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# Synthetic Polymers Provide a Robust Substrate for Functional Neuron Culture

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**Substrates for neuron culture and implantation are required to be both biocompatible and display surface compositions that support cell attachment, growth, differentiation, and neural activity. Laminin, a naturally occurring extracellular matrix protein is the most widely used substrate for neuron culture and fulfills some of these requirements, however, it is expensive, unstable (compared to synthetic materials), and prone to batch-to-batch variation. This study uses a high-throughput polymer screening approach to identify synthetic polymers that supports the in vitro culture of primary mouse cerebellar neurons. This allows the identification of materials that enable primary cell attachment with high viability even under “serum-free” conditions, with materials that support both primary cells and neural progenitor cell attachment with high levels of neuronal biomarker expression, while promoting progenitor cell maturation to neurons.**

Damage to the adult central nervous system caused by physical injuries, inflammation, or cancer cannot regenerate on its own.<sup>[1]</sup> As a consequence, surgical treatments such as tissue transplantation and nerve grafting, have been used for the reparation of damaged regions, but encounter limitations with regard to appropriate donor sites and shortages of material and are prone to infection.<sup>[2]</sup> Tissue engineering has become a promising alternative to conventional transplantation methods with a variety

of scaffolds used as a support for neuron regeneration.<sup>[3]</sup> To minimize immunological issues, the use of a patient's own cells during tissue engineering is usually considered optimal. This requires in vitro expansion of cells, and using defined cell-culture substrates would aid the regulatory approval process.<sup>[4]</sup> Currently, the number of substrates, e.g., fibronectin, collagen, polylysine, that support in vitro culture and specifically neuronal expansion is limited; with laminin perhaps the most widely used surface coating for in vitro studies.<sup>[5]</sup>


In addition, it is often difficult to maintain primary neuron cultures under serum-free conditions,<sup>[6]</sup> especially on synthetic substrates (e.g., poly(L-lactic acid) or poly-Lysine).<sup>[6c,7]</sup> Thus to conduct

research on neural regeneration as well as to achieve clinical translation, factors that influence neuron growth need to be understood, ideally with neurons that are cultured in chemically defined media to minimize the influence of growth factors and/or extracellular matrix proteins—which again would cause problematic regulatory issues. Serum-free media such as neurobasal medium (Gibco, Life Technologies) supplemented with B27 and L-glutamine has been found to be suitable,

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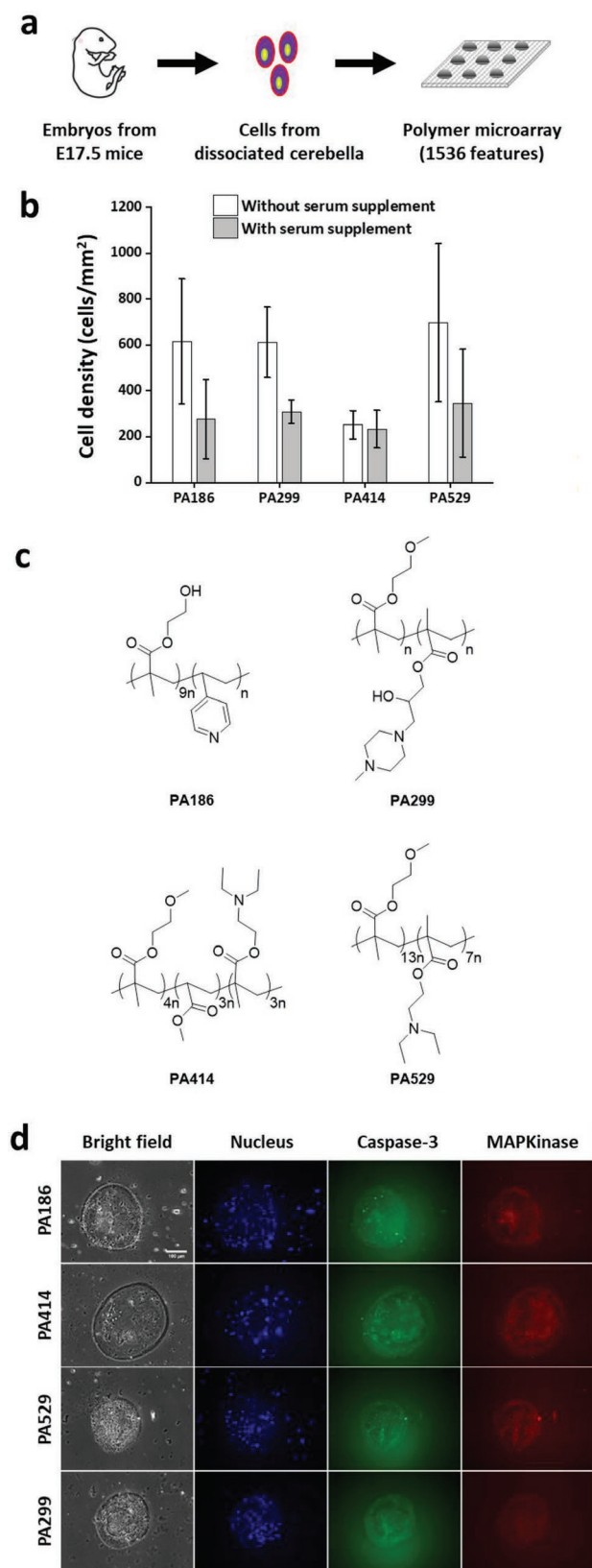
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**Figure 1.** a) Dissociated primary cerebellar cells from mouse embryos were cultured (24 h) on polymer microarrays. b) Three “lead” polymers (PA186, PA414, and PA529) along with PA299 (as a cell binding control)

