Corning® Synthemax® II SC Subsrate as an Ideal Surface for Human Neural Stem Cell Culture

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SnAPPShots

A brief technical report from the Corning Applications Group

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Introduction

Neural stem cells (NSC) are multipotent, self-renewing cells that have the ability to differentiate into multiple cell types found in the brain and spinal cord. These cells are of special interest for better understanding the central nervous system (CNS) as well as the investigation of potential therapies for CNS damage or various neurological disorders, such as Alzheimer's and Parkinson's. Culturing NSC adherently requires specialized coatings to maintain attachment and prevent spontaneous differentiation. These coatings can be expensive, contain undefined biological components, and have lot to lot variability. Corning Synthemax II-SC Substrate is a completely synthetic coating comprised of a proprietary peptide cross-linked to a unique matrix which allows the user to coat their preferred format of cultureware. Synthemax II-SC can be used to maintain and expand human neural stem cells (hNSC) over multiple passages, without the loss of their multipotent state. This paper compared Corning's synthetic substrate to a commonly used biological NSC coating, poly-L-ornithine with laminin. We looked at the ability of the surfaces to maintain undifferentiated neural stem cells over 5 passages while maintaining a consistent doubling time, expression of multipotency markers, and the ability to differentiate into astrocytes, oligodendrocytes and neurons.

Methods and Materials

Coating and Culture

Corning CellBIND® 6 well Multiple well plates (Corning, Cat. No. 3335) were coated with either Synthemax II-SC or poly-L-ornithine with laminin prior to initiating cell culture. For Synthemax II plates, 2 mL of a 0.025 mg/mL solution of Synthemax II (Corning, Cat. No. 3535XX1) was added to each well of the 6 well plate. Plates were allowed to incubate at room temperature for 2 hours prior to aspiration and use. For poly-L-ornithine/laminin plates 2 mL of 20 µg/mL poly-L-ornithine (Sigma, Cat. No. P3655) was added to each well and incubated at 37°C for 1 hour. After incubation, wells were rinsed with sterile water twice before adding 2 mL of 10 µg/mL of laminin (Sigma, Cat. No. L2020). Plates were incubated at 37°C for 2 hours, then rinsed with phosphate buffered saline (Corning cellgro®, Cat. No. 21-031-cm) prior to use. Human neural stem cells (Invitrogen, Cat. No. N7800-100) were thawed into StemPro® NSC SFM (Invitrogen, Cat. No. A10500901) media following the protocol provided by the vendor. Cells were seeded onto 1 plate per surface at $4x10^4$ cells/cm^2 in 2.5 mL of media per well. Media changes were performed every other day and cells were harvested on day 4 with StemPro® Accutase® (Invitrogen, Cat. No. A11105-01). Each plate was harvested and counted on the Vi-CELL (Beckman Coulter) by pooling 2 wells from each plate together to achieve 3 counts per surface. Once counted, the cells were used to re-seed a new plate of the same surface for continuous passage studies following the same seeding and culture protocol already described. The entire 5 passage study was repeated 3 independent times.

Multipotency Assessment

At each passage, harvested neural stem cells were assessed for multipotency via immunocytochemistry. Cells were fixed by washing with PBS twice, fixing in 4% paraformaldehyde (Boston Bioproducts, Cat. No. BM-698) for 10 minutes at 4°C. Cells were washed again and finally stored in PBS until ready to stain and assess via flow cytometry. To demonstrate that there is no loss in multipotency over passages, fixed hNSCs at passage 1 and 5 were assessed for the expression of the hNSC markers Nestin and Sox2. Approximately $1x10^6$ cells per sample were permeabilized with a 0.1% saponin (TCI, Cat. No. S0019) in HBSS (Corning, cellgro Cat. No. 21-023-cm) that contained 0.05% sodium azide (Fisher, Cat. No.ZS2271) by centrifuging at 200xg for 7 minutes. The supernatant was then aspirated and replaced with 200 µL of fresh permeabilization buffer. Finally, 10 µL of antibody or isotype control was added (Table 1). Samples were incubated at room temperature for 30 minutes, washed 2 times with HBSS and resuspended in 400 µL of HBSS to be analyzed on the MACSQuant flow cytometer (Miltenyi Biotech).

