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Akt and Src mediate the photocrosslinked fibroin-induced neural differentiation

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Neural transplantation is a promising modality for treatment of neurodegenerative diseases, traumatic brain injury and stroke. Biocompatible scaffolds with optimized properties improve the survival of transplanted neural cells and differentiation of progenitor cells into the desired types of neurons. Silk fibroin is a biocompatible material for tissue engineering. Here, we describe thin-film scaffolds based on photocrosslinked methacrylated silk fibroin (FBMA). These scaffolds exhibit an increased mechanical stiffness and improved water stability. Photocrosslinking of fibroin increased its rigidity from 25 to 480 kPa and the contact angle from 59.7° to 70.8°, the properties important for differentiation of neural cells. Differentiation of SH-SY5Y neuroblastoma cells on FBMA increased the length of neurites as well as the levels of neural differentiation markers MAP2 and βIII-tubulin. Growth of SH-SY5Y cells on the unmodified fibroin and FBMA substrates led to a spontaneous phosphorylation of Src and Akt protein kinases critical for neuronal differentiation; this effect was paralleled by neural cell adhesion molecule elevation. Thus, FBMA is an easily manufactured, cytocompatible material with improved and sustainable properties applicable for neural tissue engineering. ***NeuroReport*** **31: 770–775 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Neuronal cell transplantation for treatment of brain trauma, stroke and neurodegenerative diseases requires a precise regulation of cell differentiation by soluble ligands, cell–cell communication and extracellular matrix (ECM). Differentiation and survival of transplanted cells presumes optimal stiffness, microstructure and spatial organization of ECM. Compared to ECM proteins, natural materials such as fibroin produced by silkworm larvae demonstrated the advantageous mechanical properties, controlled biodegradation and biocompatibility [1,2].

The SH-SY5Y neuroblastoma cell line can be differentiated into neuron-like cells using all-trans-retinoic acid (ATRA) [3,4]. Differentiation is regulated via signaling pathways that include neuron-specific proteins, adhesion, reorganization of the cytoskeleton and exit from the cell cycle. The critical phosphoinositol-3-kinase/protein kinase B (PI3K/AKT) axis is required for neurite growth, cell survival, polarization, axon formation and elongation [5]. Signals from ECM (meditated through integrins) and neural cell adhesionmolecule (NCAM) involve focal adhesion kinase (FAK) followed by activation of Src and PI3K/AKT [6].

Stiffness of the solid support can regulate ECM-derived signaling and influence adhesion, proliferation, migration and differentiation [7]. Soft substrates are required for neuronal differentiation of stem cells [8], while harder surfaces stimulate dendrite growth, branching and axon elongation [8,9]. Film rigidity positively correlated with growth on fibroin films with mechanical stiffness 10–70 MPa [9]. Stiffness can influence the activity of ERK1/2, Akt and FAK [10], the stability of α5 and β1 integrin subunits and the assembly of focal contacts. In particular, stiffness of the substrate controls signaling through the FAK/Src pathway [10,11]. Cells grown on a fibroin matrix

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possess elevated α5β1 and α5β3 integrins and intercellular adhesion molecule [12]. A comparison of neurogenic activity of silk-derived films showed that a greater stiffness provided activated signaling cascades that led to the formation to form a branched network [2]. Importantly, stiffness greater than 50 kPa contributed to an increased neurite length, the hard substrate and ATRA improved cell differentiation [13].

Recently, we have developed a method for generating the photocrosslinked fibroin with improved mechanical properties, in particular [14]. The alternating crystalline and amorphous domains of fibroin include amino acid residues with –NH2 and –OH nucleophilic moieties that can be modified by electrophilic methacrylic anhydride predominantly at Lys and Arg residues. The product can be referred to as fibroin methacrylamide (FBMA) is a stiff and hydrophobic polymer with an increased contact angle [14]. The calculated isoelectric point of fibroin (pI 4.32) [15] indicates its net negative charge under physiological conditions. The strongly basic amino groups in Arg and Lys residues are transformed into uncharged N-methacryloyl derivatives, leading to a decreased pI (3.29).

