Rapid generation of functional engineered 3D human neuronal assemblies: network dynamics evaluated by micro-electrodes arrays

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Abstract

Objective. In this work we adapted a protocol for the fast generation of human neurons to build 3D neuronal networks with controlled structure and cell composition suitable for systematic electrophysiological investigations. Approach. We used biocompatible chitosan microbeads as scaffold to build 3D networks and to ensure nutrients-medium exchange from the core of the structure to the external environment. We used excitatory neurons derived from human-induced pluripotent stem cells (hiPSCs) co-cultured with astrocytes. By adapting the well-established NgN2 differentiation protocol, we obtained 3D engineered networks with good control over cell density, volume and cell composition. We coupled the 3D neuronal networks to 60-channel micro electrode arrays (MEAs) to monitor and characterize their electrophysiological development. In parallel, we generated two-dimensional neuronal networks cultured on chitosan to compare the results of the two models. Main results. We sustained samples until 60 d in vitro (DIV) and 3D cultures were healthy and functional. From the structural point of view, the hiPSC derived neurons were able to adhere to chitosan microbeads and to form a stable 3D assembly thanks to the connections among cells. From a functional point of view, neuronal networks showed spontaneous activity after a couple of weeks. Significance. We presented a particular method to generate 3D engineered cultures for the first time with human-derived neurons coupled to MEAs, overcoming some of the limitations related to 2D and 3D neuronal networks and thus increasing the therapeutic target potential of these models for biomedical applications.

1. Introduction

The study of the electrophysiological activity of neuronal cells is widely used to get insight into the complex communication and organization of neuronal circuits in health and disease. In particular, micro electrode arrays (MEAs) technology has been used for non-invasive measurements of activity from neuronal networks (mainly from rodent origin), to investigate basic mechanism of neuronal communication, to test neurotoxicity of drugs and chemicals, and to model neurological disorders (Keller and Frega 2019). However, these models are mostly animal based and the obtained results are not always directly translatable to humans (Keller and Frega 2019). Due to many different aspects, rodent models can only partly compare to healthy and pathological processes in the human brain, and some disorders cannot be replicated at all. For example, rodent neurons show functional divergence, such as a faster rate of
network maturation, making it questionable if they can accurately represent human neuronal networks (Napoli and Obeid 2016). It has been also shown that human-specific gene expression patterns are present in human neurons (Khaitovich et al. 2004, Hawrylycz et al. 2015). With the advancements in human induced pluripotent stem cell (hiPSC) technology and neuronal differentiation protocols (Ohnuki et al. 2009), investigations on neuronal network properties in health and disease became possible in a model system that more closely resembles the human brain. Nowadays, hiPSCs derived neuronal networks grown onto MEAs represent a widely accepted model system (Mossink et al. 2021a) with applicability ranging from the study of information transmission (Shew et al. 2011) and biological mechanisms (i.e. plasticity, memory loss) (Accardi et al. 2016), to the characterization of neurological disorders (Russo et al. 2018, Frega et al. 2019, Monteiro et al. 2021) and toxicological screening (Ylä-outinen et al. 2010).

The majority of these models (both animals and human-derived) are two-dimensional (2D), with limitations in representing and mimicking the complex features of a region of brain tissue. 2D neuronal networks show different electrophysiological signatures as compared to in vivo recordings. The electrophysiological activity of these networks is mainly composed by time-locked burst-like phenomena often involving the entire network, while in vivo neurons exhibit a complex dynamic, ranging from sparse to synchronous activities (Belle et al. 2018). Additionally, 2D neuronal models lack the extracellular matrix (ECM) that permits a complete morphological development and guarantees a correct exchange of nutrients and catabolites. Finally, cells cultured in 2D tend to have flat bodies and neuronal arborizations propagate only in one plane leading to a cell network organization not comparable to the in vivo situation (Duval et al. 2017).

In the past decades, several approaches to create and study 3D neuronal networks have emerged (Langer et al. 1995, Tang et al. 2006). Some of these rely on the generation of organoids, structures that try to recapitulate features of early human neurodevelopment, including the generation, proliferation, and differentiation of neural progenitors into neurons and glial cells and the complex interactions among the diverse, emergent cell types of the developing brain in 3D (Trujillo et al. 2019, Tambalo and Lodato 2020). However, the generation of these structures is subject to variability (Qian et al. 2019) and complete maturation of the cells type requires long period. In fact, organoids are composed by heterogeneous population of cells with unlikely controllable cellular composition, rate and density (Durens et al. 2020). Furthermore, variation in the size of the organoid has been observed (Qian et al. 2019, Durens et al. 2020, Fair et al. 2020). In addition, the lack of vascularization within the tissue affects the diffusion of nutrients and waste in the structure, often causing necrosis in the ‘core’ of the organoids (Wang 2018). Finally, although they are active at a single cell level, they do not necessarily have functional activity at the level of the entire network (Mayer et al. 2018) and sometimes slices are taken from the organoids to be able to record from MEA (Zafeiriou et al. 2020). Other strategies for 3D tissue construction are the scaffold-based methods. They try to mimic properties of the ECM by using materials allowing cells growth and exchange of nutrients with the external environment (i.e. biopolymers (Lavik et al. 2002), agarose (Kunze et al. 2011), collagen (Fan et al. 2017), chitosan hydrogel and gel-like substances (Alizadeh et al. 2019)). It has been shown that the use of these materials induces the spontaneous formation of 3D networks with arborizations in the 3D space, improving cellular development and better representing the in vivo condition (Willerth and Sakiyama-élbert 2007, Kunze et al. 2011). Although the methods used are very versatile, systematic electrophysiological recordings from these 3D neuronal networks have been presented only in few cases (Fan et al. 2017, Tedesco et al. 2018, Trujillo et al. 2019, Arnaldi et al. 2021, Shin et al. 2021). A disadvantage in the use of biopolymers and gels can be addressed in the relative position of neurons that are randomly sparse in the 3D scaffold making the connection between the cellular processes and the electrodes unlikely (Smith et al. 2015). Alternatively, a new experimental paradigm was proposed the first time in 2008 (Pautot et al. 2008) in which silica microbeads were used as scaffold to support 3D neuronal growth. The innovative idea derives from the possibility of being able to consider the microbeads as single units and lays the foundations for a modular approach to the construction of 3D networks. More recently, chitosan microbeads have been used as scaffold for 3D rodent neuronal networks (Tedesco et al. 2018) and it has been shown that such networks coupled to MEAs exhibited activities similar to the in vivo situation. Chitosan is a linear cationic polysaccharide derived from the deacetylation of chitin, the major component of the exoskeleton of invertebrates and of cell wall of fungi (Dash et al. 2011). Its biocompatibility, biodegradability, antimicrobial activity and structural similarity with glycosaminoglycans, naturally present in the mammalian ECM, have made chitosan one of the most used biopolymers in tissue engineering (Islam et al. 2020). Recently, we have demonstrated (Di Lisa et al. 2020) that chitosan by itself is also able to sustain primary neuronal cell adhesion and growth, allowing the formation of functional neuronal networks, without the need of pretreating with adhesion molecules, such as synthetic polypeptides. Moreover, chitosan microbeads, obtained by phase-inversion reaction, have been demonstrated to have internal micro-porosities that allow not only the exchange of nutrients but also the growth of neuronal arborizations and mechanical properties similar to the in vivo
brain (Tedesco et al 2018). Furthermore, the use of microbeads as scaffolds allows not only a better control of the structure (i.e. volume of the structure), but can also permit the insertion of functional elements, capable of controlling/regulating cell growth (Custódio et al 2015, Arnaldi et al 2020a, 2020b).

