Application note

Development of an *in vitro* model of the human brain using NANOSTACKS™

Introduction

The use of multicellular, complex *in vitro* models of the human brain can facilitate the study of physiological and pathological aspects of the human central nervous system (CNS). Additionally, neural *in vitro* models including multiple cell types can be used as platforms for drug testing, in order to assess the neurotoxic potential of novel compounds with increased accuracy compared to traditional monocellular models (1).

Amongst the variety of cells constituting the CNS, neurons and astrocytes play a key role. In particular, the main function of neurons is to carry electrical signals and form a functional neural network, whilst astrocytes provide trophic and metabolic support (2).

The neuroblastoma cell line SH-SY5Y can be used to model human neurons. In particular, SH-SY5Y can be used in an "undifferentiated" form, resembling immature neurons, or in a "differentiated" form, modelling adult neurons (3).

In this work, a NANOSTACKS™-based *in vitro* model of the human brain was developed using both differentiated and undifferentiated SH-SY5Y cells as a neuronal component, whilst an astrocytoma cell line was utilized to model astrocytes.

The neural model based on undifferentiated SH-SY5Y was then used to test the compound methylglyoxal, and a neurite outgrowth assay was performed on the model including differentiated SH-SY5Y cells to test the drug clomipramine.

Methods

Cell seeding on NANOSTACKS™

Unless otherwise specified, during the course of this work the cell culture medium used was composed of Dulbecco's Modified Eagle's medium (11995065; Thermo Fisher Scientific) including 10% foetal bovine serum, 1X MEM Non-essential Amino Acids Solution (M7145-100ML; Merck) and 1X Penicillin-Streptomycin (P0781; Merck). Prior to cell seeding, NANOSTACKS™ were placed in wells of 24-well plates, one NANOSTACK™ per well. Then, SH-SY5Y and U-138 MG cells (formerly known as 1321N1 cells) were seeded at a seeding density of respectively 5 x 10^3 and 6 x 10^3 cells per NANOSTACK™. In particular, during the seeding procedure, a droplet of 75 µL of cell suspension was placed on the membrane of the NANOSTACKS™, and the seeded cells were then incubated at 37 °C, 5% CO_2 for 2 hours. Subsequently, 825 µL of cell culture medium was added to the wells containing the cell-seeded NANOSTACKS™, reaching the working volume of 900 µL.

Compound testing on undifferentiated SH-SY5Y

The day following the cell seeding, SH-SY5Y and U-138 MG seeded on NANOSTACKS™ were combined in a coculture by placing the seeded platforms on top of each other into 24-well plates. The cell culture medium volume per well was increased to 1.5 mL, and the compound methylglyoxal was added at the following concentrations: 0 (vehicle control), 0.01, 0.1, 1, 10 and 50 µM. SH-SY5Y in monocultures on NANOSTACKS™ were used as a control. 2 days later, cell viability of SH-SY5Y in both cocultures and monocultures was assessed by performing a CellTiter-Glo® (G7571; Promega) assay according to the vendor's instructions.

Compound testing on differentiated SH-SY5Y

SH-SY5Y cells were seeded on NANOSTACKS™ on day 0, as previously described. On day 1 and every 2-3 days, cell culture medium was exchanged with a differentiation medium composed of neurobasal medium (21103049; Thermo Fisher Scientific), 1X B27 supplement (17504044; Thermo Fisher Scientific), GlutaMAX™ (35050061; Thermo Fisher Scientific), 1X Penicillin-Streptomycin, and retinoic acid (R2625; Merck) at a concentration of 10 µM. On day 7, U-138 MG were seeded on NANOSTACKS™ as previously described, at a cell seeding density of 5 x 10^3 cells per NANOSTACK™. On day 8 the differentiation of

SH-SY5Y was completed, and U-138 MG and differentiated SH-SY5Y on NANOSTACKS™ were combined in a coculture. The differentiation medium volume per well was increased to 1.5 mL, and the compound clomipramine was added at the following concentrations: 0 (vehicle control), 10, 50 µM. Monocultures of differentiated SH-SY5Y on NANOSTACKS™ were used as a control. On day 10, SH-SY5Y cells were fixed in 4% V/V paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS).