however, cells plated at low densities show significantly reduced viability.<sup>[8]</sup>

Hence, we set out to discover new synthetic substrates that support neuron cell culture looking at both primary mouse neurons<sup>[9]</sup> and mouse embryonic stem cell–derived neural progenitor cells (NPCs).<sup>[10]</sup> Primary neurons are an archetypal source for cell replacement therapy in human neurodegenerative diseases, while the electrical responses of NPCs are well characterized in a range of neurobiological studies,<sup>[11]</sup> and as such are highly relevant for the evaluation of new biomaterials suitable for functional neuron culture.

To identify synthetic polymers for culturing functional neurons, polymer microarrays were applied, a high-throughput tool that allows the parallel screening of hundreds of polymers that in association with high-content microscopy allows the independent evaluation of the interactions of all polymer features with the cells of interest.<sup>[12]</sup> Contact<sup>[13]</sup> and inkjet printing<sup>[14]</sup> methods have both been used for the fabrication of polymer microarrays, with polymers identified for numerous biological applications including bacteria-repellent coatings for medical devices, controlled expansion of human embryonic stem cells, thermal harvesting of mesenchymal stem cells, and activation of platelets.<sup>[13,14]</sup> In comparison to other synthetic substrates used for neuron culture, e.g., polylysine and polyornithine, polyacrylate-based polymers permit tuning of polymer properties and molecular weight, while providing the possibility of crosslinking of the linear polymers into 3D tissue engineering scaffolds.

In this study, we targeted the identification of polymeric substrates that would support the “serum-free” culture of neurons with the medium NS21 with no additional supplements with a polymer microarray screen conducted to identify those polymeric substrates that best supported neuron attachment, growth, differentiation, and “communication.” The “lead” polymers were scaled-up and shown to support neuron growth with multiple biomarkers expressed at significantly higher levels than equivalent cells grown on laminin-coated substrates.

Dissociated primary cerebellar cells were isolated from E17.5 mice and used for microarray screening studies (Figure 1). The polymer microarray of 1536 features (that included four replicates for each polymer composition) was fabricated by contact printing of pre-synthesized polymers with cell attachment quantified by counting of stained nuclei.<sup>[13a]</sup> The chemical compositions of the 15 “hit” polymers that supported cell attachment with or without serum supplement are shown in Figure S1 in the Supporting Information. It is worth noting that all of the polymers contained functional groups that would be positively charged under physiological conditions due to the presence of tertiary amines, pyridines, and imidazole groups—that would interact with the surface of cells.<sup>[15]</sup>