In this work we show an integration of different techniques and technologies to generate engineered human 3D neuronal networks that can be coupled to MEAs for chronic electrophysiological recordings. To obtain controllability over cell type, density and structure and to ensure rapid functional investigations, we adapted a widely used hiPSCs differentiation protocol (i.e. Ngn2 induction (Frega et al 2017)) to be combined with chitosan microbeads as scaffold. We monitored and characterized the electrophysiological activity of such human 3D neuronal networks, and we compared the exhibited activity with 2D cultures grown on MEA coated with adsorbed chitosan.

2. Material and methods

2.1. Human induced pluripotent stem cells generation and maintenance

We used an already characterized rtTa/Ngn2 positive hiPSC line (Frega et al 2017) kindly provided by Frega et al (2019) in frozen vials (Passage 10). HiPSCs were reprogrammed via episomal reprogramming from fibroblast of an healthy donor (30 year-old female, Coriell Institute for Medical Research, GM25256). Afterword, rtTa/Ngn2-positive lentiviral vectors are used to stably integrate the transgenes into the genome of the hiPSCs. By using these transfer vectors, the hiPSC line can be created for which the expression of murine neurogenin-2 can be induced by supplementing the medium with doxycycline. Detailed information about the protocol can be found (Frega et al 2017). After rapid thawing, 6 × 10⁶ cells were equally distributed on three wells of a six well-plate pre-coated with Matrigel (Corning, #356237) and cultured in Essential 8 Flex Medium (Thermo Fisher Scientific) supplemented with 1% pen/strep (Thermo Fisher Scientific), 50 μg ml⁻¹ G418 (Sigma Aldrich) and 0.5 μg ml⁻¹ puromycin (Sigma Aldrich) (in the following we will refer to this supplemented medium simply as E8F). Medium was refreshed every 2 d. Cells were split twice a week (when reaching 70% confluence) using ReleSR (Stem Cell Technologies). During the 24 h after splitting, the medium was also supplemented with 100x Revitacell (Thermo Fisher Scientific). Well-plates were kept in incubator at 37 °C, 5.5% CO₂. The detailed protocol can be found (Frega et al 2017).

2.2. Rat astrocytes

To obtain astrocytic feeder, brain cortices were collected from E18 Sprague-Dawley rat embryos (Charles River). Briefly, cortices were enzymatically dissociated and single-cell suspension was then plated in T-75 flasks. Cells were propagated once 80% of confluence was reached and frozen after the first passage. More detail can be found (Aprile et al 2019). Cells were shipped in frozen vials. Astrocytes were thawed and seeded in T-75 flask with DMEM high glucose (Invitrogen, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% pen/strep. Cells were kept in incubator to grow and proliferate. After 7–10 d, astrocytes reached confluence and were split in two flasks using trypsin-EDTA 0.05% (Thermo Fisher Scientific). Cells were finally maintained in incubator and used in the following 14 d when confluence is reached again.

2.3. Chitosan microbeads

Microbeads were fabricated following the protocol described in detail (Tedesco et al 2018) using chitosan (low molecular weight 50–190 kDa, 77% deacetylation degree, viscosity 92 cP at c = 1% w/v in 1% acetic acid, Sigma-Aldrich). Briefly, chitosan was dissolved in 0.1 M acetic acid solution (2%, w/v). Chitosan microbeads were produced two days before seeding by air-assisted jetting technique, using a microencapsulation unit (Nisco Encapsulation Unit VAR J30, conical nozzle, 0.25 mm diameter). Chitosan solution was sprayed under controlled pressure (120 mbar) and flow rate (0.6 ml min⁻¹) into a gelling solution (2%, w/v, NaOH solution in 60%, w/v, EtOH) and left overnight to complete the gelling step. The obtained microbeads were centrifuged (1200 rpm for 5 min) to remove gelling solution, rinsed with 10 ml of ultrapure water four times and resuspended in 70% ethanol for sterilization. On the seeding day, ethanol was removed by centrifugation, the microbeads were washed three times with sterile water (time between washing steps of 5 min) and finally resuspended in cell culture medium. The production yield was evaluated using optical microscopy and diameter of beads was 120 μm ± 30.

