Injury, Int. J. Care Injured 50 (2019) 278–285

Contents lists available at ScienceDirect

Injury

journal homepage: www.elsevier.com/locate/injury

Biofunctionalized peptide-based hydrogel as an injectable scaffold for BDNF delivery can improve regeneration after spinal cord injury

Zahra Hassannejad[a,b], Shayan Abdollah Zadegan[b], Alexander R. Vaccaro[c], Vafa Rahimi-Movaghar[b,*], Omid Sabzevari[d,e,**]

[a] Pediatric Urology and Regenerative Medicine Research Center, Children's Hospital Medical Center, Tehran University of Medical Sciences, Tehran, Iran
[b] Sina Trauma and Surgery Research Center, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Orthopaedic Surgery, Rothman Institute, Thomas Jefferson University, Philadelphia, PA, USA
[d] Toxicology and Poisoning Research Centre, Tehran University of Medical Sciences, Tehran, Iran
[e] Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
Self-assembling peptide
Laminin
BDNF
Spinal cord injury
Clip compression injury model
Rat

ABSTRACT

Background: The complex pathophysiological events occurring after traumatic spinal cord injuries (TSCI) make this devastating trauma still incurable. Peptide amphiphile (PA) hydrogels are nanobiomaterials displaying desirable properties for application in regenerative medicine because they are absorbable, injectable, allowing biofunctionalization, controlling release of trophic factors and mimic extracellular matrix (ECM). In this study, we explored the potentiality of the IKVAV-functionalized PA hydrogel to provide a permissive environment for cell migration and growth as well as sustained release of BDNF at the lesion after severe compression injury model.

Methods: The IKVAV-functionalized PA was synthesized by automated solid-phase approach and its secondary structure was evaluated by Circular dichroism (CD) spectroscopy. The potential of IKVAV-functionalized PA to self-assemble into nanofibers and hydrogel formation were assessed using transmission electron microscopy (TEM). Release profiles of BDNF from hydrogel and the bioactivity of the released BDNF from hydrogel were determined using ELISA and DRG bioassay, respectively. Severe spinal cord injury was induced using clip compression at T7-T8 vertebral segment. Twenty four hours post-injury the animals were treated by either IKVAV PA hydrogel, BDNF-loaded IKVAV PA hydrogel, BDNF solution or saline. Two and six weeks later, animals were sacrificed and the lesion site was evaluated based on GFAP, CD68 and ß III tubulin immunoreactivity. Also, locomotor recovery was assessed during 6 weeks using Basso, Beattie, Bresnahan (BBB) scoring test.

Results: The IKVAV PA arranged into nanofibrous structure and provided a sustained release of BDNF over 21 days while preserved the bioactivity of BDNF. Also, BDNF loading influenced the hydrogel nanostructure resulting in aligned orientation of nanofibers. Injection of BDNF-loaded IKVAV PA hydrogel resulted in a considerable axon preservation and astrogliosis reduction at 6 weeks post-injury without showing any inflammatory reaction. However, the BBB score was not statistically different between different treatment groups.

Conclusion: Although the locomotor functional recovery was not observed in this study, the axon preservation and minimal inflammation in animals treated with BDNF-incorporated hydrogel indicate the potentiality of the designed intervention for further evaluations in the path of developing efficient therapies for severe spinal cord injury.

Introduction

Traumatic spinal cord injury (TSCI) has a devastating effect on the patient's life, as well as their family and society [1,2]. Current clinical interventions, including early cord decompression and administration of high dose methylprednisolone [3], have not yielded good prognosis. The secondary pathophysiological events which immediately initiate after primary insult, including

* Corresponding author at: Sina Trauma and Surgery Research Center, Tehran University of Medical Sciences, Tehran, Iran.
** Corresponding author at: Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
E-mail addresses: v_rahimi@sina.tums.ac.ir (V. Rahimi-Movaghar), omid@tums.ac.ir (O. Sabzevari).

https://doi.org/10.1016/j.injury.2018.12.027
0020-1383/

necrotic/apoptotic cell death, excitotoxicity, ionic dysregulation, inflammation, astrogliosis and fluid-filled cyst formation, are the major obstacles to nerve tissue regeneration after TSCI. As some portion of neurons can survive even one year post-injury [4], new strategies have been proposed to preserve spared neural tissue, and to bridge the injury site by using biomaterials with sustained release of neurotrophic factors for conversion of the inhibitory environment of the lesion to a permissive one.

The main problem associated with implantation of preformed polymeric scaffolds is the dissection and removal of some tissue from the lesion site to accommodate the implantation. Recently, injectable hydrogels such as agarose [5], blend of hyaluronan and methylcellulose [6], fibrin [7,8] and self-assembling peptide nanofibers [9–13] have been evaluated to improve the regenerative potential of TSCI through minimizing the aforementioned disadvantage of preformed scaffolds. However, there are only limited amount of studies using injectable hydrogels in the compression/contusion TSCI models, which are the most similar animal TSCI models to human traumatic spinal cord injuries [9,10,13]. Tysseling-Mattiace et al., showed that injection of PA functionalized with laminin motif IKVAV (IKVAV PA) within the lesion inhibited glial scar formation and promoted axon elongation after moderate cord compression in mice [9]. They also demonstrated the efficacy of IKVAV PA on enhancing the plasticity of serotonergic fibers after cord compression in rats [13]. Furthermore, Cigognini et al., showed that injection of self-assembling peptides functionalized with bone marrow homing motif immediately after injury can stimulate upregulation of growth associated protein 43 (GAP-43), trophic factors and extracellular matrix (ECM) remodeling proteins at one week post-injury and promote functional recovery [10].

Meanwhile, therapeutic potential of neurotrophic factors has been well recognized for over two decades [14–16], however, attempts to translate that potentiality to the clinic have been disappointing, largely due to obstacles in delivery, including inadequate protein dose and kinetics released as well as protein preservation at the lesion site. In order to solve these problems considerable efforts have been made to incorporate neurotrophic factors within hydrogels through different covalent and non-covalent binding approaches. It is worth noting that the method of loading proteins into hydrogels may potentially be harmful for proteins; for example incorporation of proteins in the hydrogel matrix during gelation and subsequent cross-linking of the matrix using bifunctionalized cross-linker or ultraviolet irradiation can potentially harm the conformation of the encapsulated proteins [17]. Physical incorporation of proteins within hydrogels may diminish these drawbacks. Self-assembling peptides are useful injectable hydrogels for such applications due to their stimulus-sensitive assembly and their chemical characteristics [18].