Immunostaining

Differentiated SH-SY5Y were stained according to the following protocol. All steps were carried out at room temperature, unless otherwise stated. Firstly, cells were permeabilised by submerging the NANOSTACKS™ in a 0.1% V/V solution of Triton X-100 in DPBS for 10 min. Subsequently, cells were washed three times for 5 min in DPBS. Then, after a brief submersion in DPBS, cells were submerged for 1 hour in a "blocking solution", composed of 1% W/V bovine serum albumin in a 0.1% V/V solution of Tween-20 in DPBS. After the blocking step, cells were submerged overnight at 4 °C with a 1:1000 dilution of β-Tubulin III antibody (ab18207; abcam) in blocking solution. Then, cells were washed briefly in DPBS 3 times, and subsequently submerged for 1 hour with a 1:1000 dilution of secondary antibody (ab150077; abcam) in blocking solution, shielded from light. Subsequently, cells were washed for 5 min in DPBS, and then submerged with a solution of 1.4 µg/mL Hoechst 33342 (H3570; Thermo Fisher Scientific) in DPBS for 12 min, shielded from light. Then, cells were washed in DPBS 3 times for 5 min, and finally stored in DPBS.

Neurite outgrowth assay

To assess the effect of clomipramine on SH-SY5Y seeded on NANOSTACKS™, a neurite outgrowth assay was performed according to the following protocol. Firstly, n = 3 images of random fields at a resolution of 20X were acquired per NANOSTACK™ using a fluorescent microscope. Then, using the software FIJI (version: 1.54f) a square-shaped region of interest (ROI) was identified and maintained for each image, and the total length of neurites within the ROI was quantified. The area within the ROI positive to Hoechst was also quantified, obtaining the total nuclear area per ROI. The total length of neurites per ROI was then normalised with the total nuclear area per ROI.

Statistical analysis

Each datapoint reported in this application note was derived from measures obtained from a minimum of n = 3 cell-seeded NANOSTACKS™.

Results

Undifferentiated SH-SY5Y were cocultured with U-138 MG on NANOSTACKS™, and the drug methylglyoxal was tested on the model, using a monoculture of undifferentiated SH-SY5Y on NANOSTACKS™ as a control. In particular, methylglyoxal was used at concentrations 0.01, 0.1. 0.5, 1, 5 and 10 mM, and 48 h later the viability of undifferentiated SH-SY5Y was analysed (Fig. 1A-B). The IC50 associated to the coculture model (0.78 mM) was 27.9 % higher than the IC50 obtained from the monoculture (0.61 mM, fig. 1A), indicating that U-138 MG astrocyte-like cells exert a protective effect on undifferentiated SH-SY5Y. In particular, the viability of undifferentiated SH-SY5Y was found to be lower in monocultures relatively to cocultures at concentrations of Clomipramine of 1, 5 and 10 mM (Fig. 1B), and the difference was statistically significant.

