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Research paper

Neuronal-glial populations form functional networks in a biocompatible 3D scaffold

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HIGHLIGHTS

- We examine mixed CNS cultures in a biocompatible 3D scaffold.
- We compare 3D cultures with conventional monolayer cultures.
- Glial morphology in 3D cultures is more consistent with that seen *in vivo*.
- 3D cultures form functional neuronal networks.
- 3D cultures exhibit spontaneous local field potentials.

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ABSTRACT

Monolayers of neurons and glia have been employed for decades as tools for the study of cellular physiology and as the basis for a variety of standard toxicological assays. A variety of three dimensional (3D) culture techniques have been developed with the aim to produce cultures that recapitulate desirable features of intact. In this study, we investigated the effect of preparing primary mouse mixed neuron and glial cultures in the inert 3D scaffold, Alvetex. Using planar multielectrode arrays, we compared the spontaneous bioelectrical activity exhibited by neuroglial networks grown in the scaffold with that seen in the same cells prepared as conventional monolayer cultures. Two dimensional (monolayer; 2D) cultures exhibited a significantly higher spike firing rate than that seen in 3D cultures although no difference was seen in total signal power (<50 Hz) while pharmacological responsiveness of each culture type to antagonism of $GABA_AR$, NMDAR and AMPAR was highly comparable. Interestingly, correlation of burst events, spike firing and total signal power (<50 Hz) revealed that local field potential events were associated with action potential driven bursts as was the case for 2D cultures. Moreover, glial morphology was more physiologically normal in 3D cultures. These results show that 3D culture in inert scaffolds represents a more physiologically normal preparation which has advantages for physiological, pharmacological, toxicological and drug development studies, particularly given the extensive use of such preparations in high throughput and high content systems.

1. Introduction

Monolayer cultures of murine primary neurons form spontaneously active networks *in vitro*, and are commonly used in pharmacological, toxicological and electrophysiological studies [3,4]. In this regard, microelectrode arrays (MEAs) are particularly well suited to physiological, pharmacological and toxicological studies [4,5,16] of such cultures since they permit non-invasive, long term (weeks–months) monitoring of developmental and

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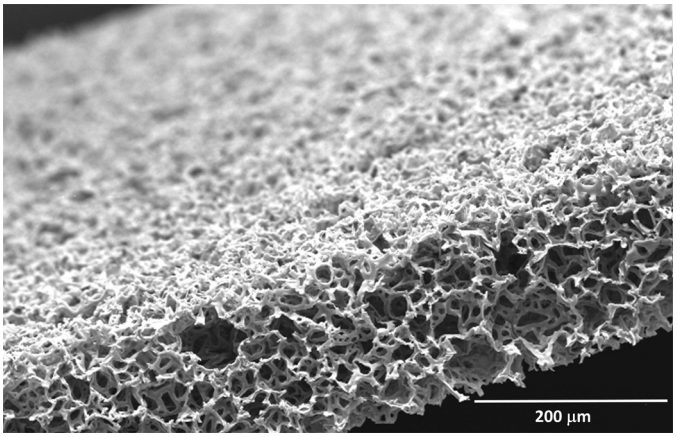

Fig. 1. Scanning electron micrograph of Alvetex 3D scaffold in transverse section. The material is 90% porous and comprised of voids (36–40 μm diameter) with interconnecting pores (13–15 μm diameter) engineered into a 200 μm thickness membrane.

treatment-induced changes in neuronal network function [5]. However, conventional monolayer cultures do not reliably reproduce some physiological features of central nervous system tissue *in vivo* or, as acute brain slices *in vitro/ex vivo* in particular synchronous large amplitude local field potentials are absent [5]. This limitation can be addressed in part *via* organotypic slice cultures, but is further complicated by requirements for a media interface and the anatomical changes that are found in slice cultures within a few days *in vitro*.

In order to exploit the potential of MEAs but address the limitations described above, it has been speculated that three dimensional (3D) cell culture approaches could be used. To this end, numerous substrates have been successfully developed which are largely gel-based and contain extracellular matrix (ECM) components to influence cell development [1]. The ideal 3D matrix is biocompatible, persistent, easily manipulated (*i.e.*, non-gel), can be coated with ECM components as required, seeded with defined cells and either grown *in situ* on an MEA or independently before assay using an MEA (*i.e.*, comparable to use of acute brain slices [7]).

Alvetex 3D cell culture scaffold [6] is commercially available, polystyrene-based and of 200 μm thickness (Fig. 1). The scaffold contains voids of variable sizes in which seeded cells can develop and can be shaped to fit MEAs and easily moved from culture vessels to MEAs. Additionally, small Alvetex circles (~6 mm diameter) require fewer seeded cells than a monolayer seeded MEA (~40 mm diameter); an important consideration for high cost cultures (*e.g.*, stem cells [13]) and in the reduction of animal use in research [9]. Related scaffolds have been used to support neuronal-glial co-culture in a peripheral nerve preparation [2] but a viable and functional central nervous system preparation is lacking.