This study aimed to provide sustained release of brain-derived neurotrophic factor (BDNF), a neuroprotective protein that belongs to the neurotrophin, using self-assembling peptide hydrogel which was functionalized with laminin epitope, IKVAV. Therapeutic efficacy of the proposed intervention was evaluated using a severe compression injury model.

Materials and methods

Synthesis and characterization of hydrogel-forming peptides

CH3(CH2)14CO-AAAAGGGEIKVAV PA, the peptide amphiphile incorporating the neuroactive pentapeptide epitope from laminin, isoleucine-lysine-valine-alanine-valine (IKVAV), was synthesized via automated solid-phase peptide synthesis. The analytical characterization was conducted using high-performance liquid chromatography (HPLC) and mass spectrometry, which showed more than 85% of purity. Lyophilized hydrogel peptides were stored at −20 °C. The secondary structure of the synthesized IKVAV PA in aqueous medium was evaluated by Circular dichroism (CD) spectroscopy. The CD spectra were obtained on Aviv 62SD spectrometer using a 1 mm quarz cuvette at room temperature (RT). PA solutions were diluted at a final concentration of 0.2 mg/ml and the spectra were collected between 190 and 260 nm by using 1 nm step and 0.5 s dwell time. Background was determined from ammonium bicarbonate buffer, and automatically subtracted from the sample data.

Preparation of IKVAV PA hydrogels

In order to prepare 1% IKVAV PA hydrogel, 2 mg of lyophilized IKVAV PA was dissolved in 200 μl ammonium bicarbonate buffer (0.1 M, pH 7.6) and was homogenized by sonication for 15 min. In order to prepare BDNF-loaded IKVAV PA hydrogel, first 80 μl of 1% IKVAV PA hydrogel was prepared and 20 μl of 0.05 mg/ml BDNF solution was immediately added to the prepared hydrogel and maintained at the room temperature for 30 min until the gelation was completed.

TEM imaging of IKVAV PA hydrogel

Transmission electron microscopy (TEM) was performed, using a CM 200 FEG STEM Philips-M.E.R.C operating at the voltage of 200 kV, in order to analyze peptide capacity to self-assemble into nanofibers as well as degradation of the hydrogel over time. The IKVAV PA hydrogel was diluted with distilled water and dripped on a copper grid, the excessive sample was removed and air-dried for 1 min at RT. Subsequently, uranyl acetate solution (10 μl of 1%) was dripped onto the grid and after 1 min the excess staining solution was removed with filter paper and was allowed to dry in the air before introduction into the microscope.

BDNF release study

Release profiles of BDNF from IKVAV PA hydrogels were measured through incubation of 100 μl of BDNF-loaded hydrogels within 900 μl of artificial cerebrospinal fluid (aCSF) composed of 149 mM NaCl, 3 mM KCl, 0.8 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1.5 mM Na_2HPO_4, and 0.2 mM NaH_2PO_4 (pH 7.4) [19]. The BDNF-loaded hydrogel was then placed in an orbital shaker at 37 °C. The supernatant containing the released BDNF and degradation products was removed and replaced completely with fresh pre-warmed aCSF at the following time points: 1, 3 h, 1, 3, 7, 10, 14, and 21 d and stored at -20 °C. The protein content of the supernatant was determined by ELISA assay (Human BDNF Immunoassay, R&D Systems®, USA) and reported as a cumulative amount of BDNF released over time.

Bioactivity assessment of released BDNF through neurite outgrowth from DRG explants

The bioactivity of BDNF released from the hydrogel at 21 d was determined using a DRG bioassay as described previously [20], with some modifications described below. DRGs were removed from one-day-old neonatal Wistar rats and pooled in ice-cold media (DMEM with 1% fetal bovine serum (FBS), 2% B-27 serum-free supplement, 1% penicillin–streptomycin and 1% L-glutamine). The DRGs were then placed on 10 mm diameter glass cover slips coated with poly-L-lysine (50 μg/mL in sterile water) and laminin (5 μg/mL in PBS) in a 4-well plate (3 DRGs per each cover slip) and were kept overnight in an incubator at 37 °C and 5% CO2. The slips were then treated with DMEM/10% FBS and 0.5 ml of the BDNF release study supernatant the following day. For the controls,

0.5 ml of aCSF with and without the same concentrations of BDNF (5 ng/ml) in release study supernatant was added to the wells instead, as negative and positive controls, respectively. The DRGs were incubated for an additional 48 h at 37 °C in 5% CO2. Subsequently, the cultures were fixed in 4% paraformaldehyde in PBS (0.1 M, pH 7.4) for 15 min. and immunostained with anti-ß III tubulin antibody (1:400, Sigma).

Animals and surgery procedure

A total of 28 adult male Wistar rats weighing 160–200 g and bred in an in-house animal facility were obtained from Tehran University of Medical Sciences (Tehran, Iran). Experiments and animal care were carried out in compliance with Tehran University of Medical Sciences (TUMS) guideline and all animal protocols were reviewed and approved by the state animal ethical committee. Animals had free access to food and water and were randomly selected and divided into different treatment groups including: 1. animals injected by saline (n = 7), 2. animals injected by IKVAV PA hydrogel (n = 7), 3. animals injected by BDNF solution in saline (n = 7), 4. animals injected by BDNF-loaded IKVAV PA hydrogel (n = 7). Rats were anesthetized using a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). All animals underwent laminectomy at the T7-T8 vertebral segment, the spinal cord was compressed dorsoventrally by the extradural application of a 134 g aneurysm clip (AESCULAP® MINI-CLIP) for 1 min (severe compression injury). Subsequently, the skin was sutured, a 5 ml injection of saline was given subcutaneously and animals were kept under a heat lamp to maintain body temperature. Gentamycin and cefazolin was administered once daily (1 mg/ kg and 75 mg/kg, respectively) subcutaneously for one week. Rats that exhibited any hindlimb movement 24 h after the injury were excluded from the study; totally 5 animals were excluded from the study due to hindlimb movement after injury.

Injection was conducted 24 h after TSCI using a Hamilton syringe (29-gauge needle) fixed at a micromanipulator. The penetration depth of the syringe within the cord was 1 mm which was controlled using the stereotactic apparatus. At the end of injection, the syringe was left in the cord for an additional one minute. Animals were monitored for autophagia and their bladder was manually expressed until recovery of the voiding reflex. Induction of injury and injection process are shown in Fig. 1.