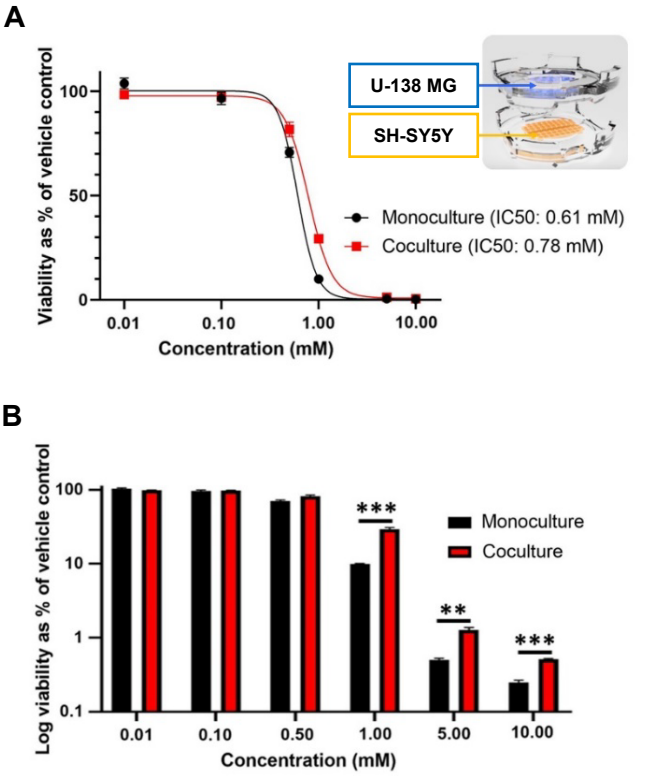

Figure 1. *Viability of undifferentiated SH-SY5Y upon drug testing with methylglyoxal.* ***A:*** *Dose response curves of coculture and monoculture models.* ***B:*** *Bar graph indicating viability of undifferentiated SH-SY5Y in monocultures and cocultures. Data are reported as mean ± SEM.*