Herein, we report the activation of signaling pathways in the course of differentiation of SH-SY5Y cells adhered to the films made of fibroin and FBMA. A pronounced activation of PI3K/Akt signaling and Src tyrosine kinase was detectable in SH-SY5Y cells adhered to FBMA compared to the cells grown on native fibroin.

Materials and methods

Reagents

Methacrylic anhydride (94%), diphenyl-(2,4,6-trimethylbenzoyl)phosphine oxide (TPO, 97%) and 3-(trichlorosilyl)propyl methacrylate (90%) were purchased from Sigma-Aldrich (Darmstadt, Germany). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99%) was from P&M – Invest (Moscow, Russia). Fibroin was obtained from silk threads LLC «Optikum» (Moscow, Russia). Eagle's minimal essential medium (EMEM) and F12 media, NeuroMax supplement (50×), L-glutamine and poly-L-lysine (PLL) were from PanEco (Moscow, Russia); Neurobasal-A from Invitrogen. Fetal bovine serum (FBS) was from HyClone. ATRA was from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Gentamycin, mouse monoclonal anti-CD56 antibodies (clone 56C04), Alexa 488 phalloidin and Hoechst 33342 were from Thermo Scientific (Waltham, Massachusetts, USA). Alexa Fluor 647 conjugated to anti-β-tubulin antibodies were from BioLegend (San Diego, California, USA). The CF 488A conjugated goat secondary antibody was from Sigma.

Film fabrication

FBMA was synthesized as described by us [14]. A solution of 2% (w/v) FBMA in HFIP (photoinitiator TPO, 5 wt % of the monomer) was filtered through a cellulose filter and centrifuged at 13 680*g* for 6 min. Film samples were obtained using the casting method on the glass slides pretreated with 1.5% 3-trichlorosilylpropyl methacrylic anhydride followed by solvent evaporation and UV illumination. Specimens were washed with water and ethanol to produce strong elastic films (several micrometers thick) firmly attached to the glass surface. Control samples from the pristine fibroin were prepared using the above procedure.

Coating with poly-L-lysine

A sterile aqueous PLL solution (0.05 mg/ml) was applied to 24 mm round coverslips and kept at room temperature for 1 h. The excess of PLL was removed by washing the glasses with water.

Measurements of the contact angle

Measurements were performed on dried films using 10 droplets of water. Each contact angle was measured at 20°C within 10 s using Meazure v.2.0.1 software.

Cell culture

The human neuroblastoma SH-SY5Y cell line (American Type Culture Collection) was cultured in EMEM/F12 medium (1:1) containing 15% FBS, 2 mM *L*-glutamine and 100 μg/ml gentamycin. Cells reaching 70–80% confluence were plated onto pristine fibroin, FBMA or PLL films (10^4 cells/cm^2). Differentiation was carried out using phase 0, phase 1 and phase 2 media [8]. For phase 0, the EMEM/F12 (1:1) was supplemented with 15% FBS, 2 mM *L*-glutamine and 100 μg/ml gentamycin. To study the interaction of neurons with the support, 10^5cells were plated in 35 mm Petri dishes onto the glass coverslips coated with films. After 24 h, the phase 0 medium was replaced with the phase 1 medium (DMEM) containing 25 mM glucose, 4 mM *L*-glutamine, 100 μg/ml gentamycin and no pyruvate). The medium was freshly supplemented with 10 μM ATRA. After 3 days, the phase 1 medium was replaced with the phase 2 medium (Neurobasal-A containing 2 mM *L*-glutamine, 2% NeuroMax 50× and 100 μg/ml gentamycin). Every two days, one half of the medium was replaced with the fresh phase 2 medium.