Histology and immunolabeling

Two and six weeks post-injury, animals were transcardially perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The spinal cords were dissected and post-fixed for 3 days at 4 °C. About 2 cm length of spinal cord was dissected longitudinally through its dorsal-to-ventral axis and embedded within paraffin blocks. For immunohistochemistry, the spinal cords were sectioned at 6 μm thickness. The sections were deparaffinized and were incubated in PBS containing 5% normal donkey serum, 2% BSA and 0.1% Triton X-100 for one hour at RT. The samples were then incubated with primary antibodies: anti-GFAP (1:400, Sigma), anti-ß III tubulin (1:200, Sigma) and anti-ED1 (1:400, Sigma) overnight at 4 °C in humidified chamber, and then incubated in either Rhodamine (TRITC)-conjugated donkey anti-rabbit IgG (1:400, Jackson) or rhodamine (TRITC)-conjugated donkey anti-mouse IgG (1:400, Jackson) for 2 h at RT.

The density of GFAP-positive astrocytes was quantified as a pixel density using the thresholding feature of NIH-ImageJ software and calibrated to total stained area [21].

Locomotor functional testing

Two trained evaluators scored each animal per session for 6 weeks post-injury. Hindlimb locomotor function was assessed with the 21-point Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB) [22]. The scale measures a wide range of hindlimb function, from complete paralysis to normal walking behavior, by assessing hind limb joint movements, stepping, trunk position and stability, coordination of forelimb and hindlimb, paw placement, toe clearance, and tail position. Rats were assessed in an open field (diameter of 50 cm) for 4 min at a similar time of day for each testing.

Statistical analysis

The Data are presented as mean ± standard deviation (SD). Multiple group comparisons were made using one-way analysis of variance (ANOVA). The P value less than 0.05 was considered as significant.

Results

Synthesis and characterization of IKVAV PA hydrogel

IKVAV PA molecules were well dissolved in ammonium bicarbonate buffer through sonication for 15 min and the solution formed a hydrogel after 30 min maintenance at RT. The hydrogel formation was reversible, so that applying mechanical forces (vortex or sonication) cause disassembly of the structure and dissolution of the IKVAV PA molecules, however the hydrogel could reform on standing at RT. The circular dichroism (CD) spectra of the hydrogel showed two prominent peaks: a positive peak centered around 198 nm and a negative peak around 219 nm, which confirms the formation of ß-helices secondary conformation during self-assembling [23] (Fig. 2).

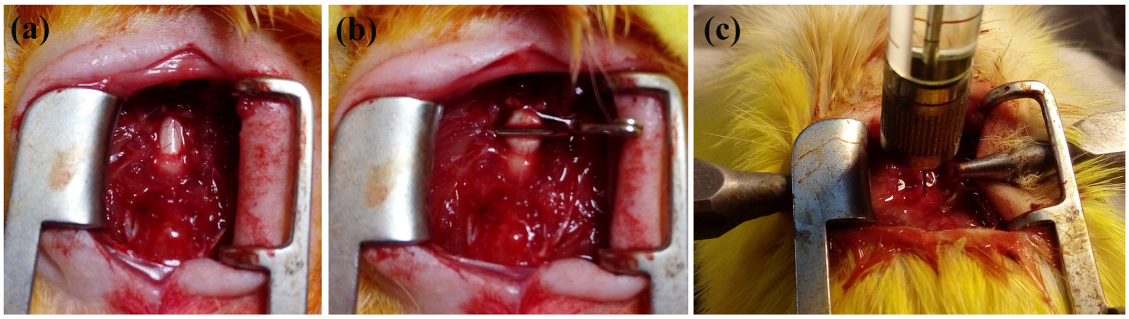

Fig. 1. The steps of animal surgery for injury induction using clip compression: (a) laminectomy at T7-T8 vertebral segment, (b) dorsoventral compression of cord using extradural application of a 134 g aneurysm clip for 1 min (severe compression injury) and (c) injection at 24 h post-injury using a Hamilton syringe (29-gauge needle) fixed at a micromanipulator with the penetration depth of 1 mm which was controlled using the stereotactic apparatus.

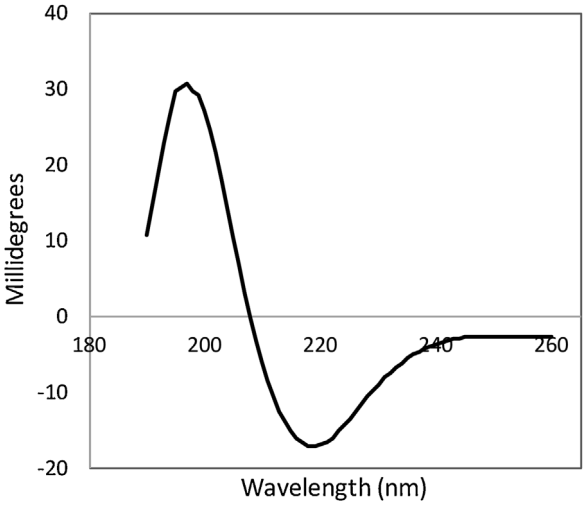

Fig. 2. The circular dichroism (CD) spectra of the hydrogel showing two prominent peaks: a positive peak centered around 198 nm and a negative peak around 219 nm, which confirms the formation of ß-helices secondary conformation during self-assembling.

TEM imaging of IKVAV PA hydrogels

Nanofibrous structure of the IKVAV PA hydrogel before and after incorporation of BDNF within the hydrogel network was visualized by TEM, and as shown in Fig. 3a and b BDNF incorporation within the hydrogel network organized the nanofibers as aligned structures. Furthermore, pore size and porosity of the BDNF-loaded IKVAV PA hydrogel network were increased after 21 days of incubation within the aCSF at 37 °C (Fig. 3c).

BDNF release study

The BDNF release from IKVAV PA hydrogel under physiological conditions were quantified with an ELISA assay. The results for this study are shown in Fig. 4. The general release profile consists of a burst release of the BDNF over first 24 h, followed by a near-linear release by 3 days and afterward reached to plateau.

