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RESEARCH ARTICLE

Ten-eleven translocation methylcytosine dioxygenase 3-loaded microspheres penetrate neurons in vitro causing active demethylation and neurite outgrowth

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Summary

Epigenetic processes, such as DNA methylation and other chromatin modifications, are believed to be largely responsible for establishing a reduced capacity for growth in the mature nervous system. Ten-eleven translocation methylcytosine dioxygenase 3 (Tet3)-, a member of the Tet gene family, plays a crucial role in promoting injury-induced DNA demethylation and expression of regeneration-associated genes in the peripheral nervous system. Here, we encapsulate Tet3 protein within a clinically tolerated poly(lactide-co-glycolide) microsphere system. Next, we show that Tet3-loaded microspheres are internalized into mHippoE-18 embryonic hippocampal cells. We compare the outgrowth potential of Tet3 microspheres with that of commonly used nerve growth factor (NGF)-loaded microspheres in an in vitro injury model. Tet3-containing microspheres increased levels of nuclear 5-hydroxymethylcytosine indicating active demethylation and outperformed NGF-containing microspheres in measures of neurite outgrowth. Our results suggest that encapsulated demethylases may represent a novel avenue to treat nerve injuries.

KEYWORDS
DNA demethylation, microspheres, NGF protein, peripheral nervous system, regeneration

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1 | INTRODUCTION

Epigenetic processes relate to changes in gene expression that are independent of mutations or changes in DNA sequence (Duncan et al., 2014; Weinhold, 2006). Epigenetic mechanisms activate or inactivate the expression of target genes. DNA methylation as well as histone methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation are currently known as covalent epigenetic modifications (Kouzarides, 2007; Weinhold, 2006). DNA methylation is regarded as the best-known epigenetic mechanism. The addition of a methyl group (CH3) to the 5-position of cytosine occurs commonly in CpG islands and its general function is to reduce gene expression (Zhang et al., 2013). The opposite process, DNA demethylation, leads to removal of the methyl group from nucleotides in DNA. Both DNA demethylation and methylation play key roles in mammalian development, cell differentiation, as well as in cognition and neuroregeneration.

Recent studies indicate that DNA demethylation is a fundamental mechanism reprogramming the cellular state of mature mammalian neurons to permit axonal regeneration. Active DNA demethylation, which is stimulated upon axonal injury, is necessary for proper axon regeneration and may act to derepress critical pro-regenerative genes (Weng et al., 2017). The locus-specific reversal of DNA methylation is promoted by enzymes named Ten-eleven translocation methylcytosine dioxygenases (Tets) (He et al., 2011; Ito et al., 2011). Tet1, Tet2, and Tet3 have been found to iteratively oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC), respectively, allowing cell-cycle-independent removal of DNA methylation (Rasmussen & Helin, 2016). It has been shown that Tet3 protein is upregulated following peripheral nerve injury and that it is required for proper axon regeneration (Weng et al., 2017). However, the efficacy of applying endogenous Tet3 in neurite outgrowth has not been investigated.

Current approaches for nerve repair after traumatic injury commonly utilize scaffolds carrying/enriched in cells, extracellular matrix molecules, growth factors, and other signaling molecules in order to encourage nerve growth (Busuttil et al., 2017; Sensharma et al., 2017). Neural tissue engineering has identified a number of biomolecules, including proteins and their related peptides, capable of promoting nerve regeneration both in vitro and in vivo (Tian et al., 2015). Neurotrophic factors are amongst the best studied. They regulate cell division, cell survival, and neurite outgrowth; and accordingly, are essential signals for full restoration of functions (Chao et al., 2006). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4, and glial-derived neurotrophic factor are the principal neurotrophic factors used in peripheral nerve regeneration approaches. Despite being naturally released in the process of nerve outgrowth from the nerve endings, they have been isolated and applied in peripheral nerve tissue engineering approaches (Bashaw & Klein, 2010; Grinsell & Keating, 2014). These factors were used individually or in combination with the use of different delivery methods, such as dispersion in matrices, microspheres, and hydrogels.

Poly (D, L-lactide-co-glycolide) (PLGA) microspheres have previously been used in studies on the nerve regeneration potential of encapsulated NGF (de Boer et al., 2010; Kemp et al., 2011). With excellent bioavailability, low toxicity, and high biocompatibility, PLGA microsphere-based protein delivery systems have already been FDA approved for certain cancer immunotherapies. While PLGA microspheres are biodegradable and allow sustained and controlled release of bioactive molecules over extended periods of several weeks or even months, they also prevent premature degradation or metabolic turnover of the cargo molecules (Han et al., 2016). Taking advantage of the exceptional in vivo properties of the PLGA microsphere-based delivery systems, we have used this approach to fine-tune the neuronal outgrowth process by the activity of DNA demethylase Tet3.

We investigated whether Tet3-loaded microspheres improve outgrowth responses of physically damaged mHippoE-18 embryonic hippocampal cells in comparison to NGF-loaded microspheres. We initially described the conditions necessary to package recombinant proteins in microspheres. Then, we examined whether recombinant Tet3 packaged in microspheres is transported intracellularly and reaches to the nucleus. Finally, we compared 5hmC levels in control cells with of injured cells as well as with those treated with NGF- or Tet3-loaded microspheres to determine if Tet3 is capable of demethylating the nuclear genome in support of neurite outgrowth.

