Plant Derived Nanofibrillar Cellulose (NFC) Hydrogel Supports Robust Human Neuronal Network Formation *in vitro*

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Background

Human stem cell based neuronal cultures are seen as a promising tool for studying e.g. disease mechanisms, drug response or developmental biology *in vitro*. However, traditional flat 2D cultures on top of rigid surface fail to reproduce *in vivo* like brain complexity, like high cell density and connectivity with surrounding cell-cell and cell-extra cellular matrix (ECM) contacts. Due to these aspects, the 2D culture can lead to unnatural cell polarization on the flat culture. Different types of 3D cell cultures aim to overcome some of these limitations, by offering cells artificial extracellular matrix (ECM) and more *in vivo* mimicking environment.

Highly biocompatible nanocellulose based hydrogels have been proven to be potential for 3D culturing of various cell types (Lou et al. 2014; Bhattacharya et al. 2012). These plant derived nanofibrillar cellulose (NFC) hydrogels mimic native soft tissue ECM in fiber size and in mechanical properties, thus providing cells more *in vivo* like growth environment.

In this study pre-differentiated human pluripotent stem cell derived neurons where cultured as encapsulated within the NFC hydrogels, for testing the NFC hydrogel as culturing matrix.

Materials and Methods

Human embryonic stem cell (hESC) derived neuronal cells were cultured for two weeks as encapsulated within the NFC hydrogel, GrowDex® (UPM Kymmene Oyj, Finland). Studied hydrogel concentrations were 1.50 and 1.0 wt%. Two different gel volumes, 80 and 60 µl, were studied on 96-well plate format.

Human neurons were derived from hESC line Regea 08/023. Briefly, stem cells were pre-differentiated for eight weeks using previously published method (Lappalainen et al. 2010). The cell concentration used in hydrogel studies was five million cells per ml of hydrogel.

The formation of the neuronal networks inside the hydrogels was evaluated by immunocytochemical staining against neuronal markers Microtubule-Associated Protein 2 (MAP-2) and β-Tubulin III (β-Tub), as previously described (Koivisto et al. 2017). Immunocytochemical samples were imaged with an Olympus IX51 inverted and Zeiss LSM 780-confocal microscope. Image processing was performed using Adobe Photoshop CS4, Huygens Essential and ImageJ –softwares. Confocal data was visualised as shown in (Fig.1).

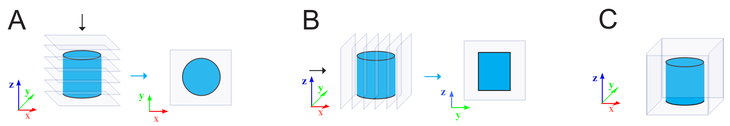

Fig.1. Image prosessing of the confocal stacks. Neuronal networks inside the hydrogels were visualized using maximum intensity projections (A,B) and 3D rendering (C).

1. NFC hydrogel was stable 3D matrix

Sample preparation, cell plating, cell culture and analysis were successfull with the NFC hydrogel. The smaller hydrogel volume caused some loss of the hydrogel during washes in the immunocytochemical staining. Overall, the samples were stable during cell culture and analysis (Fig. 2).

A

80 µl	Severe gel loss	Moderate gel loss
GrowDex 1.5 wt%	0 %	0 %
GrowDex 1.0 wt%	0 %	0 %

60 µl	Severe gel loss	Moderate gel loss
GrowDex 1.5 wt%	33 %	0 %
GrowDex 1.0 wt%	0 %	33 %

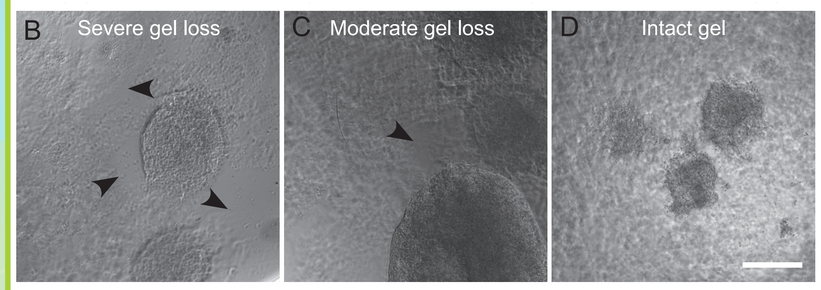

Fig.2. Samples with gel loss as percentage (A) in both studied hydrogel volumes and figures describing the classification (B,C,D). Arrow heads show areas of gel loss. Images are taken after immunocytochemical staining and mounting. Scale 200 µm.