Bioactivity assessment of released BDNF through neurite outgrowth from DRG explants

Although desirable release profile has been demonstrated using the ELISA assay, it is important to investigate the bioactivity of the

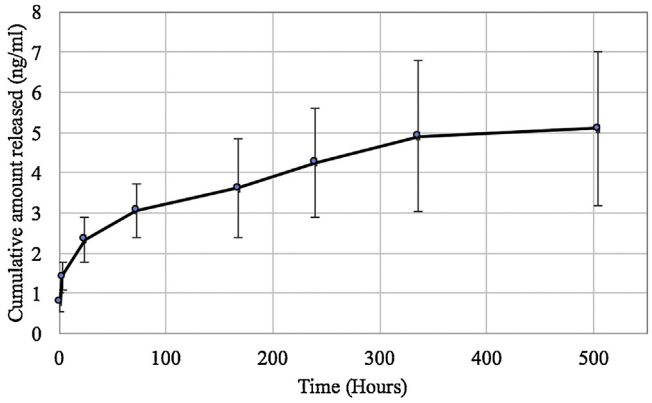

Fig. 4. The general release profile consisted of a burst of BDNF release over the first 24 h, followed by a near-linear release by 3 days and afterward reached to plateau.

released BDNF, since the hydrophobic and electrostatic interactions of the BDNF with PA may result in the conformational changes of the BDNF and therefore protein denaturation. The ability of the released BDNF to enhance neurite outgrowth from DRG explants was assessed and compared with that in aCSF as a control. DRG explants had no contact with the hydrogel, but were exposed to the released BDNF and degradation products of the hydrogel over 48 h of culture. Immunostaining against ß III tubulin revealed the extent of neurite outgrowth from DRG explants which were cultured in various conditions (Fig. 5). Although there was seldom neurite outgrowth from cultured DRG explants in the medium supplemented with aCSF, neurite outgrowth was significantly higher in the other two groups where the media were supplemented with either BDNF solution or released BDNF from hydrogel.

Histology and immunolabeling

The spinal cord sections were stained with anti-ß III tubulin, which stains neurons and axons. In Fig. 6, it can be seen that injection of BDNF-loaded IKVAV PA hydrogel resulted in a considerable axon preservation at 6 weeks post-injury. Although axon preservation after BDNF injection was greater than control, it was not as much as that in BDNF-loaded IKVAV PA hydrogel group. There was no significant difference in the extent of axon preservation between IKVAV PA hydrogel injected and saline injected animals.

We also assessed the responses of glial cells in different treatment groups. Astrocytes are the most abundant non-neuronal

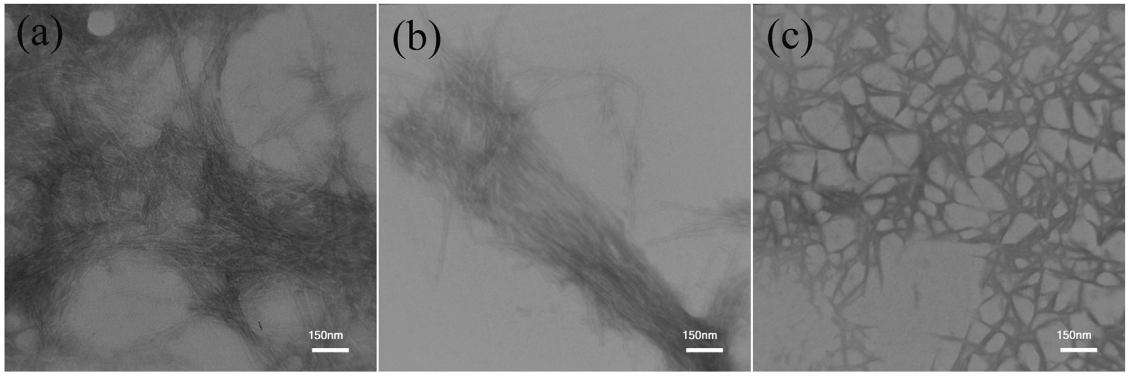

Fig. 3. Transmission electron microscopy (TEM) of the IKVAV PA hydrogel negatively stained with 2% uranyl acetate reveals the nanofibrous structure of the hydrogel (a). Incorporation BDNF caused the alignment of nanofibrous structure (b). Degradation over 21 days hydrogel incubation in physiologic condition increased the porosity of the IKVAV PA hydrogel (c).

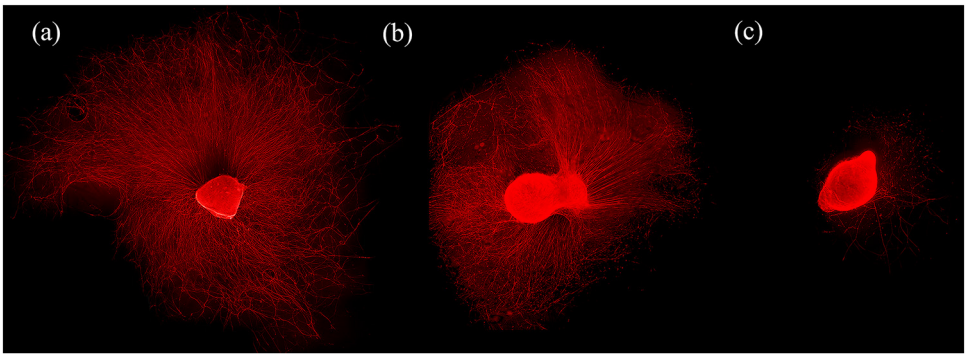

Fig. 5. Released BDNF from IKVAV PA hydrogel stimulated neurite outgrowth from DRG explants (a) which was comparable with the effect of BDNF solution with the same concentration on neurite outgrowth (b). However, there was seldom neurite outgrowth from DRG explant when aCSF was added to cell culture medium (c).