Differentiation

Passage 5 cells were used to assess the potential of the NSCs to differentiate into astrocytes, oligodendrocytes, and neu-

Table 1. Multipotency Markers

Antibody/Isotype Control	Vendor and Cat. No.
Nestin Fluorescein Mouse IgG1 (1:20)	R&D Systems® IC1259F
Fluorescein Isotype Control Mouse IgG1 (1:20)	R&D Systems IC002F
SOX2 Allophycocyanin Mouse IgG2A (1:20)	R&D Systems IC2018A
Allophycocyanin Isotype Control Mouse IgG2A (1:20)	R&D Systems IC003A

Table 2. Differentiation Markers

Cell Type	Antibody/Isotype Control	Vendor and Cat. No.
Astrocyte	GLAST Phycoerythrin Mouse IgG2a (1:10)	Miltenyi Biotec 130-095-821
	Phycoerythrin Isotype Control Mouse IgG2a (1:10)	Miltenyi Biotec 130-091-835
Oligodendrocyte	A2B5 Phycoerythrin Mouse IgM (1:10)	Miltenyi Biotec 130-093-581
	Phycoerythrin Isotype Control Mouse IgM (1:10)	Miltenyi Biotec 130-093-177
Neuron	MAP2B Alexa Fluor® 488 Mouse IgG1, κ (1:10)	BD 560399
	Alexa Fluor 488Isotype Control Mouse IgG1, κ (1:10)	BD 557721

rons. For astrocyte differentiation, cells were seeded onto their respective surfaces at a concentration of $4x10^4$ cells/cm^2 in Corning® CellBIND® Surface 24 well Multiple well plates (Corning, Cat. No. 3337) in 1 mL of culture medium. Two days after seeding, the media was changed to glutaGRO™ DMEM (Corning, cellgro Cat. No.10-101-cv) supplemented with 1% N-2 supplement (Invitrogen, Cat. No. 17502-048) and 1% fetal bovine serum (Corning cellgro,® Cat. No. 35-010-cv). The media was changed again 2 days later and the cells were assessed for marker expression 5 days after seeding. For oligodendrocyte differentiation, hNSCs were seeded using the same protocol as the astrocytes but the differentiation medium was Neurobasal media (Invitrogen, Cat. No. 21103-049) supplemented with 2% B-27 supplement (Invitrogen, Cat. No. 17504-044), 1x glutaMAX™ (Invitrogen, Cat. No. 35050-061), and 30 ng/mL of T3 supplement (Sigma, Cat. No. T5516) and the cells were assessed for marker expression 6 days after seeding. For neuron differentiation, cells were seeded at $5.5x10^4$ cells/cm^2 in 1mL of culture media on poly-L-ornithine/laminin coated plates only. Two days after seeding, the media was changed to Neurobasal media supplemented with 2% B-27 supplement and 1x glutaMAX. The media was changed again 2 days later and dcAMP (Sigma, Cat. No. D0627) was added to a final concentration of 1 mM. Neurons were assessed for marker expression 7 days after seeding. For cellular staining, cells were fixed then permeabilized and blocked with a 0.1% triton, 1% BSA, 5% FBS solution in HBSS for at least an hour at room temperature. After an hour, the buffer was removed and replaced with 200 µL per well of HBSS with 1% BSA. To the buffer 20 µL of conjugated antibody or isotype control was added (Table 2) to each well. One microliter of 2 mg/mL hoechst stain was added to stain cell nuclei. Samples were stored at 4°C for 40 minutes, then washed twice with HBSS and visualized using the Evos fl microscope (AMG).