supported robust primary cerebellar cell attachment (both with and without serum supplement) with cell densities determined by counting DAPI (4',6-diamidino-2-phenylindole)-stained nuclei ( $n = 8$ ). c) Chemical structures of the four polymers PA186, PA414, PE529, and PA299. d) Examples of polymers showing cell attachment and expression of Caspase-3 and MAPKinase (see quantification of MAPKinase expressions in, Table S1, Supporting Information). Nucleus (blue,  $\lambda_{Ex/Em} = 364/454$  nm), Caspase-3 (green,  $\lambda_{Ex/Em} = 490/520$  nm), MAPKinase (red,  $\lambda_{Ex/Em} = 548/562$  nm). Scale bar: 50  $\mu$ m.

Mitogen-activated protein kinase (MAPKinase) is involved in cell growth, proliferation, and signal transduction,<sup>[16]</sup> with some MAPKinases (e.g., p38 MAPKs) involved in the activation processes for neuron differentiation,<sup>[16a]</sup> and thus was used here as a marker during screening. Caspase-3 is a critical mediator of cellular apoptosis and was used to evaluate cell viability.<sup>[17]</sup> Detailed screening identified three “lead” polymers (PA186, PA414, and PA529) that were observed to bind cells (both with and without serum supplements, Figure 1b), while showing low Caspase-3 levels and expressing high levels of MAPKinase even without serum supplementation (see Figure 1d and Table S1, Supporting Information). Other polymers such as PA299, while providing good levels of cellular attachment, showed low levels of MAPKinase expression.

The three “lead” polymers (PA186, PA414, or PA529) have different chemical compositions but also some similarities: PA186 contains hydroxyethyl methacrylate (HEMA) that will generate a layer of water of hydration on the surface, while 4-vinylpyridine will be protonated at physiological pH, thus promoting cell binding. The same is true of PA529 that contains 2-(dimethyl-amino)ethylmethacrylate (DEAEMA)—protonation will drive cell binding. PA414 contains methoxyethyl methacrylate (MEMA), DEAEMA, and methacrylic acid (MA). The surface of mammalian cells contains both cationic and anionic groups and thus a zwitterion polymer would be expected to be both highly solvated as well as attractive to mammalian cells. Thus, the cell binding can be rationalized in all cases—but with subtle differences that explain cellular binding alterations/behavior.

The three “lead” polymers were re-synthesized en masse (several grams) using free radical polymerization with

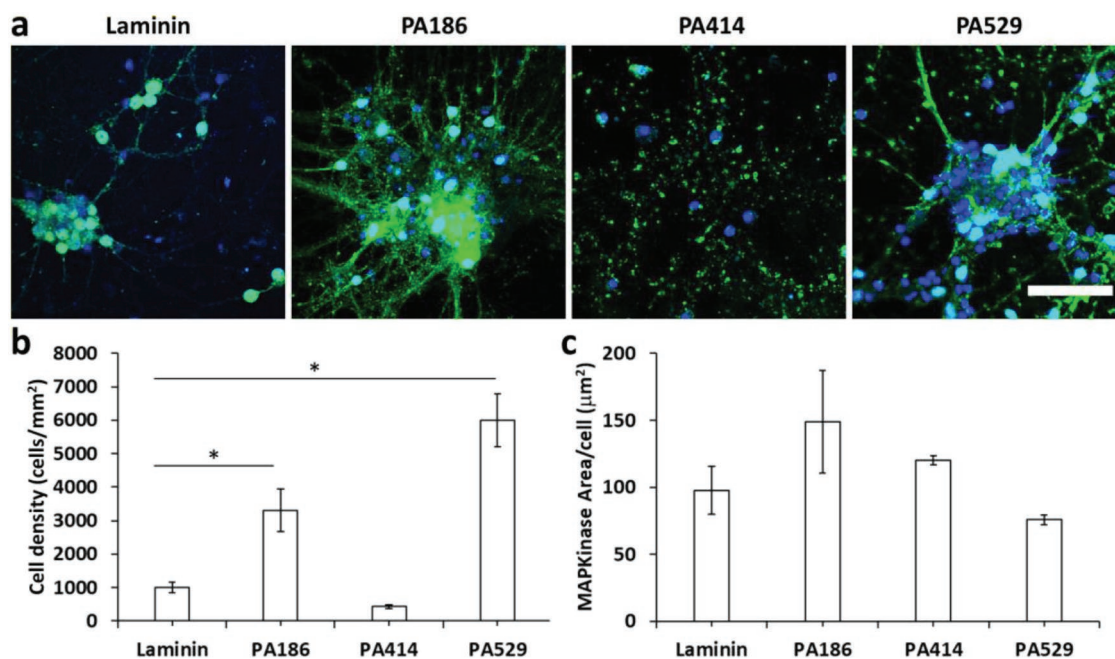
**Table 1.** Characterization of the “hit” polymers with the details of the specific functional groups of the polymers.  $M_n$ ,  $M_w$ , and dispersity ( $\mathcal{D}$ ) were quantified by gel permeation chromatography (dimethylformamide) with calibration using poly(methyl methacrylate) as standards.