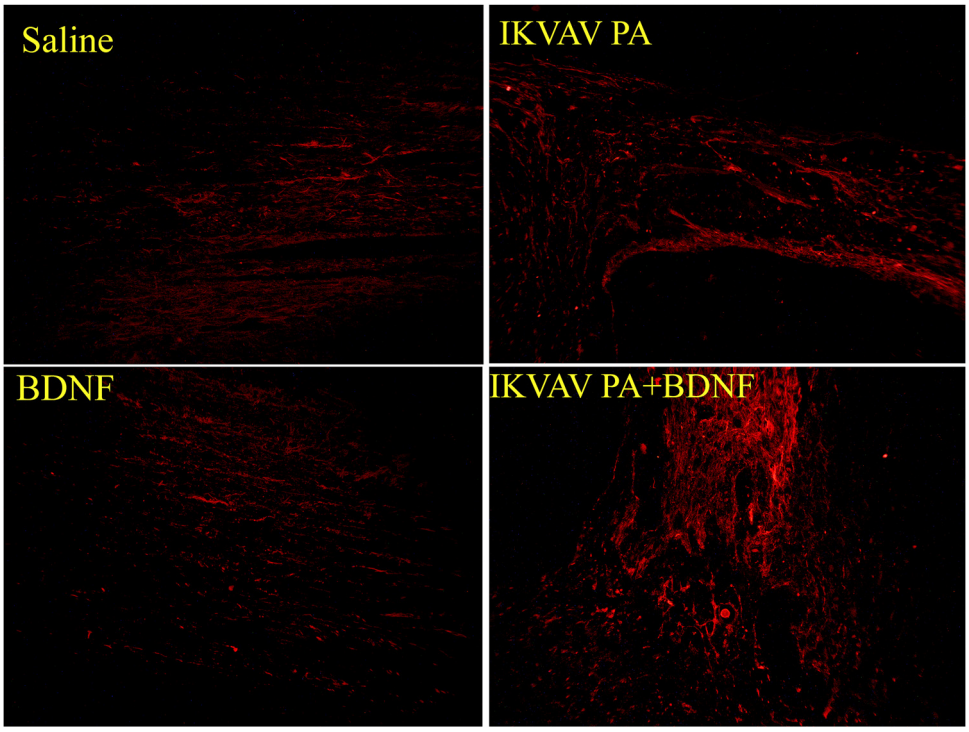

Fig. 6. Anti-ß III tubulin immunoreactivity at the lesion site of the animals treated with saline, IKVAV PA hydrogel, BDNF solution and BDNF-loaded IKVAV PA hydrogel at 6 weeks post-injury. Images were taken from the injury epicenter. Injection of BDNF-loaded IKVAV PA hydrogel resulted in a considerable axonal preservation at 6 weeks post-injury. Axonal preservation after BDNF injection was greater than control, but it was not as much as animals injected by BDNF-loaded IKVAV PA hydrogel. There was no significant difference in the extent of axonal preservation between IKVAV PA hydrogel injected and saline injected animals; IKVAV-functionalized PA hydrogel (IKVAV PA), BDNF-loaded IKVAV PA hydrogel (IKVAV PA + BDNF).

cells in CNS tissue and changes regarding astrocytes after injury range from reversible alterations in gene expression, cellular hypertrophy, cell proliferation to long lasting scar formation which certainly depend on the type and severity of injury. In this study, the extent of glial fibrillary acidic protein (GFAP) gene expression was evaluated as the marker of astrocyte reactivity after injury. As seen in Fig. 7, a dense scar tissue, mainly composed of GFAP-positive astrocytes, was observed in animals injected with saline solution. The fluorescent intensity of GFAP stains were quantified using the ImageJ Software. When the GFAP intensity was compared between the IKVAV PA hydrogel, BDNF solution and BDNF-loaded IKVAV PA hydrogel treated spinal cords and the saline-treated spinal cords, the GFAP intensity was significantly higher in the saline-treated spinal cords (Fig. 7a).

In order to monitor the immune response of host tissue to the intervention, particularly the presence of microglia/macrophage, ED1 immunoreactivity in lesion site was monitored 6 weeks after injury in animal groups treated by saline or BDNF-incorporated hydrogel. Infiltration of the microglia/macrophage due to injury was observable in both treatment groups (Fig. 8).

Locomotor functional recovery

In order to assess the effect of treatment on locomotor recovery, BBB open field motor testing was performed weekly (Fig. 9). Immediately after injury, animals had no hindlimb function (score of 0). One week after injury, animals had regained slight or extensive movement of two or three hindlimb joints (BBB score of 2–3). At 6 weeks, most animals had regained sweeping motion or plantar paw placement of their hindlimbs (BBB score of 8). In the group of animals injected by the BDNF-loaded hydrogel, one animal was the same as other groups with sweeping motion or