Immunofluorescence

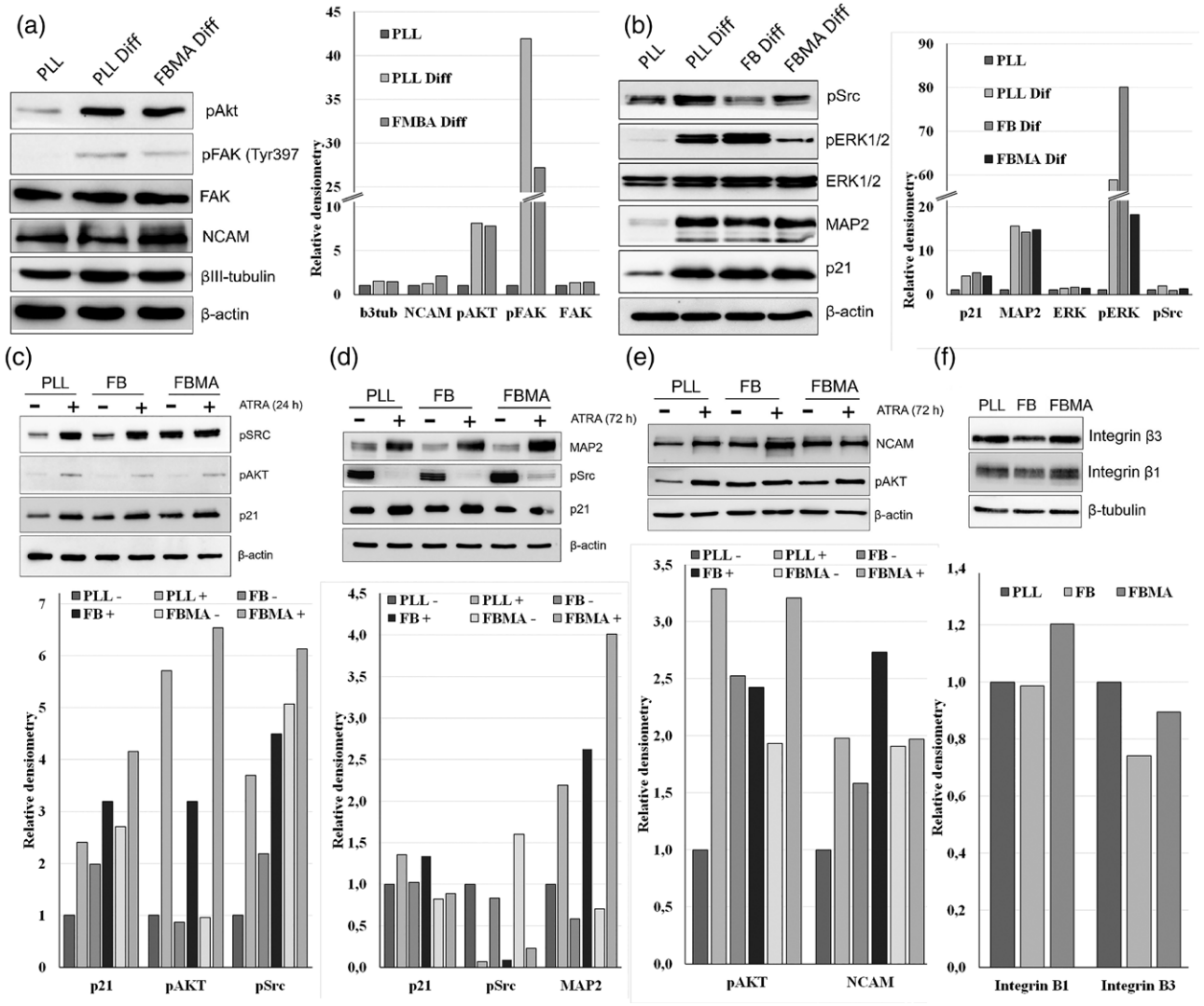
Cells were fixed with 4% paraformaldehyde in PBS 4, 24 and 72 h after culture in the phase 0 medium, washed with PBS, permeabilized in 0.1% Triton X-100/PBS/0.1% FBS for 30 min at 4°C, and washed twice with PBS/0.1% FBS. To identify actin microfilaments and the nuclei, cells were incubated with Alexa488 phalloidin and 1 μg/ml Hoechst 33342, respectively. The area of cell clusters and individual cells was divided by the number of nuclei to obtain average individual cell area. To assess cell differentiation, samples were fixed and permeabilized, incubated with PBS/1% FBS/0.1%

Tween-20 (PFT) and treated for 1 h with mouse anti-CD56 monoclonal antibodies (NCAM; clone 56C04; 1:100 in PFT). Then samples were treated for 1 h with goat anti-mouse IgG H+L conjugated to CF 488A; 1:200), washed with PFT and stained with antibodies to β-III tubulin conjugated with Alexa Fluor 647 (BioLegend; 1:100) and Hoechst 33342. Images were captured on an Eclipse Ti-E microscope with the A1 confocal module (Nikon Corporation) and a Plan Fluor 40×1.3 objective. Average neurite length was determined by measuring total neurite length and dividing by the number of nuclei.