Here, we investigate and characterize functional murine neuronal networks cultured in Alvetex by using MEA and immunocytochemical approaches to demonstrate their relevance, efficiency and economy as viable and attractive tissue culture models. We compare the electrophysiological features of monolayer and 3D cultures in addition to their pharmacological responsiveness. We present immunocytochemical findings to reveal morphological differences in astroglial cells under different conditions and differences in bioelectrical activity suggesting a more physiologically normal profile for 3D cultures.

2. Materials and methods

Chemicals were from Sigma–Aldrich. Tissue culture media, supplements, secondary antibodies and immunochemistry reagents were from Life Technologies (Invitrogen) unless otherwise stated. Alvetex was supplied by Reinnervate (Co. Durham, UK) and a single-hole punch used to make 6 mm diameter circles which were placed in a 48 well-plate and washes of 5 min each in 70% ethanol, Dulbecco's phosphate buffered saline (PBS; twice; pH7.4) and DMEM. 0.3 mL DMEM + 10% fetal bovine serum (FBS) was added to each well and plates equilibrated at 37 °C 5% CO_2 overnight. MEAs and coverslips for immunocytochemistry were coated with 25 μg/cm^2 poly-D-lysine in deionised water for 5 min and dried for 2 h. 1 mL (MEAs) or 0.5 mL (coverslips) DMEM + 10% FBS was added and equilibrated at 37 °C 5% CO_2 overnight.

2.1. Embryonic mouse cortical neuronal cultures

Primary embryonic mouse cortical neuroglial cultures were based on [15]. E14 embryos were obtained from timed mated NIHS mice in compliance with the UK Animals (Scientific Procedures) Act, 1986, decapitated, brains removed, cortices dissected and meninges removed in Dulbecco's PBS minus calcium and magnesium plus 33 mM glucose (PBS-G). Cortices were mechanically dissociated in 10 mL PBS-G, allowed to settle for 5 min before the supernatant was decanted and centrifuged (200 × *g;* 5 min) at room temperature. The resulting cell pellet was resuspended in 10 mL DMEM:F12 (1:1) with: L-glutamine 2 mM, HEPES 5 mM (pH7.4), glucose 33 mM, 6.5 mM $NaHCO_3$, 100 IU/mL penicillin–streptomycin, supplemented with: insulin 25 μg/mL, transferrin 100 μg/mL, putrescine 60 μg/mL, progesterone 20 nM and Na_2O_4Se 30 nM. Resuspended cells were counted using trypan blue dye exclusion and diluted to 1×10^6 viable cells/mL in media consisting of MEM supplemented with 5% heat inactivated horse serum, L-glutamine 0.5 mM, glucose 15 mM and gentamicin sulfate 10 μg/mL (neuronal medium). Cell suspension was diluted in neuronal medium and added to MEA dishes and multi-well plates at ~2.5×10^5 cells/cm^2 of culture surface. 50% of medium was replaced with fresh neuronal medium and on DIV 3, 5, 7, 10, 12, 14 and every other day thereafter for the life of the culture. Monolayer cultures on MEAs were sealed using plastic rings holding a 12.5 μm thick Teflon semi-permeable membrane to prevent evaporation but allow gas exchange. All results reported were obtained from cultures at DIV14-21, consistent with previous reports showing the establishment of complex neuroglial networks that exhibit robust bioelectrical activity at this stage of development [16].

2.2. Immunocytochemistry

Cells on coverslips and Alvetex were fixed by transfer to 24 well plates containing 0.5 mL/well PBS, three brief washes with 0.5 mL PBS and 10 min incubation with 0.5 mL PBS containing 3.7% formaldehyde. Wells were then washed in PBS (thrice) and 0.5 mL PBS + 0.02% Triton-X100 added for 2 min before washing with PBS + 5% FBS (thrice) and incubation 2 h with 0.5 mL of PBS + 10% goat serum. Serum solution was aspirated and 0.5 mL primary antibody diluted in PBS, or PBS alone (control), added to wells overnight at 4 °C. All wells were then washed with 0.5 mL PBS + 5% FBS (thrice) for 2 min. 0.5 mL fluorophore-conjugated goat anti-rabbit or anti-mouse secondary antibody in PBS was added to all wells and incubated for 2 h at room temperature before washing with 0.5 mL PBS (thrice) for 2 min. 0.5 mL 10 μg/mL Hoechst 33342 blue solution was added for 5 s before flooding with PBS and brief rinsing and draining with 0.5 mL PBS (thrice). Coverslips and Alvetex were removed from wells, mounted on slides using fluorescence mounting medium (Vector Laboratories) and sealed using nail varnish before visualisation using epifluorescence microscopy. Primary antibodies (supplier and dilution) used were: GFAP (Dako,