2.4. PDMS structure and chitosan coating on MEAs

One day before cell seeding, MEAs were washed and sterilized in the oven (120 °C for 2 h). The polydimethylsiloxane (PDMS) confinement structures were fabricated with a standard 1:10 ratio between curing agent and elastomer. After mixing the components, we deposited a thin layer of PDMS onto a petri dish. Consequently, we started the curing treatment by placing the dish in the oven at 70 °C for 40 min. Therefore, we obtained a stick layer of PDMS (height ≈ 1 mm) from which we obtained several PDMS rings. We used two different punches to cut the PDMS into rings (inner diameter = 5 mm; thickness = 1 mm) and we used a cutter to manually remove a slice of the ring (figure 1(b)—left) to let the reference electrode of the MEAs uncovered. All these rings were washed, sterilized in 70% ethanol, dried
and placed onto sterile and dried MEAs. We used sterile tweezers to place the ring in the correct position and adhesion was achieved thanks to the stickiness of the cured PDMS. For each MEA we stacked six PDMS structures to create an inner small chamber (height of about 6 mm) on the electrode area (figure 1(b)-right).
More detailed information about the assembly of the PDMS structure on the MEA are shown in the supplementary figure 1 (available online at stacks.iop.org/JNE/18/066030/mmedia). One day before seeding, the active area delimited by the PDMS structure was coated with a 60 µl drop of 1% (w/v) chitosan solution. Chitosan solution was previously prepared by dissolving chitosan powder in 1% of acetic acid, filtering it through 0.5 µm PTFE syringe filter (Corning) and sterilizing it (120 °C for 20 min in autoclave) and stored at 4 °C until usage. After coating, the devices were then placed in incubator at 37 °C and 5.5% CO₂ overnight. Before plating, MEAs (with PDMS structure placed in it) were washed three times with Dulbecco’s phosphate-buffered saline (DPBS, Thermo Fisher Scientific, time between washing steps of 5 min) and left to dry for 30 min under the laminar-flow hood.

2.5. Neuronal differentiation and 3D neuronal network assembly

We directly induced the differentiation of hiPSCs into excitatory cortical Layer 2/3 neurons by overexpressing the neuronal determinant Neurogenin 2 (NgN2) factor upon doxycycline treatment (Frega et al 2017). Figure 1(a) summarizes the main steps to create neuronal network on MEA from hiPSCs. HiPSCs were detached from a well after reaching confluence (6 × 10⁶ cells) using ReleSR (Stem Cell Technologies) and were redistributed equally as single cells in E8F medium with 4 µg ml⁻¹ Doxycycline (Sigma Aldrich) on two wells of a 6-wells plate. We will refer to this step as day after differentiation (DAD) 0 (figure 1(a)—DAD0). The day after, the medium was entirely replaced with DMEM/F12 (ThermoFisher Scientific) supplemented with 100X N2-supplement (Thermo Fisher Scientific), MEM non-essential amino acid solution 100X (Sigma Aldrich), 10 ng ml⁻¹ human-NT-3 (BioConnect), 10 ng ml⁻¹ human-BDNF (BioConnect), 4 µg ml⁻¹ Doxycycline and 1% pen/strep (figure 1(a)—DAD1). Cells will proliferate and reach confluence in both wells therefore, during DAD2 (figure 1(a)—DAD2) medium needs to be replaced only if cells death/ detachment is observed. On DAD3, the medium was changed in Neurobasal (Thermo Fisher Scientific) supplemented with B-27 supplement (Thermo Fisher Scientific), GlutaMAX (Thermo Fisher Scientific), human-BDNF, human-NT-3, 4 µg ml⁻¹ doxycycline and 1% pen/strep. From now on we will refer to this medium composition as ‘complete medium’. At this day (DAD3/DIV0), early stage induced neurons (iN) were detached from the well and were used in combination with rA, previously stored in a flask in the incubator, to create both 2D and 3D models (figure 1(a)—DAD3/DIV0). In particular, iN and rA were detached by using Accutase (Sigma Aldrich) and trypsin-EDTA 0.05% respectively and, were collected in two separate 15 ml Falcon tubes (Corning) (figure 1(c)). We then prepared a solution containing a total of ~800 000 cells ml⁻¹ where iN and rA were in 1:1 ratio. Consequently, we seeded a 60 µl drop of solution containing 50 000 cells in the active electrodes area of the MEAs delimited by the PDMS structure to achieve a final cell density of ~2500 cells mm⁻² (figures 1(c)–(e)). After the monolayer deposition, we left the MEAs in the incubator for 3 h to allow cells adhesion.

In parallel, we moved ~150 000 beads from the tube containing chitosan microbeads to a new 15 ml falcon tube. Therefore, 3 h before 3D assembly, DPBS was replaced with complete medium by applying three washing steps, each composed of centrifugation, aspiration of the supernatant and resuspension in 2 ml of complete medium. Then, iN and rA (from an 1:1 solution previously prepared) were added in the chitosan-beads tube to obtain a 1:5 ratio between beads and cells (figure 1(d)). Consequently, this tube was kept tilted in the incubator and gently shook every 20 min to ensure adhesion between cell and beads. After 3 h, the tube was put in vertical position for at least 30 min. Finally, the supernatant was removed, and the cell-bead mix was re-suspended in 400 µl of complete medium.