Immunoblotting

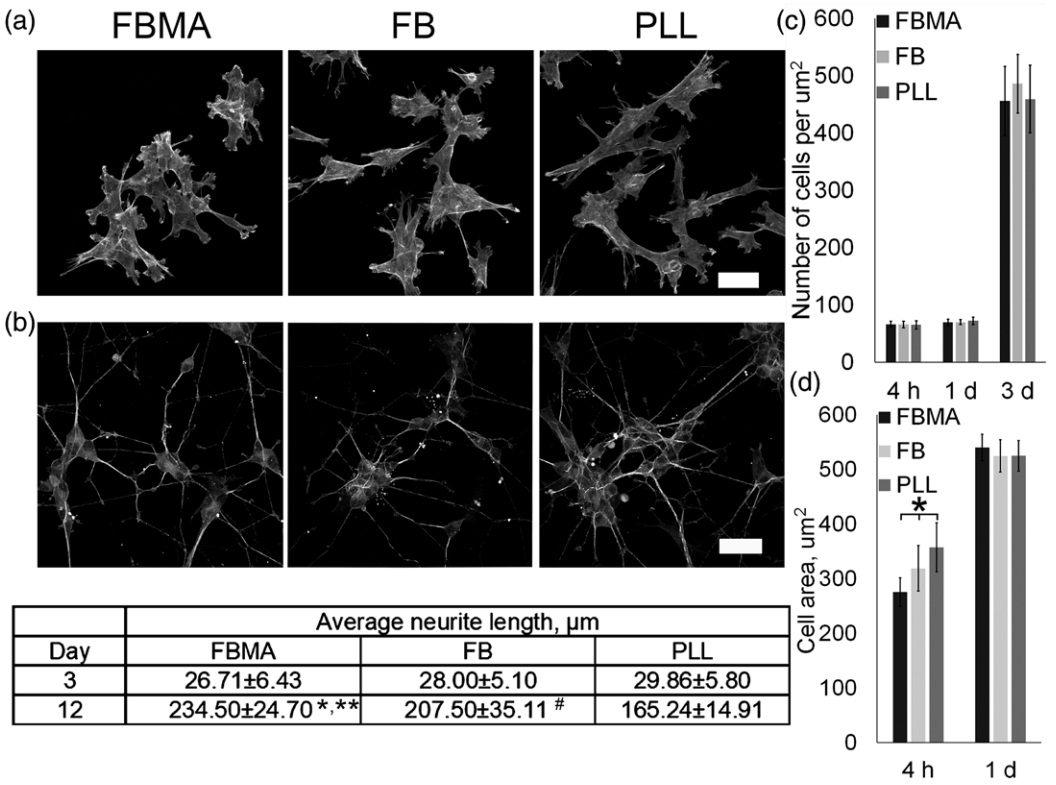
The SH-SY5Y cells were differentiated as above, washed with ice cold saline, lysed for 30 min on ice and processed for electrophoresis in a 8–10% polyacrylamide gel and electroblotting [16]. Primary antibodies were the following: pSrc (Tyr416, clone D49G4), pAkt (Ser473, clone D9E), $p21^{Waf/Cip1}$ (clone 12D1), pFAK (Tyr397, clone D20B1), FAK kinase (clone D5O7U) and β-actin (loading standard). Secondary antibody was conjugated with horseradish peroxidase. Proteins were visualized with an Enhanced Chemiluminiscence reagent (Cell Signaling) using an Image Quant LAS 4000 system (GE Healthcare).