Results and Discussion

In order to provide a xeno free surface to support the growth of hNSCs, Corning's Synthemax II self-coat surface was evaluated for it's ability to maintain undifferentiated human NSC's for five passages. Figure 1 shows typical NSC morphology and similar confluence between cells grown on poly-L-ornithine/laminin as compared to the Synthemax II-SC surface on day 4 of culture. Throughout the 5 passage study,

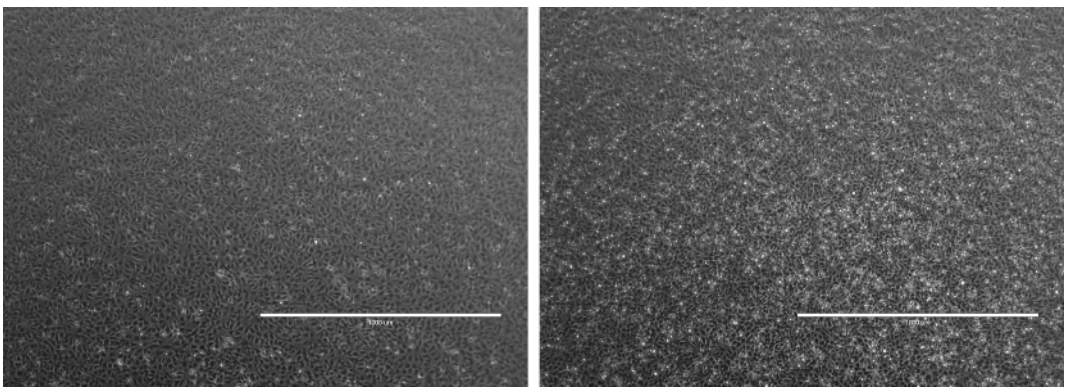


Figure 1. The 40x microphotographs show representative images of cell morphology and confluence on day 4 of culture. Cells appear similar regardless of which surface they are cultured on.

cells on Synthemax II maintained a consistent doubling time of 36 ±4 hours which is less than the vendor's suggested doubling time of 40 to 50 hours (Figures 2 and 3). In order to confirm Synthemax II self-coat's ability to maintain undifferentiated NSC's over at least 5 passages, Nestin (Figures 4 and 5) and Sox2 (Figures 6 and 7) markers were

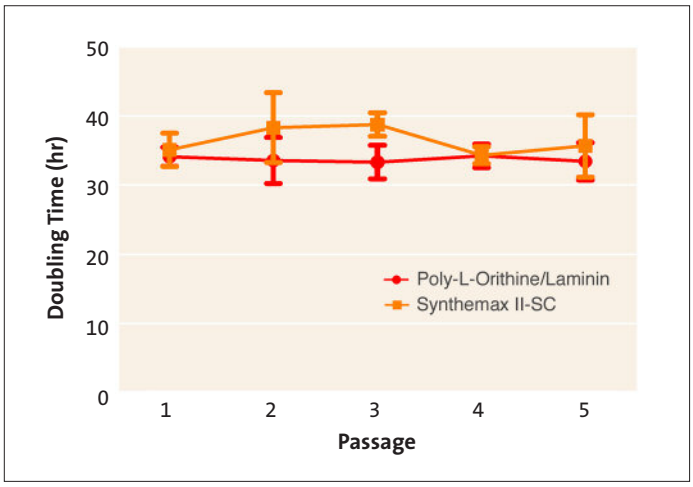


Figure 2. Average NSC doubling time for 5 passages on each surface. N=9 for each passage.

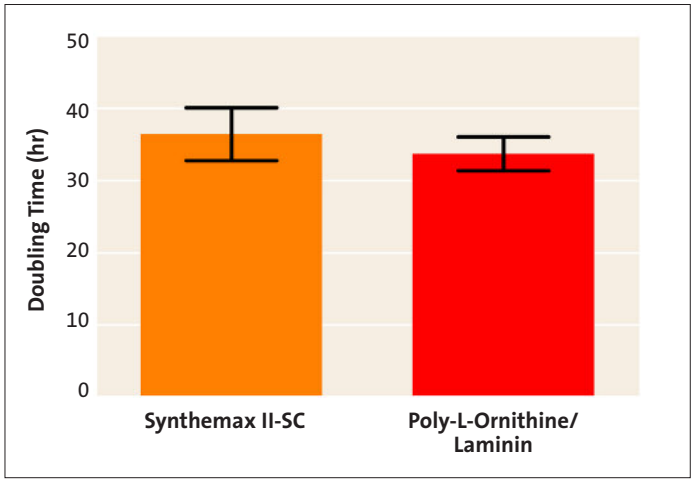


Figure 3. Average NSC doubling time on each surface. N=45.