Polymer	Yield [%]	$M_n$ [kDa]	$M_w$ [kDa]	$\mathcal{D}$	Functional groups	
PA186	89	41	58	1.4	Hydroxy	Pyridine
PA414	95	50	72	1.4	Tertiary amine	–
PA529	95	183	565	3.0	Tertiary amine	–

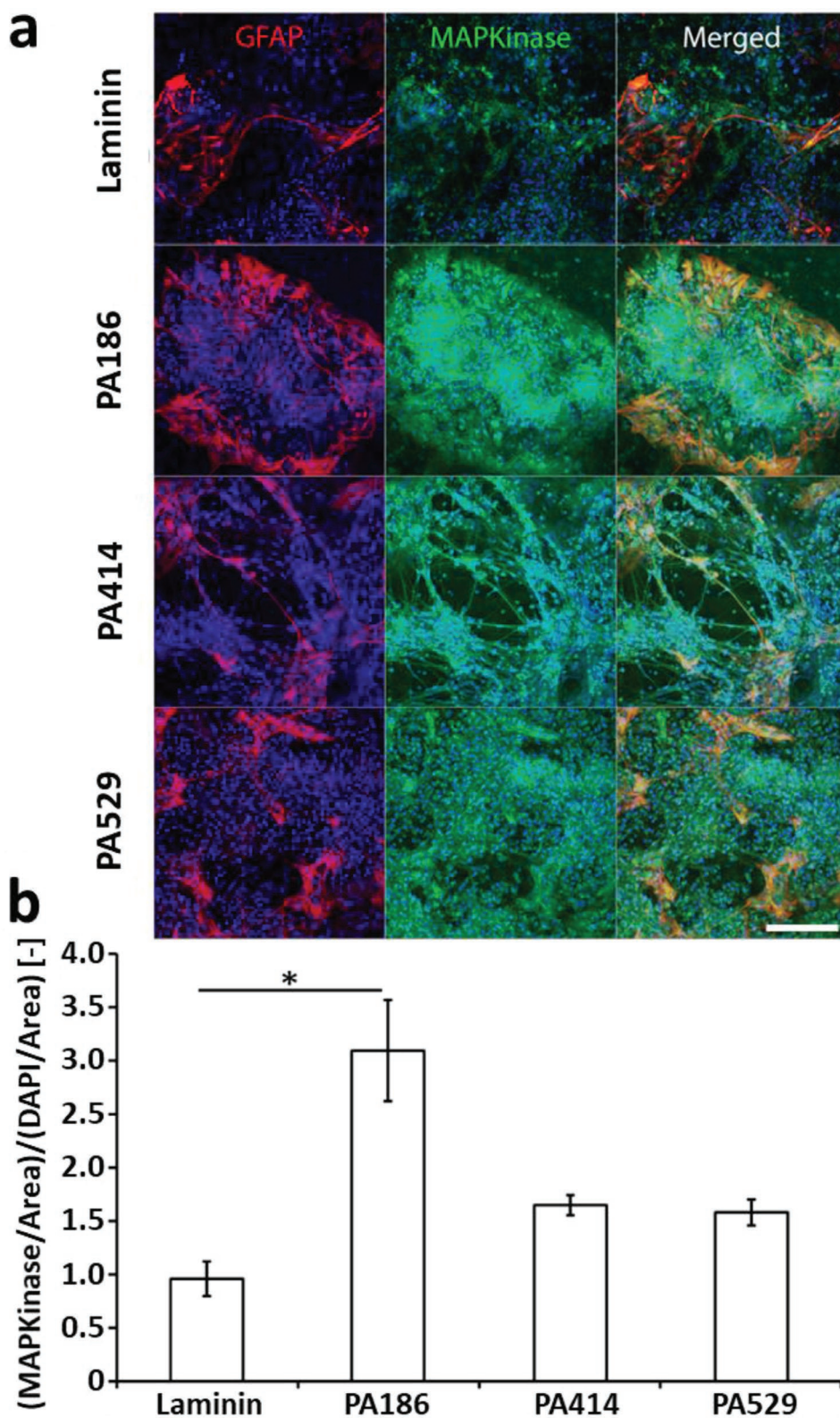
characterization data shown in **Table 1**. Studies undertaken by coating of these polymers onto glass coverslips and evaluation with the culture of primary neuron cells. The polymer coatings with thickness of 193, 372, and 433 nm (for PA186, PA414, and PA 529, respectively) were determined using atomic force microscopy (AFM; see Figure S2, Supporting Information). The expression of MAPKinase by the cells growing on these three polymers under “serum-free” conditions was similar to those observed for cells growing on laminin-coated coverslips with serum-containing media (**Figure 2**).