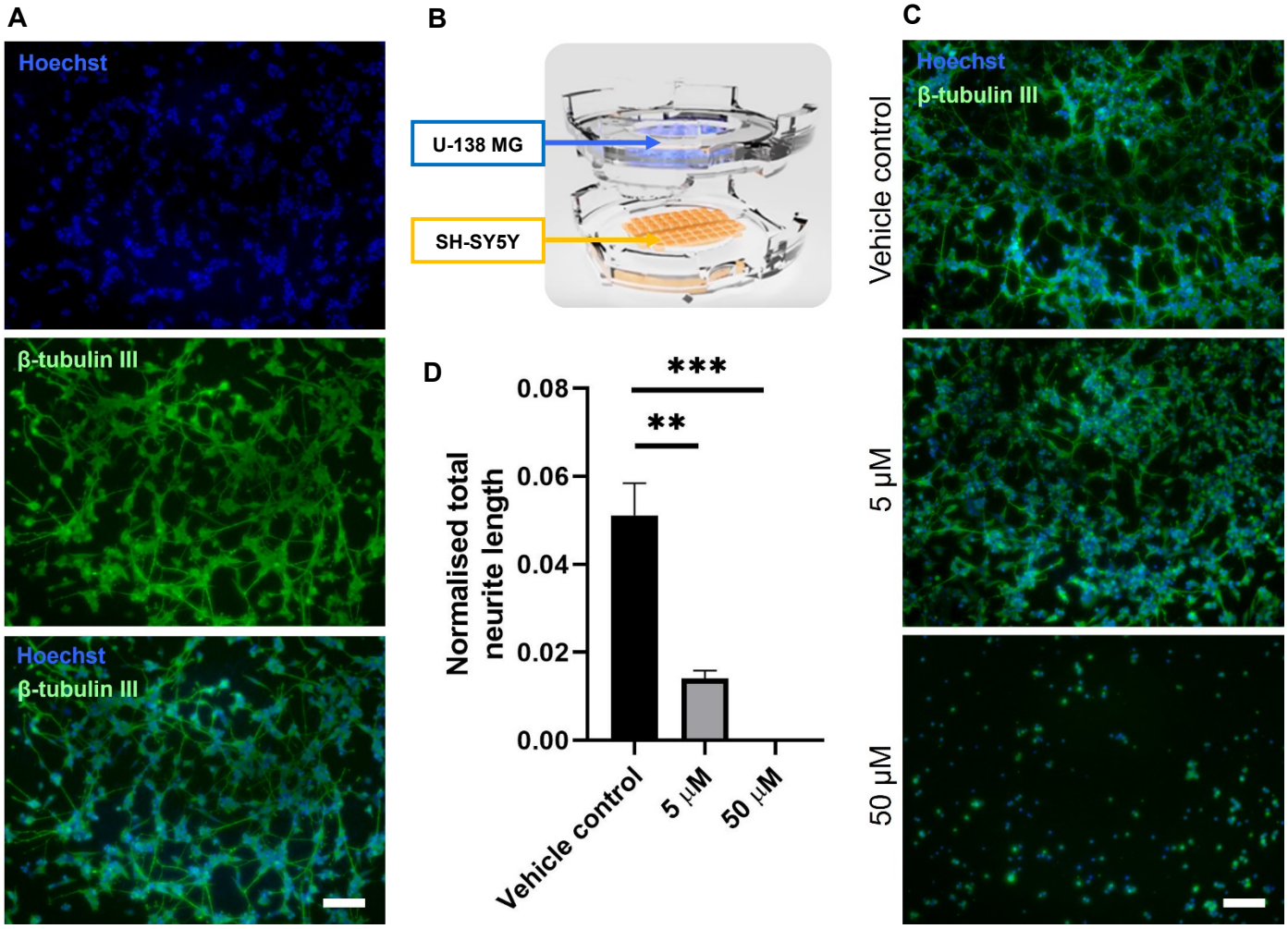

Figure 2. *Clomipramine testing on a neural model based on differentiated SH-SY5Y and U-138 MG.* ***A:*** *Representative images of Hoechst (blue) and β-tubulin III (green) immunostaining of differentiated SH-SY5Y included in the model, associated with the vehicle control of the clomipramine testing, 2 days after drug testing. Magnification: 20X. Scale bar: 100 µm.* ***B:*** *Scheme of the coculture.* ***C:*** *Representative images of immunostaining of differentiated SH-SY5Y with β-tubulin III (green) and Hoechst (blue), associated with the vehicle control (top) and with concentrations of clomipramine of 5 µM (middle) and 50 µM (bottom). Magnification: 20X. Scale bar: 100 µm.* ***D:*** *Neurite outgrowth assay results reported as mean ± SEM.*

In addition to the development of a model based on undifferentiated SH-SY5Y, a differentiation protocol was performed to differentiate SH-SY5Y towards a more mature neuronal phenotype. Then, the differentiated SH-SY5Y cells were cocultured with U-138 MG on NANOSTACKS™, and the drug clomipramine was tested on the model at a concentration of 5 µM and 50 µM (Figure 2). Upon completion of the compound testing, immunostaining of differentiated SH-SY5Y revealed the presence of neurites extending from cell bodies in the vehicle control and 5 µM conditions, whilst neurites were not observable in the 50 µM condition (Fig. 2C). Quantification of neurite outgrowth indicated that the length of the neurites normalised to nuclear area was higher in the vehicle control than in the 5 µM and 50 µM conditions (Fig. 2D), and the difference was statistically significant ($p < 0.05$).

Conclusions

This work demonstrated that the astrocytoma cell line U-138 MG and the neural cell line SH-SY5Y are compatible with NANOSTACKS™, and can be combined to build a coculture model of the human brain. The model can also be used for toxicity testing, as demonstrated in this work using the compounds methylglyoxal and clomipramine. In the model inclusive of undifferentiated SH-SY5Y, the presence of U-138 MG reduced the neurotoxicity of methylglyoxal relatively to the SH-SY5Y monoculture control. This result is in line with the work of De Simone *et al.* (4), although in their study a different astrocytoma cell line, D384, was used in place of U-138 MG.

In this work SH-SY5Y were also used in a differentiated form, providing a model of human neurons closer to the phenotype of primary adult

cells (3). The neural phenotype of differentiated SH-SY5Y was confirmed upon immunostaining of β-tubulin III, which revealed a network of neurites. Finally, the model including differentiated SH-SY5Y was compatible with neurite outgrowth assays, demonstrating the neurotoxicity of clomipramine. This work therefore demonstrates the feasibility of performing imaging-based assays to assess the neurotoxicity of compounds on NANOSTACKS™-based multicellular *in vitro* models.

References

1.Woehrling, E., Hill, E. & Coleman, M. Development of a neurotoxicity test-system using human post-mitotic, astrocytic and neuronal cell lines in co-culture. *Toxicology* 231, 110–111 (2007).

2.Chiareli, R. A. *et al.* The role of astrocytes in the neurorepair process. *Frontiers in Cell and Developmental Biology* 9, (2021).

3. Kovalevich, J. & Langford, D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Neuronal Cell Culture* 9–21 (2013).

4. De Simone, U., Caloni, F., Gribaldo, L. & Coccini, T. Human co-culture model of neurons and astrocytes to test acute cytotoxicity of neurotoxic compounds. *International Journal of Toxicology* 36, 463–477 (2017).

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