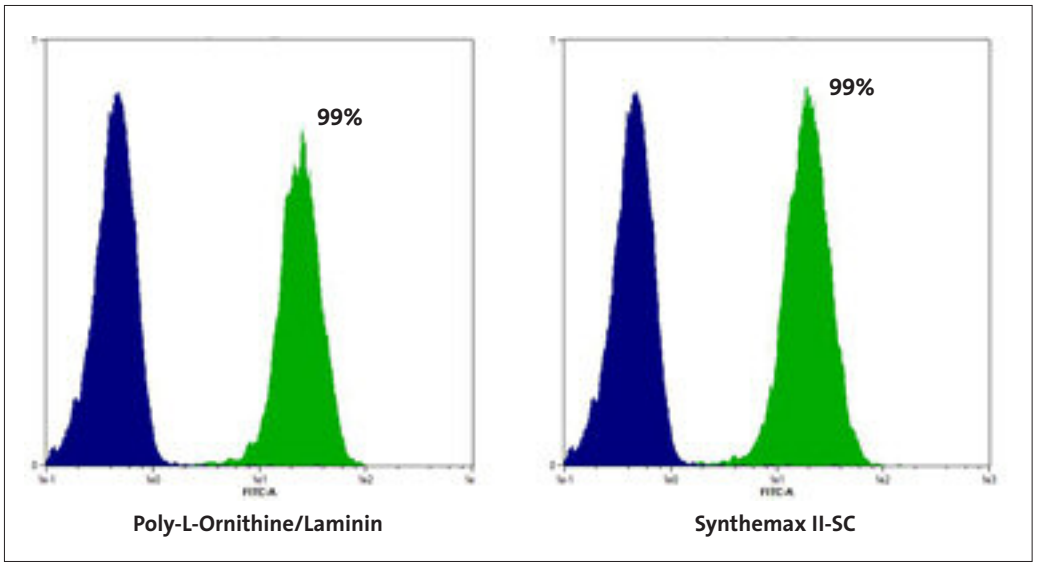


Figure 4. Representative Nestin expression from hNSCs cultured on Poly-l-ornithine/laminin and Synthemax II-SC.

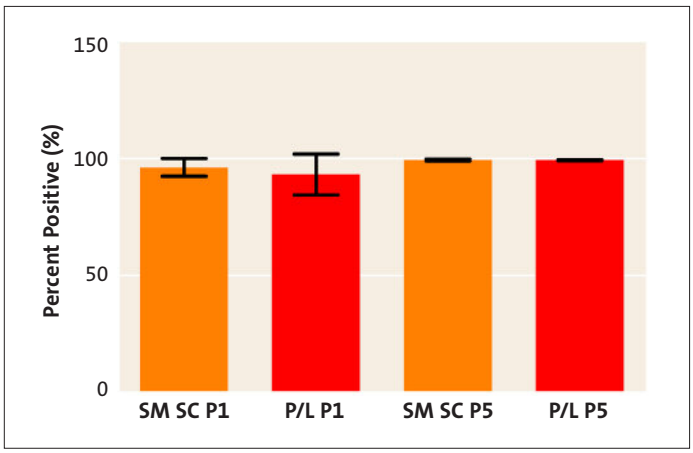


Figure 5. ANOVA with Newman-Keuls post test shows no statistical significance in Nestin expression between passages or surfaces. N=3.

used to assess multipotency via flow cytometry. The markers were chosen based on Invitrogen's recommendations and a literature search. The high expression of these markers confirms multipotency of the cells cultured for at least 5 passages on Synthemax® II-SC. Finally, to confirm that the NSCs cultured on Synthemax Self Coat II maintain their ability to differentiate into neurons, oligodendrocytes, and astrocytes, cells from passage 5 were reseeded and differentiated on poly-L-ornithine/laminin and Synthemax II-SC. Figure 8 confirms the ability of NSCs cultured for 5 passages on Synthemax II-SC to differentiate into the 3 cell types examined as well as the surface itself to support the differentiation of astrocytes and oligodendrocytes.