2 | MATERIALS AND METHODS

2.1 | Materials

PLGA (Resomer® RG 503 H, acid terminated, lactide:glycolide 50:50), dichloromethane, poly(vinyl alcohol) (PVA; 87%–89% hydrolyzed) and bovine serum albumin (lyophilized powder) were purchased from Merck KGaA. Phosphate-buffered saline (PBS; without calcium and magnesium) was acquired from CytoGen. NGF (recombinant mouse protein with an initial Met at the N-terminus) was purchased from Life Technologies. Mouse ten-eleven translocation methylcytosine dioxygenase (Tet3, recombinant protein with His-tag, host: *Escherichia coli*) was acquired from Wuhan EIAab Science Co.

2.2 | Preparation and characterization of microspheres

2.2.1 | Preparation of microspheres

PLGA microspheres were prepared by a standard double emulsion/solvent evaporation technique (Goraltchouk et al., 2006). Briefly, 150 mg of PLGA was dissolved in 1.5 ml of dichloromethane. Then, 0.15 ml of an aqueous protein solution dissolved in PBS containing

0.1% (wt/vol) BSA alone or in combination with 0.025 mg NGF or Tet3 was added to PLGA solution (Table 1). The mixture was homogenized for 1 min using a homogenizer (SONOPULS HD 2070 homogenizer; BANDELIN electronic GmbH & Co. KG) set at power: 10% and number of cycles: 6. Then, the homogenized mixture was poured to 25 ml of 1 wt.% PVA aqueous solution and homogenized for 1 min using a homogenizer set at power: 50% and number of cycles: 6. Finally, the volume was added to 25 ml of a 0.1 wt.% PVA aqueous solution and homogenized for another 1 min using a homogenizer set at power: 50% and number of cycles: six for 1 min. Then, the entire mixture was stirred for 3 h on a magnetic stirrer. After complete solvent evaporation, the microspheres were centrifuged (400*g* for 3 min at room temperature) and washed twice by centrifugation (400*g* for 3 min at room temperature) in 50 ml of sterile deionized water. Finally, the microspheres were suspended in PBS containing 0.1% (wt/vol) BSA and further diluted in culture medium (Dulbecco's modified Eagle's medium with 4500 mg/L glucose; Merck KGaA) to the concentration of 2 mg/ml (concentration determined in preliminary studies, data not shown) and used in the in vitro experiments.

2.2.2 | Imaging of microspheres

A Nikon Eclipse 50i microscope (Nikon Corporation) with a Nikon Plan Fluor 40x/0.75 Ph2 DLL WD 0.72 objective and a Fi3 digital camera was used for imaging of microspheres. NIS-Elements D (Nikon Corporation) imaging software was used to determine the average size of microspheres. At least 10 microspheres from 10 visual fields were chosen to measure their diameters for each condition. Results are presented as average ± standard deviation.

2.2.3 | Zeta potential measurements

Zeta potential of microspheres was determined using the Photon Correlation spectrometer Zetasizer Nano ZS (Malvern Panalytical Ltd.). Measurements were determined in distilled water at 25°C. The values were calculated from the Helmholtz–Smoluchowski equation (Sze et al., 2003). From 7 to 12 measurements were collected for each condition. Results are presented as average ± *SD*.

TABLE 1 The protein composition of the prepared microspheres

Sample	BSA (ml)	NGF (mg)	Tet3 (mg)
PLGA-BSA	0.15	–	–
PLGA-BSA-NGF	0.15	0.025	–
PLGA-BSA-Tet3	0.15	–	0.025

Abbreviations: BSA, bovine serum albumin; NGF, nerve growth factor; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation methylcytosine dioxygenase 3.

2.3 | Cell culture

The mHippoE-18 hippocampal cells (CELLutions Biosystems) were maintained in 25-cm^2 tissue culture flasks in DMEM (Merck KGaA) culture medium supplemented with 10% fetal bovine serum (Merck KGaA) and antibiotics: 100 U/ml penicillin with 100 µg/ml streptomycin (Merck KGaA) under standard conditions (37°C, 5% CO_2, <90% humidity). Confluent monolayers were passaged using 0.25% trypsin solution (Merck KGaA), and the obtained cell suspension was used to start new cultures or perform experiments described below. Before each experiment, cell viability and monolayer confluence were verified by 4% trypan blue (Merck KGaA) exclusion assay and observed under an inverted microscope (Motic AE2000; Motic), respectively.