As is usual in any high-thought assay, initial validation was used to select “leads” that were subsequently evaluated in more detailed assays. Subsequent studies using NPCs (on the “lead” polymers PA186, PA414, and PA529) showed similar results to the primary neurons, with all three polymers supporting NPC growth (without serum) with comparable levels of MAPKinase expression to cells grown on laminin with serum supplement. One polymer PA186 consistently “allowed” cells to show higher levels of MAPKinase than the other two polymers and the “gold standard” laminin (see **Figure 3**).

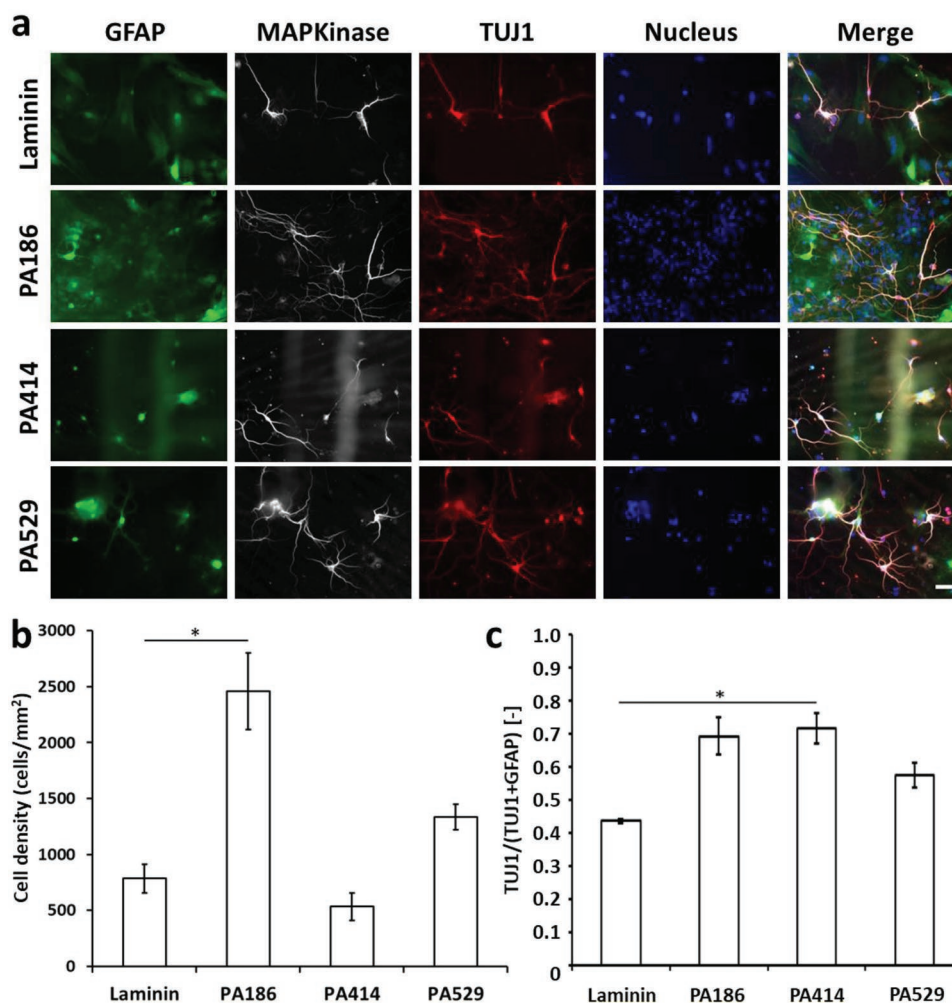
Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed by numerous cell types of the central