At this point, we started the assembly of the 3D neuronal network on the MEA previously plated with the monolayer culture of iN and rA. First, 40 µl of medium were removed from the MEAs and were replaced with a 40 µl drop of the cell-bead solution, to obtain a scaffold of cells and beads on top of the monolayer culture (figure 1(g)). Each 3D structure was composed by 15,000 beads and 75,000 cells. Cultures were then kept in the incubator for 2 h and, after this time 100 µl of complete medium was added to avoid evaporation during the night. The next day (DIV1), the entire medium was replaced with complete medium supplemented with Ara-C (2 µM, Sigma Aldrich) (figure 1(a)–DIV1). MEAs were then stored in the incubator and checked daily. At DIV3, 50% of the remaining medium was removed and new complete medium was added to fulfill the small chamber created by the PDMS insert (~200 µl). From this DIV on, half of the medium was changed every two days and from DIV 7, complete medium was supplemented with 2.5% of FBS (figure 1(a)–DIV7).

2.6. Data acquisition and analysis

We used MEA60 from MultiChannel System (MCS) to record the network activity of 2D and 3D neuronal populations. These devices present 60 electrodes (TIN/SiN, 30 µm electrode diameter, 200 µm spaced) arranged in an 8 × 8 square grid without the four corners. The electrophysiological activity was acquired with the 2100 System (MEA 2100-System, MCS) and signals were sampled at 10 kHz. Sterility was maintained using an autoclavable cap on top of the MEA chamber while normal conditions were maintained by pre-heating the head stage at 37 °C.
and by delivering humidified air with 5.5% CO₂ over the MEA thanks to a plastic box placed on top of the recording system.

Recordings lasted 15 min and data were analyzed offline using MATLAB (The Mathworks) scripts. Raw data were first subjected to high-pass filtering (cut-off frequency = 200 Hz) then to spike detection and finally analyzed as point processes. For each channel, noise was estimated by dividing the whole recording in 60 s bins and the noise with the lowest standard deviation was chosen among the bins.

2.6.1. Spike detection
We implemented Precise Time Spike Detection algorithm as detailed (Maccione et al 2009). Spike were extracted if the peak was five times higher than the noise's standard deviation and the peak-to-peak amplitude was six times the noise's standard deviation, considering 1 ms of Peak Lifetime and refractory period. For the analysis we considered as active electrodes the ones that showed at least 0.1 spikes s⁻¹. For each recording we quantified the array wide firing rate (AWFR) by dividing the recording in 100 ms bin and counting all the spikes detected in each bin, the mean firing rate (MFR) as the sum of all spikes recorded in one electrode divided by the recording time, averaged across only active electrodes. Inter spike interval (ISI) was obtained for each active electrode and calculated in bin of 10 ms for 10 s as probability to have an event after a certain amount of time.

2.6.2. Burst detection
Burst detection algorithm was applied to each channel. We considered burst as a series of at least five spikes in which the ISI among them was not longer than 100 ms. Therefore, we quantified the array wide firing burst and the mean burst rate as we described for the spikes analysis. Inter burst interval (IBI) was obtained as the ISI but considering the time between the end of a burst and the start of the next one. Burst duration (BD) was obtained by calculating the duration (ms) of each burst. We evaluated the percentage of random spikes (PRS) as the total number of spikes in the culture that does not belong to a burst.

2.6.3. Network burst detection
We defined network burst (NB) as synchronous burst activity among channels. We detected a NB whenever we observed bursts in 20% of the active channel with an IBI ≤ 100 ms (Bologna et al 2010).

2.6.4. Network synchronous activation
We implemented an algorithm for the network synchronous activation (NSA) detection. We first evaluated the AWFR of a culture in 200 ms bins, then we detected the peaks higher than the mean AWFR plus two times the standard deviation. Afterwards, for each peak we verified that in the peak bin there were synchronous activation between electrodes. We imposed the condition that at least 20% of the active electrode must fire with an ISI ≤ 100 ms. If the condition is met, the algorithm searches for the starting point of the NSA. Therefore, we searched for the first point in the AWFR that was below threshold before the detected peak. We then divided the time between the two points in 25 ms bin and we choose the starting point as the first bin in which at least 20% of the electrodes were firing. If no bins met the criteria, peak were discarded from the NSA detection. For the ending point of the synchronous activation, we used the first point below the AWFR mean after the peak, we divided the time between these points in 25 ms bins. Consequently, we looked for four consecutive bins without activity and we assigned the end of the NSA to the first active bin preceding that silent period. Finally, we merged the NSAs that were less than 200 ms apart.

2.6.5. NSA shape and propagation
We extracted and aligned the NSA of each culture by grouping all the spikes detected within NSA in chronological order. Mean shape was obtained by calculating the occurrences of spikes detected in 50 ms bins in a 5 s window. We defined an ignition site as the electrode correspondent to the first spike in at least 5% of detected NSA in a defined culture. To estimate signal propagation from the ignition sites to the rest of the electrodes we considered the latency from the first spike of the NSA to the first occurrence in the other electrodes. Distances were then evaluated considering the Euclidean distance between electrodes. Furthermore, we evaluated mean intra-latency and mean intra-distance for each detected NSA. Intra-latency was evaluated by considering all the inter-spike-intervals within the NSA and averaging across the total number of spikes. Mean Intra-distance was evaluated in the same way but considering the distance between electrodes of consecutive spikes.

2.6.6. Statistical analysis
We used rank-sum MATLAB function to perform non parametrical Mann–Whitney U-test since data do not follow a normal distribution. Significance differences were considered when \( p < 0.05 \).