2.4 | Tet3-uptake studies

In order to evaluate Tet3 uptake by mHippoE-18 cells, 1 ml of hippocampal cell suspension (4×10^5 cells/ml) was introduced into the wells of 12-well tissue culture plates containing sterile round glass coverslips (MedLab). Following overnight incubation (37°C, 5% CO_2, <90% humidity), the confluency of cell monolayers was verified and culture medium was replaced with 1 ml of DMEM containing Tet3-or BSA-loaded microspheres in a concentration of 2 mg/ml and incubated for 24 h under standard conditions. Next the glass coverslips with cell monolayers exposed to protein-loaded microspheres were washed with PBS and fixed (99.9% ice cold acetone, 7 min, −20°C) (POCH). Obtained slides were air dried to remove traces of fixative and blocked with blocking buffer (10% BSA in PBS) for 1 h at room temperature. The preparations were rinsed with PBS and incubated with a rabbit polyclonal IgG anti-mouse Tet3 antibody (1:75, 18 h, 4°C) (Biorbyt) in PBS/10% BSA. Following the incubation, the unbound primary antibodies were washed twice with PBS, and cells were incubated (1 h, room temperature) with a goat anti-rabbit IgG (H + L) F(ab')2 fragment antibody conjugated with CF™594 (5 µg/ml) (Merck KGaA). To visualize morphology, the cells were additionally stained with 0.5 µg/ml mixture of 4′,6-diamidino-2-phenylindole (DAPI; Merck KGaA) and 3,3′-dihexyloxacarbocyanine iodide (DiOC6; Merck KGaA) in PBS for 10 min. Cells were rinsed with PBS and each coverslip was carefully inverted and transferred on glass slide with mounting medium (Leica Biosystems). The confocal laser scanning microscopy platform TCS SP8 (Leica Microsystems) with the objective 63×/1.40 (HC PL APO CS2; Leica Microsystems) was used for microscopic imaging. Samples were imaged with the following wavelength values of excitation and emission: 485 and 490–590 nm for DiOC6, 405 and 430–480 nm for DAPI, 593 and 610–640 nm for CF™594 conjugated antibody. Leica Application Suite X (LAS X; Leica Microsystems) was used for cell imaging.

2.5 | Hippocampal neurite outgrowth assay

To evaluate the influence of Tet3 on neurite outgrowth of hippocampal cells, we performed the scratch assay, where 1 ml of

hippocampal cell suspension (4×10^5 cells/ml) was introduced into 12-well tissue culture plates containing sterile round glass coverslips (MedLab). Following overnight incubation (37°C, 5% CO_2, <90% humidity), mechanical disruption of cell monolayer was performed by using a sterile pipette tip to create a gap in the confluent monolayer. The in vitro lesion was verified by observation on an inverted microscope. After overnight incubation, culture medium was replaced with BSA-, NGF-, or Tet3-loaded microspheres in DMEM at concentration of 2 mg/ml (in duplicates) and cells were incubated for 24 h under standard conditions. Following the incubation, blocking and fixation steps were performed, as previously described. To visualize the outgrowing neurites, the preparations were stained with mouse monoclonal IgG1 α-Tau-1 conjugated with Alexa Fluor® 488 (1:100, 18 h, 4°C) (Merck KGaA). Following the incubation, cells were rinsed with PBS (3×), stained with 0.5 μg/ml of DAPI in PBS and the preparations on coverslips were transferred on glass slides with mounting medium. The confocal laser scanning microscopy platform TCS SP8 (Leica Microsystems) with the objective 63×/1.40 (HC PL APO CS2; Leica Microsystems) was used for microscopic imaging. Samples were imaged with the following wavelength values of excitation and emission: 405 and 430–480 nm for DAPI, 480 and 530–595 nm for Alexa Fluor® 488 conjugated antibody. Leica Application Suite X (LAS X; Leica Microsystems) was used to examine the average size of cytoplasmic protrusions.

2.6 | Quantification of global DNA methylation

2.6.1 | DNA isolation and purification

mHippoE-18 hippocampal cells were cultured and exposed to BSA-, NGF-, or Tet3-loaded microspheres as described previously in Sections 2.3 and 2.4. However, to gather optimal amount of hippocampal cell DNA for the quantification of its methylation, these experiments were performed in five repeats to receive a yield of 1×10^6 cells for each microsphere type. To remove the remaining microspheres, hippocampal monolayers were rinsed three times with PBS for 2 min each time and the cells were detached from the bottom of tissue culture plate by 0.25% trypsin treatment. The cell suspensions were transferred to individual tubes and centrifuged (400*g* for 10 min at room temperature). Cellular pellets were suspended in 200 μl of TRIS buffer and were used for DNA isolation according to the Genomic Mini DNA purification protocol (A&A Biotechnology). Briefly, samples were transferred to 2 ml DNAse free tubes, were treated with lysis buffer and proteinase K for 20 min, and an additional 5 min with 5 μl of RNAase A (10 mg/ml). Following incubation (5 min at 70°C) with TRIS elution buffer, 20 s vortexing and centrifugation (12,000 rpm), the supernatants were transferred to microcolumns and the purified DNA samples were stored at −20°C. The efficiency and purity of DNA samples were verified spectrophotometrically by Nanophotometer™ Pearl (Implen) and it was equal to 100–125 ng/μl, and A 260/280 ratio = 1.6, respectively.

2.6.2 | Global DNA hydroxymethylation assay

5hmC was determined using the MethylFlashTM Global DNA Methylation enzyme-linked immunosorbent assay (ELISA; Epigenetek), using high DNA affinity strip wells, capture and detection antibodies specific for 5hmC. The quantity of hydroxymethylated DNA fragments was detected colorimetrically at OD_λ = 450 nm. The percentages of 5hmC in DNA samples were calculated in reference to standard curve ranging from 0.02% to 1%. To calculate the percentage of 5hmC in total DNA the following formula was used:

$$5\text{-}hmC\% = (\text{sample OD} - \text{negative control OD}/\text{slope} \times 100\ ng) \times 100\%. \quad (1)$$

2.7 | Data analysis and statistics

Data were analyzed using a Kruskal–Wallis *H* test (one-way ANOVA on ranks, a nonparametric method). This test was selected upon evaluating the data for normal distributions. All statistical analyses were conducted using Statistica (data analysis software system), version 13.3 (TIBCO Software Inc.).