**Figure 2.** a) Dissociated cerebellar primary cells as cultured (for 10 days) on 13 mm diameter coverslips coated with Laminin, PA186, PA414, and PA529, showing MAPKinase expression (green) and cell number (DAPI, blue). Scale bar: 50 μm. b) Densities of cells attached to different substrates and c) their MAPKinase expression level.



**Figure 3.** NPCs cultured (10 days) on glass coverslips coated with Laminin, PA186, PA414, or PA529 with all cells stained with DAPI. a) NPCs expressing GFAP (red) and MAPKinase (green); b) MAPKinase expression by the NPCs. Data are represented as the mean and standard error of mean ( $n = 10$  images from five experimental runs from five cell batches), which were assessed via one-way analysis of variance (ANOVA) followed by Tukey's with statistical significance set at  $p \leq 0.01$ . Scale bar: 100  $\mu\text{m}$ .



**Figure 4.** a) GFAP-positive cells expressed GFAP (green), MAPKinase (white), and TUJ1 (red) with nucleus shown in blue. b) Density of NPCs attached to substrate. c) The ratio of TUJ1-positive cells to GFAP-positive cells grown on laminin, PA186, PA414, or PA529 coated coverslips. Data are shown as the mean and standard error of mean ( $n = 10$  images from three experimental runs consisting of three cell batches), which were statistically evaluated via one-way ANOVA followed by Tukey's with statistical significance set at  $p \leq 0.01$ . Scale bar: 50  $\mu\text{m}$ .

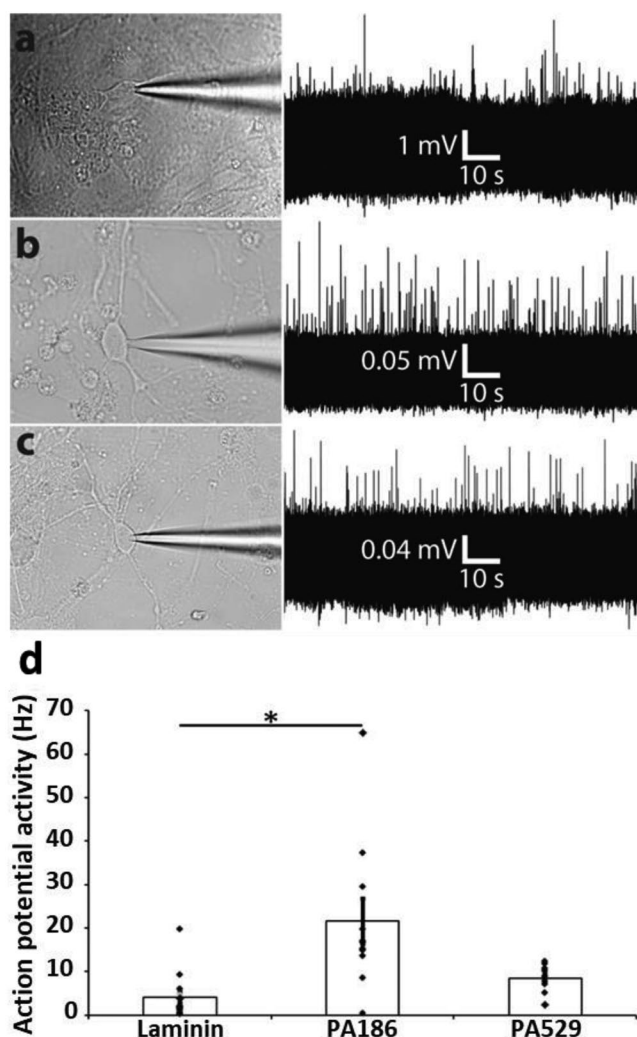
nervous system including astrocytes.  $\beta$ -III-tubulin (TUJ1) is expressed almost exclusively by neurons. Therefore, comparison of expression of these two markers allowed quantification of the maturation of NPCs to neurons. Thus, NPCs cultured on these three polymers were analyzed for the expression of GFAP<sup>[18]</sup> and TUJ1<sup>[19]</sup> studied using immunohistochemistry, to determine the maturation progression of NPCs into neural cells.