2.7. Immunofluorescence
Sample were washed with sterile PBS solution and fixed in 4% para-formaldehyde (PFA, Sigma-Aldrich) solution for 15 min at room temperature. Then we used 0.2% of Triton X-100 (Thermo Fisher Scientific) for 10 min to permeabilize the cells and non-specific binding antibodies were blocked by adding to the sample a blocking buffer solution (BBS, composed of 5% normal goat serum (Sigma Aldrich), 1% bovine serum albumin (Sigma Aldrich, 0.2% Triton X-100 in phosphate-buffered saline) for 45 min. Primary antibodies were diluted in BBS and left overnight.
at 4 °C. After that, samples were rinsed three times with PBS and stained with secondary antibody for 1 h at room temperature. Finally, DAPI (1:10 000, nuclei, Sigma Aldrich) was added at the end for 10 min and cells were stored in dark condition at 4 °C in PBS solution. GFAP (1:500, glial fibrillary acidic protein, monoclonal or polyclonal antibodies, Sigma Aldrich) and MAP-2 (1:500, dendritic microtubule-associated protein, Chemicon Millipore) were used as primary antibodies to label glia and neurons, respectively. Alexa Fluor 488 and Alexa Fluor 549 Goat anti mouse or Goat anti rabbit (diluted 1:700 and 1:1000; Thermo Fisher Scientific) were used as secondary antibodies. We acquired pictures with the upright microscope Olympus BX51M equipped with the fluorescent lamp Olympus U-RFL-T and Hamamatsu ORCA-ER digital camera.

3. Results

3.1. Human neurons form 3D neuronal networks
To build a human 3D excitatory neuronal network on MEAs we optimized the original hiPSCs differentiation protocol introduced (Frega et al 2017).
To achieve a controlled cell density and composition, we prepared independent solutions containing inN, rA and microbeads with known concentrations (figures 1(c) and (d)—tubes 1-2-4, respectively). To create 2D neuronal networks and as a first step of the 3D network construction, we deposited a monolayer of inN and rA (i.e. 1:1 ratio, figure 1(c)—tube 3) on top of the active area of a MEA. Figure 1(e) shows a picture of the seeded cells homogeneously sparse over the entire electrode area. The iN solution was obtained by using hiPSCs at the third day of differentiation, period necessary to allow the cells to lose their proliferative ability and thus enabling us to control final density. After 42 DIV, by immunocytochemistry we found a ratio between inN and rA of 1:1.2, confirming that the implemented methodology offers a good control over cell density (supplementary figure 2). Right after the monolayer deposition, we built the 3D engineered neuronal network. First, we combined inN, rA and microbeads to obtain a 1:1:0.2 ratio (figure 1(d)—tube 5). Then, we kept the tube containing cells and beads tilted in the incubator for 3 h and we gently shook it every 20 min. This step was essential to ensure a homogeneous deposition of inN and rA on the beads and to enhance reciprocal adhesion. After 3 h, we observed that cells were attached to the microbeads taken from the tube, showing that cells and beads were ready to be assembled to form a 3D network (figure 1(f)). Thus, we let the preparation precipitate by simply leaving the tube in a vertical position, avoiding centrifugation which could lead to the detachment of the cells from the beads. Finally, we placed cells and microbeads on the monolayer of inN and rA formed on the active area of MEAs delimited by the PDMS structure. The PDMS structure we created was essential to confine cells and beads, ensuring a controlled 3D network formation (figure 1(g)). During the first week of development, the 3D structure was still weak since small mechanical perturbations (i.e. change of culture medium) led to flickering of the beads, increasing the probability of creating damage to the network. During time, we observed that the diameter of the beads decreased leading to an increase in the solidity of the structure (i.e. average diameter of about 100 µm by DIV 42, figures 2(a) and (b)). Furthermore, we investigated how the 3D structure influenced the cellular distribution in the cell monolayer. While in 2D neuronal networks a homogenous distribution of cells was present (figure 2(d)), cells were sparser and unevenly distributed on the electrode area when the 3D structure was plated on top (figure 2(c)). Finally, we acquired images at different height of the 3D structure to investigate neuronal organization in 3D late in development (supplementary figure 4). It is possible to observe neurons adhering to the beads that are interconnected to each other, forming a network. In addition, the presence of micro-spaces between the beads can be seen, which should ensure exchange of metabolites. These results show that also within the 3D structure neurons are present and connected.

3.2. Electrophysiological development of 2D and 3D neuronal networks coupled to MEAs
We seeded 3D neuronal networks (n = 5) on MEAs and we evaluated their electrophysiological activity during development (17–42 DIV). To investigate whether human neurons formed 3D structure functionally connected exhibiting developmental trajectories indicative of proper maturation and patterns of activity that in principle, better recapitulate the activity in the in vivo brain area as compared to golden standard 2D models, we characterized 2D sister cultures (n = 8). All 2D networks showed spontaneous activity starting from DIV 17, which was mainly composed by isolated spikes and burst (figure 3(a)). During development, the general level of activity of the network as well as the number of electrodes involved increased. As shown by the peaks present in the AWFR (figure 3(a)), the activity increased over time and organized itself in synchronized network events (i.e. NB, NSA). 3D networks showed spontaneous activity from DIV17 composed only by sparse spikes (figure 3(b)). As observed for 2D populations, the level of activity and the number of electrodes involved increased during time, together with the appearance of synchronous patterns (i.e. NSA, figure 3(b)—DIV 42). These results indicate that both 2D and 3D cultures developed into functional interconnected neuronal networks.
To investigate and compare the development of 2D and 3D neuronal networks, we evaluated
six different features, averaged across the same experimental conditions, and we plotted their normalized trend using the maximum value reached during the development of the cultures (figure 3(c)). Considering the 2D networks, we found that spikes started organizing into burst by DIV 17, while network bursts and synchronous activation emerged by DIV 24. Overall, during development the firing and (network) bursting frequencies increased, together with an involvement of more recording electrodes and a reduction of the PRS. From DIV 32 to 35 onwards, all these parameters tended to stabilize, as shown in figure 3(c). We found that the dynamic exhibited by 2D and 3D cultures during development was similar, although 3D networks exhibited different changes of the observed parameters compared to 2D cultures. Firing activity was detected starting from DIV 17 while single channel bursting activity appeared around DIV 28. Network bursts were not detected in all samples until DIV 38. However, NSAs were present from DIV 28 and their frequency remains almost unchanged during development.