3 | RESULTS

3.1 | Characteristics of microspheres

Polymeric PLGA microspheres encapsulating BSA (PLGA-BSA), NGF with BSA (PLGA-BSA-NGF), or Tet3 with BSA (PLGA-BSA-Tet3) were prepared by water in oil in water method with PVA as a stabilizer at the external aqueous phase. The applied preparation technique has been widely studied (de Boer et al., 2010; Kemp et al., 2011). The main advantage of it is maintenance of bioactivity of encapsulated growth factors. Photographs of the microspheres obtained are shown in Figure 1a–c. Particles were spherical and irregularities were not or rarely observed.

Particles were dispersed in distilled water and submitted to analysis of size distribution. Only particles with diameter higher than 0.5 μm and with encapsulated protein were analyzed. Typically, optimal release profiles are achieved by using microspheres with diameters in the range of 10 ÷ 200 μm (Han et al., 2016). The average particle size was: 3.75 μm in diameter with 58% of the particles being between 2.5 and 5 μm for PLGA-BSA, 5.11 μm in diameter with 53% of the particles being between 2.5 and 5 μm for PLGA-BSA-NGF, and 5.58 μm in diameter with 42% of the particles being between 2.5 and 5 μm for PLGA-BSA-Tet3 as determined by the Imaging Software NIS-Elements D (Nikon Corporation) (Figure 1d,e). The Kruskal–Wallis test ($H(2, N = 1049) = 120.3656$, $p = 0.001$) indicated significant differences between PLGA microspheres containing different types of proteins. The following multiple comparison revealed differences between PLGA-BSA versus

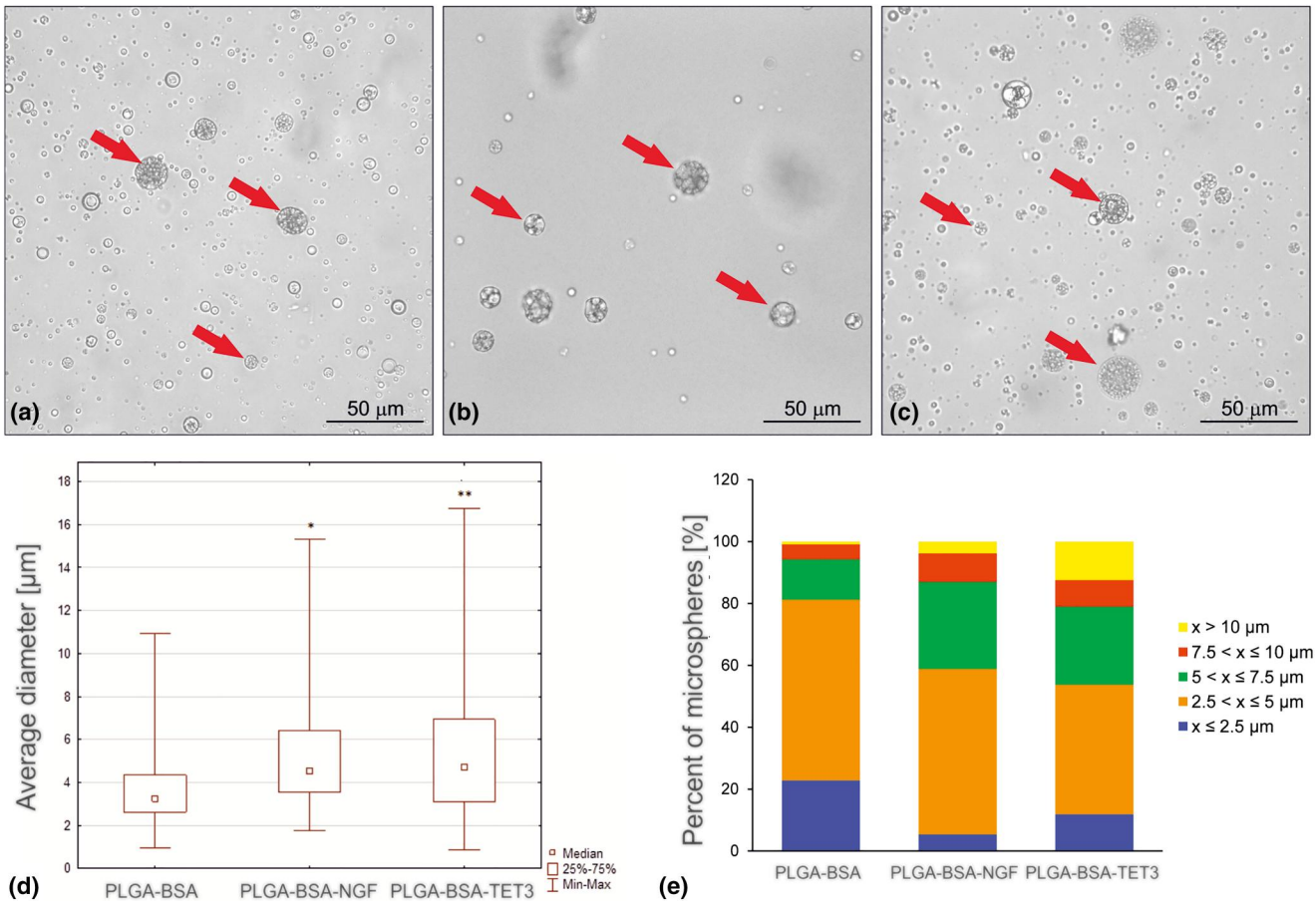

FIGURE 1 The photographs of the microspheres obtained: (a) PLGA-BSA, (b) PLGA-BSA-NGF, and (c) PLGA-BSA-Tet3. Arrows indicate exemplary microspheres containing protein, which diameters were measured. (d) Average diameter and (e) percent of microspheres obtained. * indicate significant difference $p < 0.001$ between PLGA-BSA and PLGA-BSA-NGF. ** indicate significant difference $p < 0.001$ between PLGA-BSA-NGF and PLGA-BSA-Tet3. BSA, bovine serum albumin; NGF, nerve growth factor; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation methylcytosine dioxygenase 3 [Colour figure can be viewed at wileyonlinelibrary.com]

PLGA-BSA-NGF ($p < 0.001$) and between PLGA-BSA versus PLGA-BSA-Tet3 ($p < 0.001$).