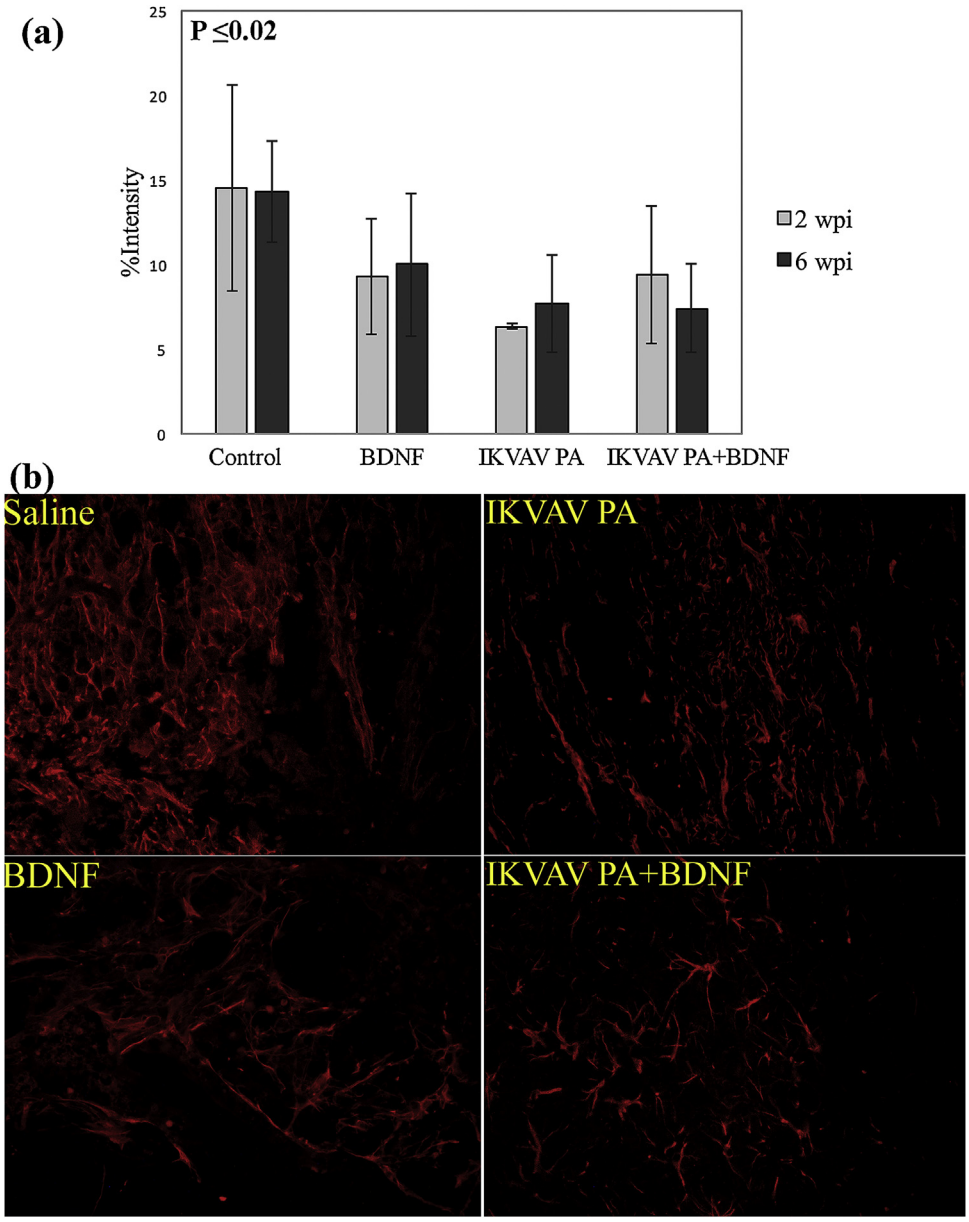

Fig. 7. The fluorescent intensity of the GFAP stains were quantified using the ImageJ Software. When the GFAP intensity was compared between animals treated with IKVAV PA hydrogel, BDNF solution and BDNF-loaded IKVAV PA hydrogel and animals treated with saline, the GFAP intensity was significantly higher in the saline-treated animals, for all treatment groups at both evaluation time (i.e., 2 and 6 weeks post-injury) $P \leq 0.02$ in comparison with saline-treated animals (a). Anti-GFAP immunoreactivity at the lesion site of the animals treated with saline, IKVAV PA hydrogel, BDNF solution and BDNF-loaded IKVAV PA hydrogel at 6 weeks post-injury (b) ; IKVAV-functionalized PA hydrogel (IKVAV PA), BDNF-loaded IKVAV PA hydrogel (IKVAV PA + BDNF).

plantar paw placement of their hindlimbs (BBB score of 8), in addition, the remained 3 animals had regained plantar placement of their hindpaw with weight support or frequent to consistent weight supported plantar steps (score of 9–11). However, the difference between treatment groups was not statistically significant.

Discussion

A unique feature of PAs is its ability to self-assemble into nanofiber when it is injected into tissue and contacts cations [24]. IKVAV PA hydrogel with or without BDNF showed an injectable property and in situ gel formation after injection and showed nanofibrous structure particularly as a result of hydrophobic interactions and entanglement of nanofibers was demonstrated in TEM images. Addition of BDNF changes the arrangement of the nanofibers which can be attributed to an increase in the weak interaction between nanofibers after protein addition. Furthermore, degradation of the hydrogel increased the porosity and shortened the nanofibers length.

In addition, the hydrogel enabled the encapsulation and sustained release of BDNF during 21 days. The obtained release profile was consistent with previous study showing that the release of neurotrophins can be controlled using degradable hydrogels [25]. The relationship between protein release kinetic and protein dimension and electrical charge has also been evaluated [26]. Gelain et al. showed that self-assembling peptide scaffolds based on RADA16-I provide slow and sustained release of cytokines and are able to preserve the bioactivity of cytokines up to 3 weeks [26]. They demonstrated that the electrostatic interactions between protein and nanofibers can further increase the retention time of proteins within the hydrogel. In this study the

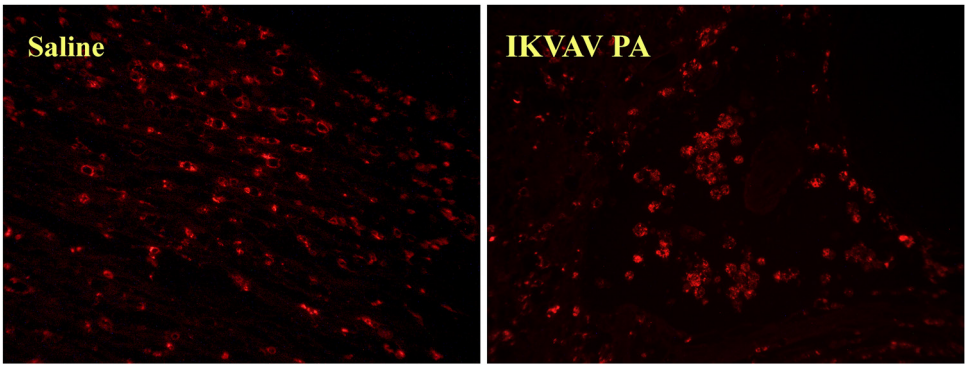

Fig. 8. Representation of immune response of host tissue to the intervention through immunohistochemistry against ED1 to visualized reactive microglia/macrophage at the lesion site at 6 weeks after injury. Infiltration of the microglia/macrophage due to injury was observable in both treatment groups; saline and IKVAV PA injection.

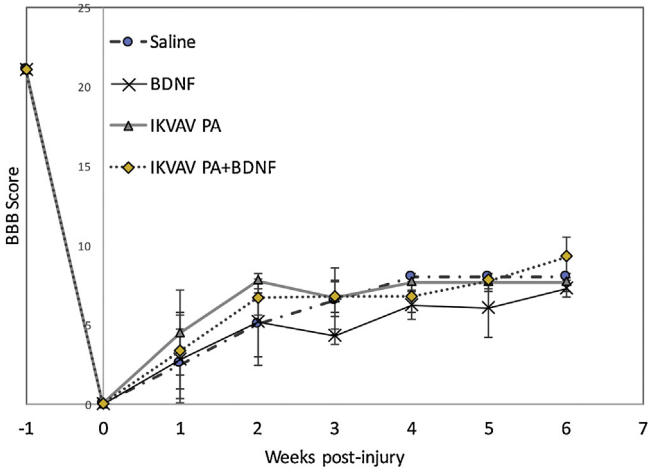

Fig. 9. Functional assessment of injured animals with open field BBB scoring test: One week after injury, animals had regained slight or extensive movement of two or three hindlimb joints (BBB score of 2–3). At 6 weeks, most animals had regained sweeping motion or plantar paw placement of their hindlimbs (BBB score of 8). In the group of animals injected by the BDNF-loaded hydrogel, one animal was the same as other groups with sweeping motion or plantar paw placement of their hindlimbs (BBB score of 8), in addition, the remained 3 animals had regained plantar placement of their hindpaw with weight support or frequent to consistent weight supported plantar steps (score of 9–11). However, the difference between treatment groups was not statistically significant.

release rate of BDNF from self-assembling peptides was monitored up to 48 h and a sharp release profile similar to our result was observed during the first 5 h and at later times the BDNF released slower [26].