3.3. excitatory 3D neuronal networks exhibit different electrophysiological activity as compared to 2D

We have found that 3D neuronal networks exhibited similar developmental behavior as compared to 2D, although with different dynamics. To better compare 2D and 3D neuronal network activities measured by MEAs, we further analyzed the network activity at a late and stable developmental stage, when the NSA rate showed a plateau phase (i.e. DIV 38). At this time point, we found significantly lower MFR and mean bursting rate in 3D networks as compared to 2D sister cultures, together with an increased PRS (figures 4(a)–(c), p < 0.02). The mean BD was also lower in 3D neuronal networks as compared to 2D, though with no statistical significance. Regarding the network burst analysis, we found significantly lower rate in 3D compared to 2D, while the duration of the NBs was not statistically different (figures 4(e) and (f)). Finally, we observed a reduced NSA rate and duration in 3D neuronal networks, although not significant (figures 4(g) and (h)). Furthermore, from the evaluation of the ISI graph it was evident that
Figure 3. Electrophysiological activity of 2D vs 3D cultures. (a), (b) Raw data recorded from one electrode (6 s), rasterplots and AWFR exhibiting 1 min of spontaneous activity during development. The rasterplot y-axis represents electrodes and each blue dot indicates a detected spikes in the given electrode. The AWFR quantifies the level of activity showed in the rasterplots and highlight the emergences of synchronous network event. (c) Comparison between 2D and 3D culture during development. All Data points represents mean values across sample with same experimental conditions and error bars represents standard deviation. Data from each sample were normalized regarding the maximum value reached during development and then averaged. Number of active electrodes and perceptual random spikes (PRS) are represented without normalization. 3D data are represented in blue ($n = 5$), 2D data are represented in green ($n = 8$).
Figure 4. Electrophysiological comparison of 2D and 3D cultures at DIV38. (a)–(h) Graphs showing the (a) mean firing rate (MFR), (b) percentage of random spikes (PRS), (c) mean burst rate (MBR), (d) mean burst duration (MBD), (e) network burst rate (NBR), (f) network burst duration (NBD), (g) network synchronous activation rate (NSAR), (h) network synchronous activation duration (NSAD) in 2D and 3D neuronal networks at DIV 38. (i), (j) Graphs showing the (i) Inter spike intervals (ISI) (10 ms bin) and (j) burst duration (BD) (50 ms bin). Data shows the mean obtained across sample with same experimental condition and error bars shows standard deviation. *p < 0.05. 2D data are represented in blue (n = 5), 3D data are represented in green (n = 8).

2D cultures fired closer spikes (ISI < 100 ms) more frequently, while in 3D networks spikes appeared spread at different intervals, including ISIs greater than 100 ms (figure 4(i)). Finally, by analyzing the distribution of the BDs we found that 3D neuronal networks showed mainly burst lasting between 250 and 500 ms, while in 2D a wider range of BDs was present (figure 4(j)). Additionally, we kept alive two cultures from both experimental paradigm until DIV60 and we recorded the activity. Supplementary figure 3 shows how the network firing proprieties remained almost unchanged from DIV38, confirming that networks reached a stable developmental stage between the 5th and 6th weeks in vitro.

Even if no statistical difference was observed in the NSA frequency, we found a specific NSA dynamic in 2D and 3D neuronal networks (figure 5). By evaluating the mean NSA shape (figure 5(a)), we found differences in the recruiting part of the NSA (initial phase) and in the NSA decay. In 2D neuronal networks, the maximal activity was reached after about 100 ms from the ignition of the event. The decay phase was fitted with a power model ($R^2 = 0.9931$). Activity decreased to 20% in about 700 ms and finally ceased after about 3 s. Instead, in 3D neuronal networks the maximal activity was reached almost immediately after the ignition (<50 ms) and persisted for about 200 ms. The decay phase was fitted with a linear model ($R^2 = 0.9708$) and the NSA lasted about 2 s. Furthermore, for each NSA we plotted the NSA duration versus total number of spikes observing differences in 2D and 3D neuronal networks (figure 5(b)). In particular, we noticed distinct NSAs clusters from different cultures (figure 5(c) different colors). In 3D, the number of spikes per NSA was smaller compared to 2D and NSAs were shorter. We then evaluated the number of NSAs and ignition sites (i.e. the electrodes from which the NSA is initiated) and we investigated the propagation of the signal during the NSAs in 2D and 3D neuronal networks. Even if no significant difference in the number of NSA and ignition sites was found (figure 5(d)), we found a clear difference in signal propagation and latency. In particular, while
2D neuronal networks exhibited a fast and continuous propagation of the signal, in 3D we found a discontinuous propagation characterized by longer latencies (up to 1.8 s), as shown by the representative isochrones (figure 5(e)). Finally, we analyzed the characteristics between intra-distance and intra-latency during NSAs. We noticed that 3D NSAs are sparser than 2D (figure 5(f)) and, more specifically, we observed that for 2D networks each cluster is bounded in a certain region in the latency-distance plane and these regions are culture specific (figure 5(g)). In the 3D samples, we noticed a wider range of both intra-latency and distance, indicating a propagation developing in a 3D space and only partly captured in the read-out layers of neurons coupled to the planar MEA. All these evidences can be considered as indirect proofs of signal propagation in the upper layers of the 3D structure.

Finally, we investigated whether changes in activity observed when moving from 2D to 3D structures were similar in rodent and human neuronal models. We found that human neurons in a 3D environment differ from the related 2D model on the same parameters observed in rodent (figure 6) (Frega et al 2014, 2019, Tedesco et al 2018). In particular, when embedded in a 3D structure, neuronal networks show a lower firing and (network) burst rate, a higher PRS and a lower (network) BD as compared to 2D cultures with the same neuronal origin.