Fig. 1



Differentiated SH-SY5Y cells show increased NCAM expression on FBMA, altered ERK/Src signaling, Src and Akt phosphorylation. (a) Increased phosphorylation of FAK and Akt in SH-SY5Y cells cultured in differentiating medium for 12 days. (b) MAP2 and p21 are elevated in differentiated cells. pSrc and pERK1/2 are decreased on FBMA compared to fibroin or PLL. (c) Src phosphorylation, p21 and pAkt. (d) Fibroin and FBMA further increase ATRA independent Src phosphorylation. ATRA induces MAP2 neuronal marker on FBMA. (e) Increased NCAM expression and spontaneous Akt phosphorylation. (f) Similar levels of β1 and β3 integrin subunits in SH-SY5Y cells on PLL, fibroin and FBMA. Western blots and corresponding densitometric analysis are representative of three independent experiments. ATRA, all-trans-retinoic acid; FBMA, fibroin methacrylamide; NCAM, neural cell adhesion molecule; PLL, poly-L-lysine.

Fig. 2



	Average neurite length, μm		
Day	FBMA	FB	PLL
3	26.71±6.43	28.00±5.10	29.86±5.80
12	234.50±24.70 *,**	207.50±35.11 #	165.24±14.91

Spreading and differentiation of SH-SY5Y cells on different substrates. (a) Cells were stained with phalloidin-Alexa 488 (actin) and Hoechst 33342 (nuclei). Scale bar, 25 μm. (b) Cells were stained with antibodies to βIII-tubulin and NCAM and counterstained with Hoechst 33342 at days 3 and 12 of culture. Bar, 25 μm. *$P<0.05$ ($n=25$) compared to PLL; **$P<0.05$ ($n=25$) compared to fibroin. #$P<0.05$ ($n=25$) compared to PLL. (c) Cell number. (d) Cell area, *$P<0.05$. FBMA, fibroin methacrylamide; NCAM, neural cell adhesion molecule; PLL, poly-L-lysine.

Blots were quantified using ImageJ 1.50i. Values were normalized to β-actin (Fig. 1a–e) or β-tubulin 3 (Fig. 1f); PLL samples were taken as 1.

Statistics

Data are presented as mean of three repeats ± SD. A one-way ANOVA test with Tukey's multiple comparisons (GraphPad Prism 8.2.0; GraphPad Software, Inc.) was used. The *P* value <0.05 between the groups was considered a statistically significant difference.

Results

We investigated SH-SY5Y cell differentiation on fibroin and FBMA using a protocol of a 3-day incubation with ATRA followed by switch to Neurobasal medium, N-2 supplement and BDNF [4], and incubation for another 9 days (total 12 days). We compared the effect of scaffolds on cell attachment, spreading and proliferation (Fig. 2a, c, d). Stiffness and the contact angle of FBMA films had little effect on cell adhesion or proliferation rates. A transient delay of cell spreading on FBMA (attributable to its higher hydrophobicity) disappeared after one day on the substrate. Next, we compared the expression of differentiation markers and neurite length after 12 days period (Fig. 2b). NCAM expression was more pronounced on FBMA compared to PLL (Fig. 1a; see also [17]). Neurite outgrowth increased 5.5-fold on PLL, 7.4 times on fibroin and 8.8-fold on FBMA by day 12 compared to day 3 (Fig. 2b).

An enhanced neurite outgrowth and expression of differentiation markers have been attributed to an increased signaling via integrins and NCAM [13,18]. While Src and ERK phosphorylation were increased on PLL and FMBA, respectively (relative to β-actin), incubation on FBMA surprisingly decreased phosphorylation of both proteins, whereas NCAM levels increased (Fig. 1a, b). These effects may contribute to longer neurites and an increased expression of MAP2 and NCAM. We observed no correlation between phosphorylation of Src/ERK and neurite length. After 1 day with ATRA, Src and Akt were phosphorylated, while on fibroin and FBMA, Src was hyperphosphorylated even without ATRA (Fig. 1c). This effect was especially evident on FBMA where ATRA-independent pSrc is comparable to ATRA-treated cells. By day 3, fibroin and FBMA stimulated Akt phosphorylation similarly as ATRA; the expression of MAP2 and

NCAM in ATRA-treated cells on FBMA was elevated (Fig. 1d, e). Also, we observed pSrc decrease (relative to β-actin) after 72 h with ATRA. A spontaneous (no ATRA) Src phosphorylation was more pronounced on FBMA compared to PLL and fibroin (Fig. 1d) indicating that FBMA activates signaling through PI3K/Akt and FAK/Src pathways even without additional differentiation signals. Nevertheless, signals without ATRA are insufficient to enhance the expression of differentiation markers (Fig. 1d).