Long lasting retention and release of BDNF from IKVAV PA in our study may be a result of electrostatic interactions between BDNF and nanofibers; since BDNF is highly charged at electrophysiological condition (pI [isoelectric point] = ~9–10) [3] and BDNF release from hydrogel is affected by the protein-nanofiber interactions which have positive and negative charges, respectively. However, at later time points it was likely that the release of the BDNF was controlled by the hydrogel degradation which resulted in an increase in pore size of the hydrogel network, as shown in the TEM images (Fig. 3c).

BDNF supports the survival and differentiation of distinct populations of neurons [27] and retrograde transport of BDNF from the lesion site to the cell body has been used to preserve supraspinal neurons after injury [28,29]. Protein inactivation due to protein aggregation and protein–peptide interactions could occur during self-assembly and nanofiber formation, or during the release process. In our study, the released BDNF stimulated the axon outgrowth from DRG explants confirms the preservation of protein conformation and function.

Dense astrogliosis, which often form following TSCI contain inhibitory molecules that can hamper the axon regeneration [30,31]. It has been shown that the inhibitory nature of the astrogliosis can be overcome through addition of growth promoting cues that serve to balance inhibitory environment of the glial scar [32,33]. In our study injection of IKVAV PA with and without BDNF reduced the accumulation of GFAP positive astrocytes at the lesion margin even 6 weeks after injury. As astrocytes are the main cells which contribute in the release of inhibitory molecules, predominantly chondroitin sulfate proteoglycans (CSPG), as well as glial scar formation, density reduction of astrocytes at the lesion reduce the strength of the inhibitory signals thus enhance regeneration. The delayed accumulation of astrocytes was also observed in a study evaluating the effect of fibrin-based scaffolds on regeneration [34]. This study showed that although fibrin injection reduces GFAP density at 2 weeks post-injury, the reduction of GFAP density was not statistically significant at 4 weeks post-injury.

Despite numerous reports discussing physical and molecular inhibitory role of astrocytes, the beneficial contribution of astrocytes in regeneration has been proposed, as well [35]. Alongside other glial cells astrocytes aid central nervous system regeneration through expression of axon growth supporting molecules and it has been shown that complete removing of astrocytes and preventing astrocyte scar formation fail to result in regrowth of transected axons after severe SCI. In our study, decrease in astrocyte density at the lesion margin can eliminate the physical barrier, meanwhile astrocyte infiltration within the lesion area in animals injected with IKVAV PA provide axon growth cues. This was also confirmed by assessment of axon density at the lesion area and it was found that injection of IKVAV PA preserved axons by 6 weeks post-injury and this effect was strengthen by sustained release of BDFN at the lesion site.

Animal treatment with IKVAV PA promoted cell migration into the lesion area without eliciting an increased inflammatory response. This fact was concluded based on the results of immunohistochemistry to detect glial cells; astrocytes and oligodendrocyte. This is consistent with previous studies which show that replacement of inhibitory environment of injured tissue with hydrogels functionalized with biologically active molecules promotes cell migration within the lesion site. Plant et al. showed that modification of N-(2-hydroxypropyl) methacrylamide (HPMA) hydrogels with RGD peptide increased infiltration of non-neuronal cells (mostly GFAP and ED1 positive cells), while no cellular infiltration was observed in unmodified HPMA hydrogel

[36]. Migration of supporting cells within the fibrin-based hydrogels which were injected in the lesion after SCI has also been reported [34].

In our study, a pentapeptide sequence of laminin was selected for functionalization of PA hydrogel. Laminin is the major component of the basement membrane and plays a crucial role in cell adhesion and axon growth and functions through its bioactive binding sites and can interact with cell surface receptors to activate signaling pathways, which affects the cell functions and nerve tissue regeneration [37]. The beneficial effect of surface modification of scaffolds by laminin, either as a whole protein [38] or its bioactive epitope [39], on cell function has been previously reported.

In our study, the difference in BBB score among various treatment groups was not statistically significant, however, it should be noted that the BBB score is not sensitive enough to report subtle changes in regeneration, particularly if regeneration of key neuronal tracts has not achieved functional connection with their targets.

Conclusion

We demonstrate that PA hydrogels can effectively control the release of BDNF over 21 days and the bioactivity of the BDNF is preserved within the hydrogel. Also, injection of the BDNF-loaded IKVAV PA hydrogel elicits a minimal inflammatory response 6 weeks after implantation. We also demonstrate that this hydrogel can mitigate the astrogliosis and enhance axon preservation after severe SCI. While tissue engineered scaffolds can be tailored to provide a permissive environment for regeneration, incorporation of neurotrophic factors may be necessary to elicit significant regeneration after SCI.

References

[1] Saadat S, Javadi M, Divshali BS, Tavakoli AH, Ghodsi SM, Montazeri A, et al. Health-related quality of life among individuals with long-standing spinal cord injury: a comparative study of veterans and non-veterans. BMC Public Health 2010;10:6-.

[2] Rahimi-Movaghar V, Moradi-Lakeh M, Rasouli MR, Vaccaro AR. Burden of spinal cord injury in Tehran, Iran. Spinal Cord 2010;48:492–7.

[3] Evaniew N, Noonan VK, Fallah N, Kwon BK, Rivers CS, Ahn H, et al. Methylprednisolone for the treatment of patients with acute spinal cord injuries a propensity score-matched cohort study from a canadian multi-center spinal cord injury registry. J Neurotrauma 2015;32:1674–83.

[4] z Hassannejad, Zadegan S, Shakouri-Motlagh A, Mokhatab M, Rezvan M, et al. The fate of neurons after traumatic spinal cord injury in rats: a systematic review. Iran J Basic Med Sci 2018;21:546–57.

[5] Jain A, Kim Y-T, McKeon RJ, Bellamkonda RV. In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. Biomaterials 2006;27:497–504.

[6] Guptaa D, Tator CH, Shoichet MS. Fast-gelling injectable blend of hyaluronan and methylcellulose for intrathecal, localized delivery to the injured spinal cord. Biomaterials 2006;27:2370–9.

[7] King VR, Alovskaya A, Wei DYT, Brown RA, Priestley JV. The use of injectable forms of fibrin and fibronectin to support axon ingrowth after spinal cord injury. Biomaterials 2010;31:4447–56.