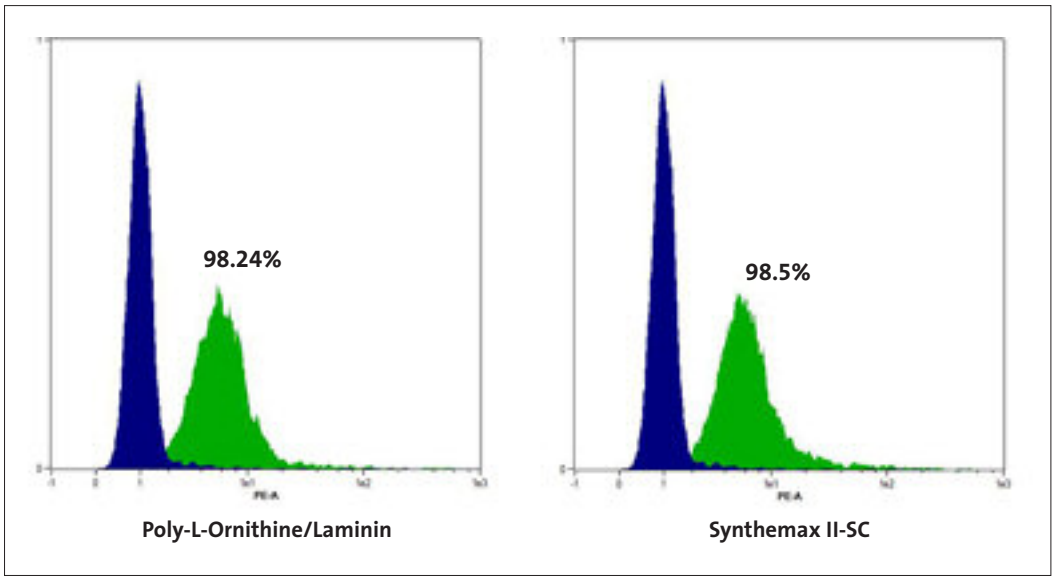


Figure 6. Representative Sox2 expression from hNSCs cultured on Poly-l-ornithine/laminin and Synthemax II-SC.

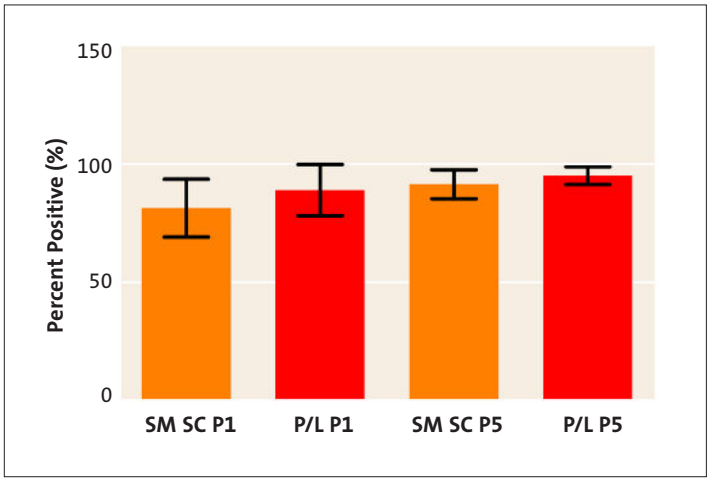


Figure 7. ANOVA with Newman-Keuls post test shows no statistical significance in Sox2 expression between passages or surfaces. N=3.