The average zeta potential was: −2.50 ± 1.18 mV for PLGA-BSA, −1.34 ± 0.93 mV for PLGA-BSA-NGF, and −1.33 ± 0.75 mV for PLGA-BSA-Tet3.

3.2 | Tet3-uptake studies

To visualize cellular uptake of PLGA-BSA-Tet3 microspheres, mouse mHippoE-18 hippocampal cells were incubated in the presence of PLGA-BSA or PLGA-BSA-Tet3 for 24 h. To detect Tet3 within hippocampal cells immunofluorescent staining was performed using IgG anti-mouse Tet3 antibody detected by fluorescently labeled α-rabbit IgG (H + L), F(ab')2 conjugated with CF™594. The labeling was performed in (a) Tet3-loaded microspheres and mHippoE-18 mouse hippocampal cells after 24 h culture (b) alone and in the presence of (c) PLGA-BSA or (d) PLGA-BSA-Tet3 (Figure 2). Confocal images demonstrated that the applied standard double emulsion/solvent evaporation technique used to prepare PLGA microspheres loaded with Tet3 protein was effective. Figure 2a,e illustrates that Tet3 protein is entrapped inside polymeric microspheres. Cells incubated alone (Figure 2b,f) or in the presence of BSA-loaded microspheres (Figure 2c,g) do not display presence of Tet3 protein. Confocal microscopy (594 nm) of cell preparations cultured in the presence of Tet3-loaded microspheres showed that PLGA-BSA-Tet3 microspheres were localized intracellularly both in cytoplasm and cell nucleus (Figure 2d,h). We have also shown that Tet3 protein appears to be transported into the nucleus via nuclear pores (arrow in Figure 2d).

3.3 | Hippocampal neurite outgrowth assay

In order to assess the regeneration potential of Tet3-loaded microspheres, we applied the scrape assay on mHippoE-18 embryonic hippocampal cells (Huebner et al., 2011). We compared the regenerative response with the Tet3 containing microspheres relative to that of those loaded with NGF—a commonly used stimulator of axon regeneration. Confocal microscopy was used in order to visualize