2. Hydrogel volume effected cell growth

Neurite outgrowth was analysed from the immunocytochemically stained samples. Cultures were classified into three categories: 1) robust neurite outgrowth, 2) moderate neurite outgrowth, or 3) neural aggregates (no outgrowth), according to the amount of visible neurites (Fig. 3). Smaller hydrogel volume was more supportive for neurite outgrowth wereas larger volume supported neural aggregates.

A

80 µl	Robust neurite outgrowth	Moderate neurite outgrowth	Neural aggeregates
GrowDex 1.5 wt%	22 %	11 %	67 %
GrowDex 1.0 wt%	11 %	22 %	67 %

60 µl			
GrowDex 1.5 wt%	0 %	67 %	33 %
GrowDex 1.0 wt%	33 %	67 %	0 %

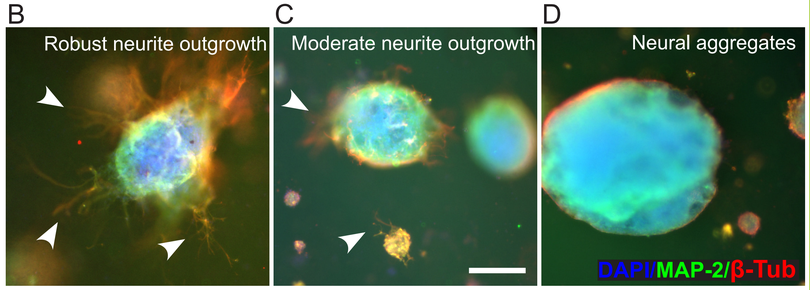

Fig. 3. Evaluation of neurite outgrowth in 3D cultures (A) and examples of classification (B,C,D). Arrow heads show areas with neurite outgrowth. Scale 200 µm.

3. Robust neurite outgrowth in 3D

Neurite outgrowth was studied more detailed using confocal imaging. Both NFC hydrogel concentrations supported robust neurite outgrowth in all directions (Fig. 4), in hydrogel volume of 60 µl.

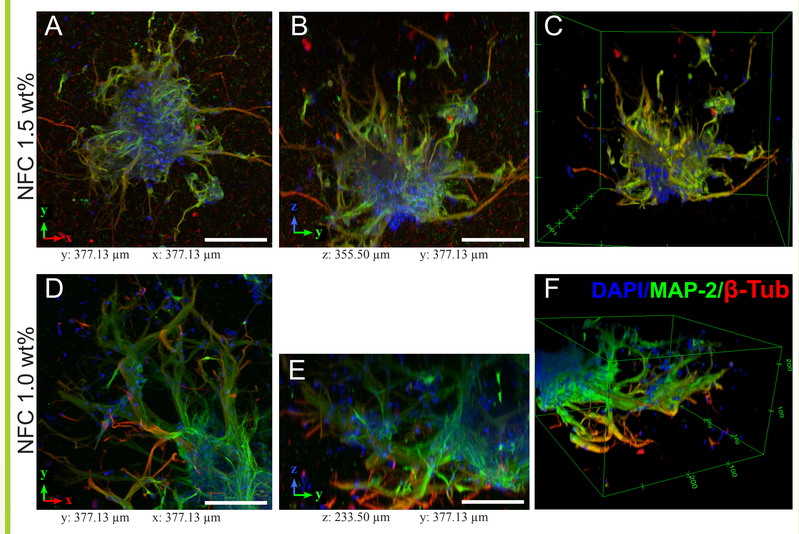

Fig.4. Maximum intensity projections (A,B,D,E) and 3D rendering (C,F) of confocal data (see. Fig. 1 for method). Hydrogel concentrations: 1.5 wt% (A,B,C) and 1.0 wt% (D,E,F). Scale 100 µm.

Conclusions

Based on these results it can be concluded that studied NFC hydrogels offered a very good 3D growth environment for human neural cells. No clear differences were seen between the two studied hydrogel concentrations, whereas the hydrogel volume was found to have an unexpected effect on neurite outgrowth. In larger hydrogel volume (80 µl) the growth as organoid like aggregates was favored and smaller hydrogel volume (60 µl) supported neurite outgrowth and cell infiltration. These findings suggest that GrowDex hydrogel is promising matrix for 3D human neural *in vitro* models.

Acknowledgements:

This study was financially supported by UPM Kymmene Oyj and TEKES (the Finnish Funding Agency for Technology and Innovation) Human Spare Parts project. Hanna Mäkelä and Eija Hannuksela are acknowledged for the cell production.

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