4. Discussion

We specifically optimized a widely used differentiation protocol (i.e. Ngn2 induction) to be suitable for the construction of a 3D assembly onto MEAs using microbeads as scaffold. This protocol induced the fast conversion of hiPSCs into a homogeneous population of cortical excitatory neurons, that organize into a functional network in three weeks (Frega et al 2017).
To ensure proper functional development, the protocol required co-culture with rat astrocytes. Different differentiation protocols have been developed to obtain astrocytes from hiPSCs, however they are extremely long-time and expensive (Krencik et al 2011, Santos et al 2017, Li et al 2018). In the original protocol, hiPSCs differentiation was initiated directly on MEA and astrocytes were added on top of the differentiating cells 3 d after Ngn2 induction. Since integration of neurons and astrocytes in 3D is required, we plated neurons, astrocytes and microbeads at a desired ratio on MEA at the same time. To ensure proper control over cell ratio and to allow neuronal network integration, we used neurons in which Ngn2 induction was initiated three days before. At this stage of differentiation hiPSCs have lost their proliferative capacity, ensuring control over the number of seeded cells. Furthermore, hiPSCs morphologically started to resemble neurons, small processes were formed although mature arborization and structural connections between neurons were still not present, thus enabling neuronal network formation. The success ratio of the protocol depends on the user’s experience and working conditions. Indeed, a proper cell-beads coupling should be established before plating and mechanical perturbations should be minimal to avoid damage of the structure. Once the protocol is properly established, the success rate (i.e. 3D neuronal networks coupled to MEA showing mature electrophysiological activity) is about 80%. The cultures originated by using the last drops of cell-beads mix solution might not survive, probably because they contain cells precipitated at the bottom of the vial (i.e. dead cells or cells not adhered to beads) and a higher percentage of larger beads. These conditions do not allow a healthy/homogeneous development of the network, leading to cell death and breakdown of the construct. This success rate can be therefore increased by plating only about 80% of the solution containing beads and cells.

The bead scaffold-based method has been already validated by us with the use of rodent cells (Frega et al 2014, Tedesco et al 2018). In particular, we have already shown network responses from stimuli delivered both from top layer of the structure proving that neurons grown in 3D with this scaffolding procedure were functionally connected. Furthermore, axonal reconstruction showed neurites propagation in different layers. The choice of chitosan microbeads as scaffold was based on several considerations.

Figure 6. Graphs showing the quantification of electrophysiological activity exhibited by 2D ad 3D rodent and human neuronal networks. Data regarding the rodent model were taken from (Tedesco et al 2018). (a) Mean firing rate (MFR), (b) mean burst rate (MBR), (c) mean burst duration (MBD), (d) percentage of random spikes (PRS), (e) network burst rate (NBR), (f) network burst duration (NBD). *p < 0.05. In blue we presented 2D models (rodent n = 3, human n = 8), in green the 3D (rodent n = 3, human n = 5).
First, it has been described that the softness of the microbeads allowed to obtain a 3D tissue with mechanical properties similar to the in vivo brain (Tedesco et al 2018). Furthermore, chitosan is easily available at low cost, and the extreme bio-compatibility and degradability (mediated by hydrolytic enzymes) makes it a good candidate for future in vivo applications. Moreover, the inter-porosity formed by beads assembly allows the flow of nutrients and waste metabolites from the center of the tissue to the outside, thus resulting in a less dense structure than a scaffold-free organoid and probably preventing long-term cell death in the core of the structure. The use of such microbeads as scaffold offers the possibility to create engineered neuronal networks. In fact, by varying the ratio between microbeads and cells, the density of the assembly can be tuned. Furthermore, making the assumption that beads were spherical and follows the cubic close packing the final dimension of the construct can be tailored by varying the total number of microbeads and/or changing the dimension of the constrain (i.e. PDMS ring). Greater control over the bead size/shape variability would certainly lead to greater replicability of the experiments and higher control of different parameters of the resulting structure. Pre-treatment with adhesion factors or encapsulation of molecules can be performed on the microbeads to confer additional features to the structure (i.e. guidance of axonal growth, controlled release of drugs) (Arnaldi et al 2020a, 2020b). Finally, the possibility to integrate different hiPSCs differentiation protocols generating homogeneous population of cells (i.e. excitatory neurons, inhibitory neurons (Mossink et al 2021b), astrocytes) will allow to build a 3D neuronal network with controlled cell density and composition, which is not currently possible.

Through imaging we monitored the development of the 3D neuronal networks. During the first week, microbeads were covered with cells not connected to each other. After the first week, we observed that microbeads decreased in diameter and the structure became more stable. In a previous work (Tedesco et al 2018), we showed that neuronal processes were able to grow passing through the beads and that perineural net-like structures were formed. Thus, the decrease in diameter might be caused by the extension of cellular processes that—both enveloping and penetrating chitosan microbeads and connecting them to each other—sustain the formation of a complex and stable network and apply force to the beads that start to slowly shrink.