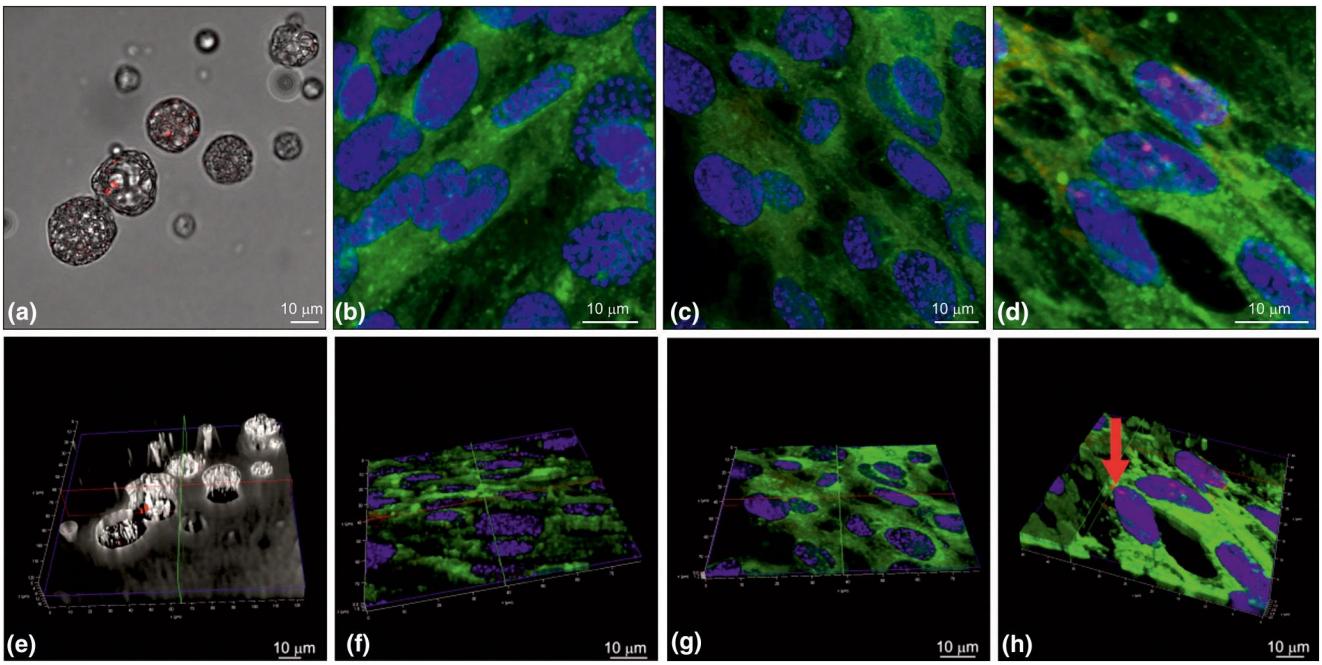

FIGURE 2 Confocal microscopy images of (a and e) Tet3-loaded microspheres (PLGA-BSA-Tet3) and mHippoE-18 mouse hippocampal cells after 24 h culture (b and f) alone and in the presence of (c and g) PLGA-BSA and (d and h) PLGA-BSA-Tet3. Merged images showing ×63 magnification snapshot and 3D view. Blue—DAPI, green—DiOC, and red—CF™594 conjugated secondary antirabbit IgG (H + L) F(ab')2 fragment antibody. Arrow indicates a microsphere spanning a putative nuclear pore. BSA, bovine serum albumin; NGF, nerve growth factor; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation 3 [Colour figure can be viewed at wileyonlinelibrary.com]

neurite outgrowth of (a) control cultures and cultures treated with (b) PLGA-BSA, (c) PLGA-BSA-NGF, and (d) PLGA-BSA-Tet3 after their scratch injury (Figure 3). Twenty-four hours after injury, the average neurite length for the control, PLGA-BSA, PLGA-BSA-NGF, and PLGA-BSA-Tet3 was 18.29, 23.20, 24.22, and 54.80 μm, respectively (Figure 4a). The Kruskal–Wallis test ($H(3, N = 224) = 101.7216$, $p = 0.001$) indicated significant differences between neurite length of mHippoE-18 embryonic hippocampal cells cultured in the varying treatment conditions ($n = 56$/condition, chosen randomly). Multiple post hoc comparisons revealed significant differences between Control versus PLGA-BSA-Tet3 ($p < 0.001$), PLGA-BSA versus PLGA-BSA-Tet3 ($p < 0.001$), and PLGA-BSA-NGF versus PLGA-BSA-Tet3 ($p < 0.001$). The most important observation was that 64% of hippocampal protrusions created by cells treated with PLGA-BSA-Tet3 remained longer than 40 μm, while the percentage of 40 μm protrusions in control cultures was equal to or below 5%. Moreover, hippocampal cells responded to PLGA-BSA-NGF with creation of 14% long protrusions ($40 < x \leq 60$ μm; Figure 4b).

3.4 | Quantification of global DNA methylation

MethylFlashTM Global DNA Methylation enzyme-linked immunosorbent assay was applied in order to determine the quantity of hydroxymethylated DNA. We compared the amount of 5hmC in (a) control cultures and cultures treated with (b) PLGA-BSA, treated with (c) PLGA-BSA-NGF and (d) treated with PLGA-BSA-Tet3 (Figure 5). Moreover, we performed this assay on uninjured cultures and to cultures with a scrape injury. The level of 5hmC in uninjured cultures was comparable for all conditions applied. Average levels were 0.033%, 0.035%, 0.032%, and 0.034% for control cultures, cultures treated with PLGA-BSA, cultures treated with PLGA-BSA-NGF, and cultures treated with PLGA-BSA-Tet3, respectively. However, in cultures with scratch injuries, we observed substantial differences between the conditions studied. The average quantity of hydroxymethylated DNA for control cultures, cultures treated with PLGA-BSA, cultures treated with PLGA-BSA-NGF, and cultures treated with PLGA-BSA-Tet3 was 0.035%, 0.038%, 0.071%, and 0.117%, respectively. The Kruskal–Wallis test ($H[3, N = 16] = 13.25{,}735$, $p = 0.0041$) indicated significant differences between the cultures. The following multiple comparison revealed differences between control versus PLGA-BSA-Tet3 ($p < 0.005$).

4 | DISCUSSION

The current understanding of nerve repair after traumatic injury supports the use of biomolecules, including proteins and their related peptides, capable of promoting nerve regeneration both in vitro and in vivo (Tian et al., 2015). NGF has been shown to provide a beneficial effect on promoting peripheral nerve regeneration (Liu et al., 2013b). It activates signaling pathways that promote not only the long-distance growth of axons but also axonal branching (Fogli