Both NCAM and integrin signaling contribute to Src and Akt activation. We detected the expression of β1 and β3 (Fig. 1f) but not α5, αV, β4 and β5 integrin subunits (not shown). We found no changes in integrin expression after 3 days (Fig. 1f) suggesting that Src/Akt activation and integrin expression are independent processes.

Discussion

In this study, we investigated the unmodified, physically cross-linked scaffolds from the pristine fibroin, and novel photochemically cross-linked FMBA. The FBMA monomer was the starting material for the preparation of polymer networks with a double-type cross-linking [14]. The material was then exposed to ethanol to change the conformation of protein chains to antiparallel β-sheets and implement the transition from an amorphous state to a semicrystalline one. The obtained polymer has covalent and physical cross-links. Materials with such a structure have higher strength and rigidity than physically cross-linked gels with that have no additional covalent cross-links. FBMA exhibits a higher stiffness compared to the pristine fibroin [14]. Storage modulus was 480 kPa for FBMA scaffolds vs 25 kPa for the pristine fibroin in saline at 37°C. FBMA films possessed a significantly higher stiffness and a slight hydrophobicity. Positively charged functional groups were neutralized during methacrylation; therefore, unlike fibroin, the net negative charge of the film was marginal. The contact angles, a measure of surface wettability, were bigger for FMBA than for fibroin (70.8° and 59.7°, respectively). Nevertheless, the values of FBMA hydrophobicity were acceptable for biomaterials [19]. The photocrosslinked FBMA has an increased stiffness and a slightly increased negative charge. Negatively charged surface can elevate neuronal adhesion due to electrostatic interaction with positively charged substrates [2]. Furthermore, negatively charged hydrophobic materials can adsorb laminin, fibronectin, vitronectin or fibrinogen from the bloodstream [20], thereby improving cell attachment and proliferation via the increased wettability and the surface charge.

Cell adhesion plays a decisive role in regulating proliferation rate and a switch between the proliferation and differentiation programs [21]. We found no significant difference in cell proliferation and adhesion between the tested substrates, although an increased contact angle could contribute to the observed differences in signaling and expression of surface NCAM.

Activation of Src and Akt are important hallmarks in neural differentiation. Akt phosphorylation is required for differentiation in various models including SH-SY5Y cells [3]. Src phosphorylation does not affect the expression of neural markers, but is required for neurite outgrowth [22]. We confirmed that ATRA induces pAkt and pSrc. Interestingly, Src was predominantly phosphorylated during the initial 24 h of differentiation and dephosphorylated by 72 h with ATRA. Meanwhile, Akt phosphorylation remained sustained for up to 12 days. Fibroin and FBMA scaffolds alone induced similar levels of pAkt and pSrc p as ATRA alone. This finding is in line with reports showing that the increased stiffness of the substrate activates Akt and Src through integrin engagement [10,11].

Mechanical properties of FBMA lead to an increased NCAM expression and phosphorylation of Akt and Src associated with neurite elongation and increased expression of differentiation markers βIII-tubulin and MAP2. NCAM expression was increased, whereas the expression of β1 and β3 integrins was unaltered. We hypothesize that the increased NCAM expression on FBMA enhances signaling through β1–NCAM interaction and subsequent activation of Akt and Src. Nevertheless, phosphorylation of Akt and Src was insufficient to differentiate SH-SY5Y cells without ATRA. Overall, optimization of mechanical properties is perspective for cell adhesion, survival and differentiation of the transplant.

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Conflicts of interest

There are no conflicts of interest.

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