[8] Petter-puchner AH, froetscher W, Krametter-Froetsher R, Lorinson D, Redl H, Griensven Mv. The long-term neurocompatibilty of human fibrin sealant and equine collagen as biomatrices in experimental spinal cord injury. Exper Toxicol Pathol 2001;58:237–45.

[9] Tysseling-Mattiace VM, Sahni V, Niece KL, Birch D, Czeisler C, Fehlings MG, et al. Self-assembling nanofibers inhibit glial scar formation and promote axon elongation after spinal cord injury. J Neurosci 2008;28: 3814 –23.

[10] Cigognini D, Satta A, Colleoni B, Silva D, Donega M, Antonini S, et al. Evaluation of early and late effects into the acute spinal cord injury of an injectable functionalized self-assembling scaffold. PLoS One 2011;6: e19782.

[11] Zhang W, Zhan X, Gao M, Hamilton AD, Liu Z, Jiang Y, et al. Self-assembling peptide nanofiber scaffold enhanced with RhoA inhibitor CT04 improves axonal regrowth in the transected spinal cord. J Nanomater 2012;2012:1–10.

[12] Guo J, Su H, Zeng Y, Liang Y-X, Wong WM, et al. Reknitting the injured spinal cord by self-assembling peptide nanofiber scaffold. Nanomed Nanotechnol Biol Med 2007;3:311–21.

[13] Tysseling VM, Sahni V, Pashuck ET, Birch D, Hebert A, Czeisler C, et al. Self-assembling peptide amphiphile promotes plasticity of serotonergic fibers following spinal cord injury. J Neurosci Res 2010;88:3161–70.

[14] Tom VJ, Sandrow-Feinberg HR, Miller K, Domitrovich C, Bouyer J, et al. Exogenous BDNF enhances the integration of chronically injured axons that regenerate through a peripheral nerve grafted into a chondroitinase-treated spinal cord injury site. Exp Neurol 2013;239:91–100.

[15] Awad BI, Carmody MA, Steinmetz MP. Potential role of growth factors in the management of spinal cord injury. World Neurosurg 2013.

[16] Novikov L, Novikova L, Kellerth JO. Brain-derived neurotrophic factor promotes axonal regeneration and long-term survival of adult rat spinal motoneurons in vivo. Neuroscience 1997;79:765–74.

[17] Zustiak SP, Leach JB. Characterization of Protein Release from Hydrolytically Degradable Poly(ethylene glycol) Hydrogels. Biotechnol Bioeng 2011;108:197–206.

[18] Tian YF, Hudalla GA, Han H, Collier JH. Controllably degradable beta-sheet nanofibers and gels from self-assembling depsipeptides. Biomater Sci 2013;1:.

[19] Jimenez Hamann MC, Tsai EC, Tator CH, Shoichet MS. Novel intrathecal delivery system for treatment of spinal cord injury. Exp Neurol 2003;182:300–9.

[20] Frick C, Muller M, Wank U, Tropitzsch A, Kramer B, Senn P, et al. Biofunctionalized peptide-based hydrogels provide permissive scaffolds to attract neurite outgrowth from spiral ganglion neurons. Colloids Surf B Biointerfaces 2017;149:105–14.

[21] Arnold SA, Hagg T. Anti-inflammatory treatments during the chronic phase of spinal cord injury improve locomotor function in adult mice. J Neurotrauma 2011;28:1995–2002.

[22] Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 1995;12:1–21.

[23] Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. Nat Protoc 2006;1:2876–90.

[24] Pugliese R, Gelain F. Peptidic biomaterials: from self-assembling to regenerative medicine. Trends Biotechnol 2017;35:145–58.

[25] Burdick JA, Ward M, Liang E, Young MJ, Langer R. Stimulation of neurite outgrowth by neurotrophins delivered from degradable hydrogels. Biomaterials 2006;27:452–9.

[26] Gelain F, Unsworth LD, Zhang S. Slow and sustained release of active cytokines from self-assembling peptide scaffolds. J Control Release 2010;145:231–9.

[27] Hempstead BL. Brain-derived neurotrophic factor: three ligands, many actions. Trans Am Clin Climatol Assoc 2015;126:9–19.

[28] Xu XM, Guénard V, Kleitman N, Aebischer P, Bunge MB. A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into schwann cell grafts in adult rat thoracic spinal cord. Exp Neurol 1995;134:261–72.

[29] Ito K, Enomoto H. Retrograde transport of neurotrophic factor signaling: implications in neuronal development and pathogenesis. J Biochem 2016;160:77–85.

[30] Yiu G, He Z. Glial inhibition of CNS axon regeneration. Nat Rev Neurosci 2006;7:617.

[31] Fitch MT, Silver J. CNS injury, glial scars, and inflammation: inhibitory extracellular matrices and regeneration failure. Exp Neurol 2008;209:294–301.

[32] Prang P, Muller R, Eljaouhari A, Heckmann K, Kunz W, Weber T, et al. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials 2006;27:3560–9.

[33] Fon D, Zhou K, Ercole F, Fehr F, Marchesan S, Minter MR, et al. Nanofibrous scaffolds releasing a small molecule BDNF-mimetic for the re-direction of endogenous neuroblast migration in the brain. Biomaterials 2014;35:2692–712.

[34] Johnson PJ, Parker SR, Sakiyama-Elbert SE. Fibrin-based tissue engineering scaffolds enhance neural fiber sprouting and delay the accumulation of reactive astrocytes at the lesion in a subacute model of spinal cord injury. J Biomed Mater Res A 2010;92:152–63.

[35] Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, et al. Astrocyte scar formation aids central nervous system axon regeneration. Nature 2016;532:195.

[36] Plant GW, Woerly S, Harvey AR. Hydrogels containing peptide or aminosugar sequences implanted into the rat brain: influence on cellular migration and axonal growth. Exp Neurol 1997;143:287–99.

[37] Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. Dev Dyn 2000;218:213–34.

[38] Rajabi M, Firouzi M, Hassannejad Z, Haririan I, Zahedi P. Fabrication and characterization of electrospun laminin-functionalized silk fibroin/poly (ethylene oxide) nanofibrous scaffolds for peripheral nerve regeneration. Journal of biomedical materials research Part B. Appl Biomater 2018;106:1595–604.

[39] Farrukh A, Ortega F, Fan W, Marichal N, Paez JI, Berninger B, et al. Bifunctional hydrogels containing the laminin motif IKVAV promote neurogenesis. Stem Cell Rep 2017;9:1432–40.