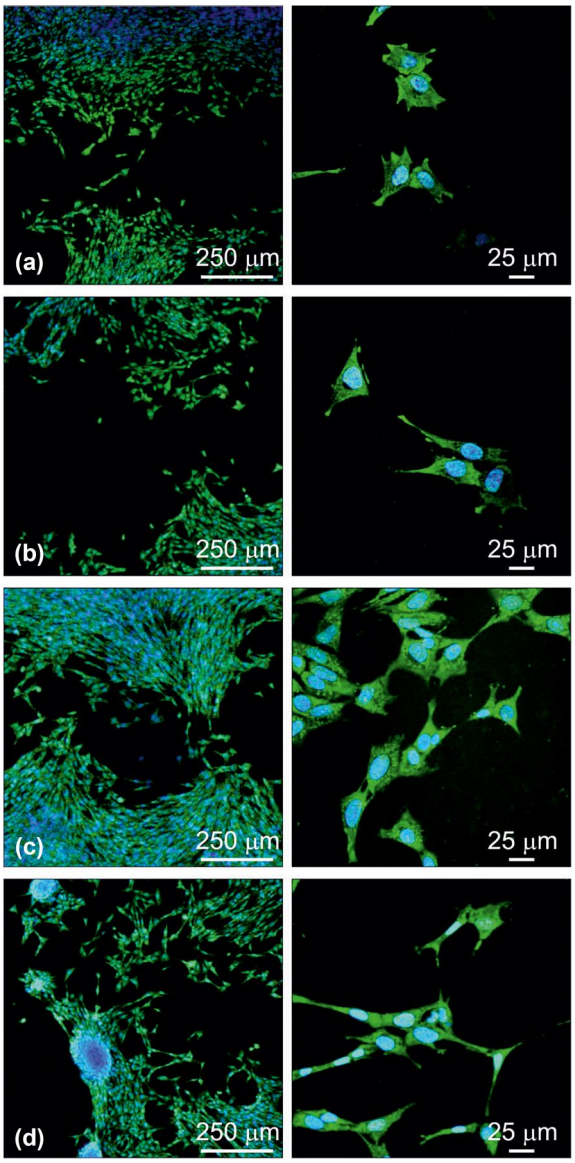

FIGURE 3 Confocal microscopy images of (a) control cultures and cultures treated with (b) PLGA-BSA, (c) PLGA-BSA-NGF, and (d) PLGA-BSA-Tet3. (Left column) Merged images showing ×10 magnification. (Right column) Images of cell layers with ×63 magnification. Blue—DAPI and green Alexa Fluor® 488 conjugated α-Tau-1 antibody. BSA, bovine serum albumin; NGF, nerve growth factor; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation methylcytosine dioxygenase 3 [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2019). Delivery of NGF to the microenvironment of nerve injury may significantly increase the morphological and/or functional recovery of injured and repaired nerves (Yildiz et al., 2011). However, it was shown that the degree of behavioral recovery following peripheral nerve injury is regulated by the duration of NGF administration (Kemp et al., 2011). NGF has a short half-life and undergoes degradation in aqueous environments at 37°C (Uebersax et al., 2007). Therefore, approaches and molecules allowing for both sustained and controlled protein release as well as protection against its degradation are needed. It was found that encapsulation of NGF in polymeric microspheres is one of the best solutions (Liu et al., 2013a). The main advantage of this technique is maintenance of bioactivity of encapsulated growth factors (de Boer et al., 2010; Kemp et al., 2011). In addition, microsphere delivery systems can be incorporated into a carrier (e.g., scaffold) allowing on localized delivery of bioactive molecules directly to the desired site. Inspired by this approach, that is, encapsulation of external stimulator of signaling pathways, we evaluated a protein responsible for globally reprograming the cellular state of mature mammalian neurons to permit neurite outgrowth at the nuclear level. We focused on Tet3 protein, a member of the Tet gene family, which plays a crucial role in promoting injury-induced DNA demethylation and the expression of regeneration-associated genes in the peripheral nervous system.

Our results provide evidence that engineered particles containing the Tet3 demethylating protein enters cells, contribute to global demethylation, and increases the length of regenerating neurites. Polymeric PLGA microspheres encapsulating BSA (PLGA-BSA), NGF with BSA (PLGA-BSA-NGF), or Tet3 with BSA (PLGA-BSA-Tet3) were prepared by water in oil in water method with PVA as a stabilizer at the external aqueous phase (Goraltchouk et al., 2006). It has been demonstrated that this method of preparation generates microspheres with diameters depending on the applied concentrations of microspheres' constituents (Adebileje et al., 2017). According to studies of Adebileje et al. (2017) the concentration of BSA has a significant but minor effect on the diameter of particles. When the concentration of either PLGA or PVA is low, increasing BSA concentration increases the particle size. However, at high values of PLGA or PVA, increasing BSA makes the particle diameter smaller. Generally, increasing PLGA in dichloromethane increases the particle size. The concentration of PVA in the external aqueous phase is regarded as a significant parameter influencing the sphere size. In general, an increase in its concentration leads to a larger particle size. One more factor that cannot be ignored is the volume ratio between the first emulsion and external aqueous phase. Increasing the volume ratio increases the sphere size.

BSA is one of the most commonly used carrier proteins for trophic factors (Liu et al., 2013a). Moreover, the applied BSA concentration of 0.1% is used in most protein release studies in order to keep proteins from aggregating, misfolding, and for absorption. Immobilization of proteins in microspheres was optimized based on the results obtained in the literature (Adebileje et al., 2017; Goraltchouk et al., 2006). The obtained average particle size was 3.75 μm for PLGA-BSA, 5.11 μm for PLGA-BSA-NGF, and 5.58 μm for PLGA-BSA-Tet3. The slight difference in diameter of PLGA-BSA-NGF and PLGA-BSA-Tet3 is potentially explained by the difference of molecular weights of the applied proteins. The molecular weight of NGF is 13.5 kDa and the Tet3 protein is 26.4 kDa.