Then, we investigated the functional properties of 3D human neuronal networks grown on MEAs. We found that hiPSCs-derived neurons formed 3D functionally active neuronal networks. Our analysis revealed that the activity exhibited by 3D neuronal networks was different as compared to 2D. Since the 3D assembly was deposited on top of a 2D neuronal network, the comparison of the activities exhibited by the two structures allowed us to investigate how the 3D layers modulate the network dynamics. 3D networks exhibited lower spiking and bursting activities as compared to 2D (figure 3). While in 2D the activity was observed in all electrodes, a reduced number of electrodes were active in 3D (figure 4(a)). This is in line with the lower cell density observed in the electrode plane under the 3D cultures. Considering that we assembled the 3D network on top of a 2D network, the low cell density suggests that a migration of cells in the upper layers of the structure occurred. We have already observed this behavior in our previous work (Tedesco et al 2018) where neurons were partially transferred onto the surface of the overhanging 3D assembly because of the higher biomimeticity of 3D chitosan-beads scaffold than the 2D MEA surfaces. Low firing and bursting activity could be also indicative of poor viability. However, we showed that neurons adhering to beads connected through arborizations were present in the inner layers of the 3D structure (supplementary figure 4), indicating that complex neuronal networks are formed in the core of the structure. Moreover, we have presented chronic and protracted physiological activity (up to 60 DIV) and we have shown that activity exhibited by 3D neuronal network at DIV60 did not differ from the one at DIV38 (figure 4 and supplementary figure 3) indicating that good viability was present. Indeed, in the case of cell death, the toxic signals of the dying cells (i.e. in the core of the structure) would cause damage to the surrounding area, resulting in a decrease or loss of activity later in development. Finally, since changes in activity patterns observed in 3D as compared to 2D rodent models (figure 6) were associated with proper 3D neuronal network formation (Frega et al 2014, 2019, Tedesco et al 2018), the fact that the same trend is observed when moving from a 2D to a 3D human model indicates that a 3D neuronal network was properly formed.

The synchronous network bursting activity (i.e. NB) characterizing 2D neuronal networks dynamic in absence of external inputs was not maintained in 3D cultures. The absence of NBs in 3D neuronal networks might indirectly indicate that neurons on the electrode plane received inputs from neurons of the upper layers of the structure, thus giving rise to a more heterogeneous network dynamic. In both 2D and 3D networks an oscillatory dynamic based on the quasi-synchronous activation of neurons on different electrodes (i.e. NSA) was observed, although with different characteristics (i.e. shape, composition, propagation). In particular, we found that NSAs propagated uniformly over the entire surface of the MEA in 2D neuronal networks, while in 3D the propagation was apparently discontinuous (as it propagates in the 3D space). Additionally, we found specific cluster of NSAs in the intra-latency/intra-distance plane in 2D networks. The fact that these clusters were cultures specific, suggests that
NSAs characteristic are influenced by intrinsic properties of the network. We were able to record and describe an NSA event in all 2D culture, because these events were generated and propagated in the electrodes plane. In 3D neuronal networks, instead no specific clusters were present and longer propagation latencies of the signals (and intra-latencies in NSAs) have been observed. This is an indirect proof that signals propagate in the upper layers and indicates the formation of a functionally connected network. Indeed, we have already shown in a rodent model using the same scaffolding methodology (Frega et al 2014) that longer propagation latencies and variable spontaneous activity were a convincing hallmark of the inherent contribution of the 3D structure to the network dynamics.

As a whole, in this work we have shown a microbeads-based method for the generation of engineered hiPSC-derived 3D excitatory neuronal networks coupled to planar MEAs for chronical evaluation of electrophysiological recording. Although the methodology to build 3D neuronal networks using microbeads as scaffold has been already presented (Pautot et al 2008, Frega et al 2014, Tedesco et al 2018) protocol adaptation for hiPSCs-derived neurons is new, as it is novel the chronical electrophysiological analysis of these particular biological samples carried out with standard MEAs (i.e. MEA with 60 planar electrodes). Electrophysiological recordings of 3D structures have been already successfully performed with MEA devices. In particular, Trujillo and coworkers have shown that hiPSCs-derived organoids exhibit activity with features similar to the neonatal EEG (Trujillo et al 2019). However, the combination of hiPSCS-derived neurons and microbeads scaffolding allows to generate engineered neuronal networks with control over several culturing variables (i.e. cell type, density and ratio, volume, good reproducibility) which is still not possible with brain organoids. Unfortunately, the electrophysiological activity exhibited by neurons belonging to the inner layers of the structure is still a hurdle. However, new technologies allowing recordings at different heights are emerging. The most recent example is shown in (Shin et al 2021) where a device capable of recording and stimulating different sites at different heights within their 3D hydrogel-based culture is presented. The use of such a device will allow to characterize the complex electrophysiological dynamics occurring in the core of the structure. The integration with our protocol would result in a more versatile system, since the inter porosity of the beads-method could guarantee the insertion of the 3D electrodes afterwards, without forcing the construction of the culture around the electrodes.

The use of hiPSCs has opened new perspectives to study neuronal disease in vitro, allowing to obtain a human model system maintaining the genetic background of the donor. It has been shown that rodent neurons show functional divergences as compared to human neurons and that genes expressed in human neurons are not present in rodent (Napoli and Obeid 2016), making it questionable the validity of such model system for human disease investigation. In our study, we also observed differences in activity when comparing human and rodent neurons organized in both 2D and 3D networks. In particular, human neuronal networks show a lower firing and (network) burst rate, a higher PRS and a longer (network) burst as compared to rodent cultures (figure 6). These results might indicate a slower human neuronal network maturation, as previously shown, and thus might be indicative of species-specific differences. However, the patterns observed can be associated with other aspects (i.e. different cellular composition and developmental stages), making a proper comparison of the two system not possible.

5. Conclusion

We presented a new experimental in vitro platform constituted by 3D engineered neuronal cultures derived from hiPSCs and coupled to MEAs. This experimental model offers control over cell type, cell ratio, and total volume of the network, allows good replicability and ensures chronical functional assessment of the electrophysiological activity. Although the use of chitosan microbeads as scaffolds had already been investigated, in this work we have integrated a neuronal differentiation protocol from iPSC, showing the possibility of creating a tissue that can be engineered from various points of view. Further and deeper characterization of these construct are needed to validate the model as possible valid approach for future brain-on-a-chip.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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Ethical statements

The experimental protocol was approved by the European Animal Care Legislation (2010/63/EU), by the Italian Ministry of Health in accordance with the D.I. 116/1992 and by the guidelines of the University
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