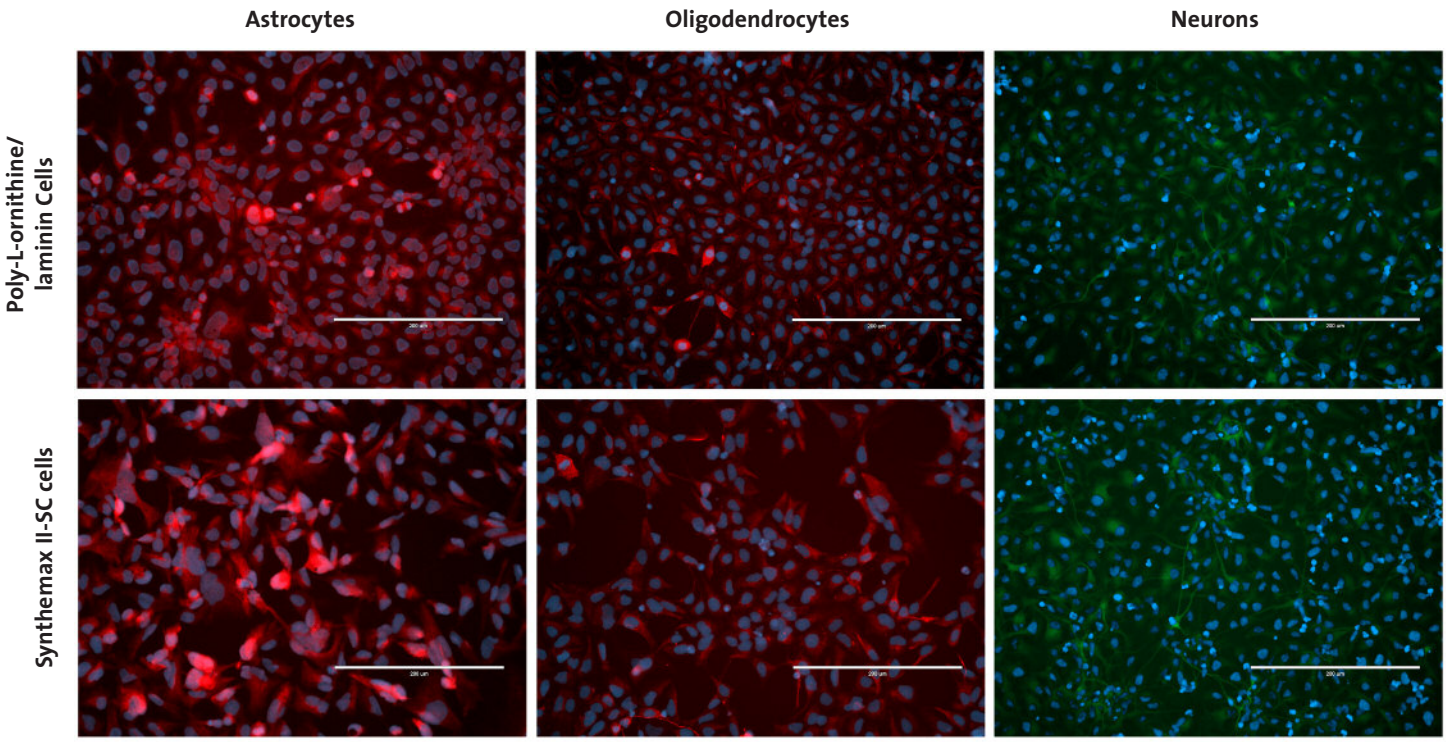
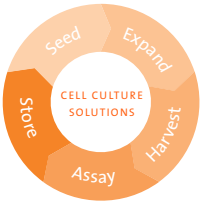


Figure 8. Representative 200x microphotographs of positively stained astrocytes (GLAST), oligodendrocytes (A2B5), and neurons (MAP2B) counterstained with hoechst that were derived from poly-L-ornithine/laminin or Synthemax II-SC passage 5 NSCs.

Conclusions

- For at least 5 passages, hNSCs grown on Corning® Synthemax® II-SC can maintain a consistent doubling time that is less than the published vendor's doubling time for these cells.
- Synthemax II-SC can maintain hNSCs that exhibit expression of multipotent stem cell specific markers Nestin and Sox2 for at least 5 passages.
- hNSCs cultured on Synthemax II-SC maintain their ability to differentiate into astrocytes, oligodendrocytes, and neurons.
- Synthemax II-SC surface can support differentiation of hNSCs into astrocytes and oligodendrocytes.



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