As a proof of concept, NPC maturation was initially characterized on the current “gold standard” substrate, laminin, over 15 days, quantifying total cell numbers and also the neuron versus non-neuron populations (see Figure S3, Supporting Information). The total number of cells  $\text{mm}^{-2}$  (quantified by nuclear staining) did not vary significantly during the 15 day culture period (Figure S3b, Supporting Information), while in contrast, the percentage of both TUJ1-positive (neuron) and GFAP-positive (non-neuron) cells increased significantly between days 1 and 10 (Figure S3c, Supporting Information). However, neither the percentage of TUJ1-positive nor the mean dendritic length varied significantly over day 10 to day 15 an incubation time of 10 days was selected for further studies.

NPCs cultured for 10 days on PA186, PA414, PA529 and laminin-coated coverslips (with no serum supplement) showed high cell numbers (except for PA414), while immunohistochemistry revealed that cells cultured on PA186 and PA414 had significantly higher TUJ1 expression than laminin, indicating the higher differentiation potential of NPCs to neurons (see Figure 4). It is worth noting that PA414 provided limited cell attachment (Figure 1) but promoted NPC maturation and expression of TUJ1.

The functionality of neurons was studied by comparing the firing frequencies of action potentials<sup>[11]</sup> of the cells cultured on the three “hit” polymers (with no serum supplement) (see Figure 5).<sup>[9]</sup> Neurons on PA186 showed significantly higher firing frequency than cells grown on PA529 or laminin. Thus, this study has revealed a novel polymer, PA186, as an excellent substrate for neuronal culture (Figure 5d).

In summary, a library of 382 polymers was screened with primary cells dissociated from the cerebellum of mouse embryos, and a high-throughput screen identified three polymeric substrates that allowed attachment of these cells



**Figure 5.** Spiking activity of neurons recorded in cell-attached configuration ( $n = 15$  cells per substrate) on: a) Laminin; b) PA186; and c) PA529. d) Average intrinsic spike activity of neurons on the substrates. Data are shown as the mean and standard error of mean ( $n = 15$  cells from five experimental runs consisting of five cell batches), which were statistically compared via one-way ANOVA followed by Tukey's with statistical significance set at  $p \leq 0.01$ . (Neurons cultured on PA414 did not survive the preparation necessary for electrophysiological recordings.)

(with no/low Caspase-3 expression), yet showing high levels of MAPKinase, even in the absence of serum supplementation. Scale-up studies conducted by culturing these primary cells on glass coverslips coated with the three polymers, PA186, PA414, and PA529, showed high cellular attachment and high levels of MAPKinase expression under “serum-free” conditions, giving similar levels to cells grown on laminin with serum supplement. Thus, these polymers have the potential to be the substrates for primary neuron culture. Culture of NPCs revealed that PA186 allowed higher MAPKinase and TUJ-1 expression and demonstrated higher intrinsic spike activity than the other “hit” polymers as well as the gold “standard,” laminin (which has hugely variable batch-to-batch limitations). This study shows that polymer PA186 has the potential to replace laminin

as a substrate for the “serum-free” culture of primary and progenitor cerebellar neurons.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

central nervous system regeneration, neuron cultures, polymer microarrays, progenitor cell maturation, synthetic polymer substrates

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