligning regenerating nerves with their target [9, 32, 33]. However, 3D scaffolds produced using this class of biomaterials lack the stability for the development of long-term 3D neural network models *in vitro*

[34]. The 2PP technique enables the reproducible fabrication of complex 3D platforms with great spatial control over the structure microarchitecture. The 2PP structured platform produced using reported inorganic-organic material may have many distinct advantages for neurobiological and electrophysiological applications. This microfabrication technique will allow the production of 3D scaffolds directly on the active area of the micro-electrode array. In accordance with this perspective and also using conductive materials for 2PP fabrication [35], neural networks coupled to micro-electrode arrays, will represent a new, powerful *in vitro* model capable of better emulating *in vivo* physiology. Taken together, this 3D platform will contribute to the development of a next class of experimental models to study neurophysiology *in vitro*, and for the development of new biohybrid microsystems.

5. Conclusion

It has been demonstrated that Zr-Si scaffolds fabricated by the 2PP technique display appropriate properties for neuronal cell adhesion even without the application of traditional cationic polymers. It was shown that the porous hybrid Zr-Si polymer materials are not toxic to the cells of the nervous system. After 14 d of *in vitro* development on scaffolds, dissociated hippocampal neural cells were positive for the specific neural marker β III-Tubulin. Dissociated hippocampal neurons were able to develop the neuronal network complex as indicated by the expression of the synaptophysin presynaptic marker, with the formation of a large number of intercellular connections to each other.

Thus, Zr-Si scaffolds provide a good artificially fabricated platform for the effective adherence of neuronal cells and are suitable for the creation of 3D neural network models to study various neurophysiological and pathological processes and neural synaptic interactions.

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