The stabilizing agent, PVA, was the main constituent responsible for the sphere surface properties (Mura et al., 2011). As PVA is a partially hydrolyzed poly(vinyl acetate) containing vinyl alcohol and vinyl acetate monomer units, it was adsorbed at the organic phase-water interface during particle formation, leading to its entrapment

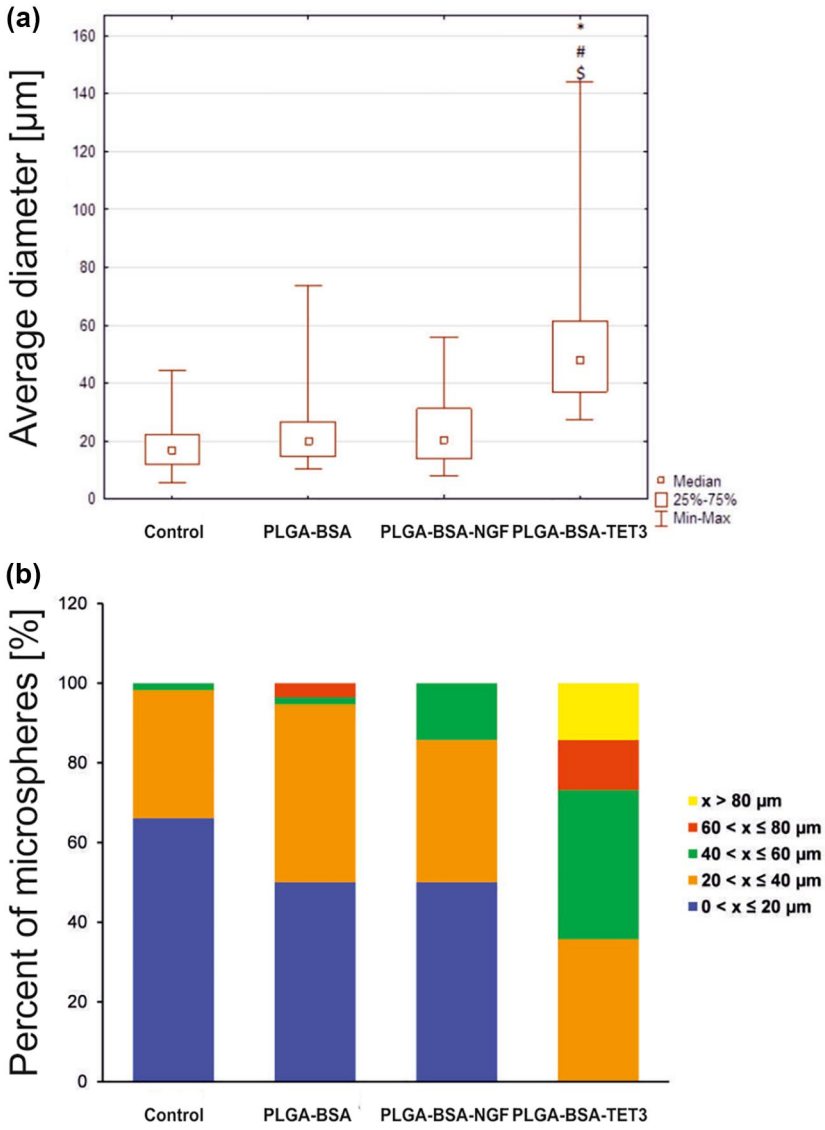

FIGURE 4 (a) Average neurite length and (b) percent of protrusion for the following cultures: Control, treated with PLGA-BSA, treated with PLGA-BSA-NGF, and treated with PLGA-BSA-Tet3. *, #, and $ indicate significant difference $p < 0.001$, $p < 0.001$, and $p < 0.001$ compared to control, PLGA-BSA, PLGA-BSA-NGF cultures, respectively. BSA, bovine serum albumin; NGF, nerve growth factor; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation methylcytosine dioxygenase 3 [Colour figure can be viewed at wileyonlinelibrary.com]

within the PLGA matrix on the sphere surface. Moreover, its particles possess high molecular weight (30–70 kDa) and therefore the layer of PVA was able to screen PLGA charges, leading to an almost neutral zeta potential values (−2.50 ± 1.18 mV for PLGA-BSA, −1.34 ± 0.93 mV for PLGA-BSA-NGF, and −1.33 ± 0.75 for PLGA-BSA-Tet3). It has been shown that the isoelectric point of BSA is 4.5–5.0 and its surface is not charged (Bronze-Uhle et al., 2016). The slight reduction in charge in PLGA-BSA-NGF and PLGA-BSA-Tet3 can be attributed to the presence of additional proteins, that is, NGF and Tet3. Surface charge of particles intended to be used in living systems determines its performance in the body, for example, interactions with cell membranes. It has been shown that near-neutral particles are not toxic in relative contrast to positively charged particles (Pillai et al., 2015).

Axonal outgrowth in the developing mammal is a complex process dictated by the interplay between activation of progenerative gene transcription programs and external stimuli (Zhou & Snider, 2006). When cells reach terminal differentiation, progenerative genes are methylated, and in consequence they experience transcriptional silencing. Thus, DNA methylation appears to protect the mammalian nervous system from widespread axonal overgrowth. It has been shown that mature neurons can reverse the transcriptional silencing of pro-generative gene in cases of injury (Mahar & Cavalli, 2018). The best example of nerve regeneration is rapid regeneration of adult dorsal root ganglion (DRG) neurons (Saijilafu & Zhou, 2012). Having a complete understanding of processes that are involved in neurite outgrowth arise not only from scientific inquisitiveness, but also is a matter of great clinical concern.

Initially, many scientists pointed at signaling molecules and their pathways as main factors involved in the initiation of axon regeneration (Saijilafu et al., 2013). Despite their substantial importance, they are only component constituents of the regeneration process. In this study, we compared outgrowth potential of commonly used NGF-loaded microspheres with that of Tet3-loaded microspheres in an in vitro injury model. The average neurite length measured 24 h after injury using confocal microscopy photographs was equal to 24.22 µm

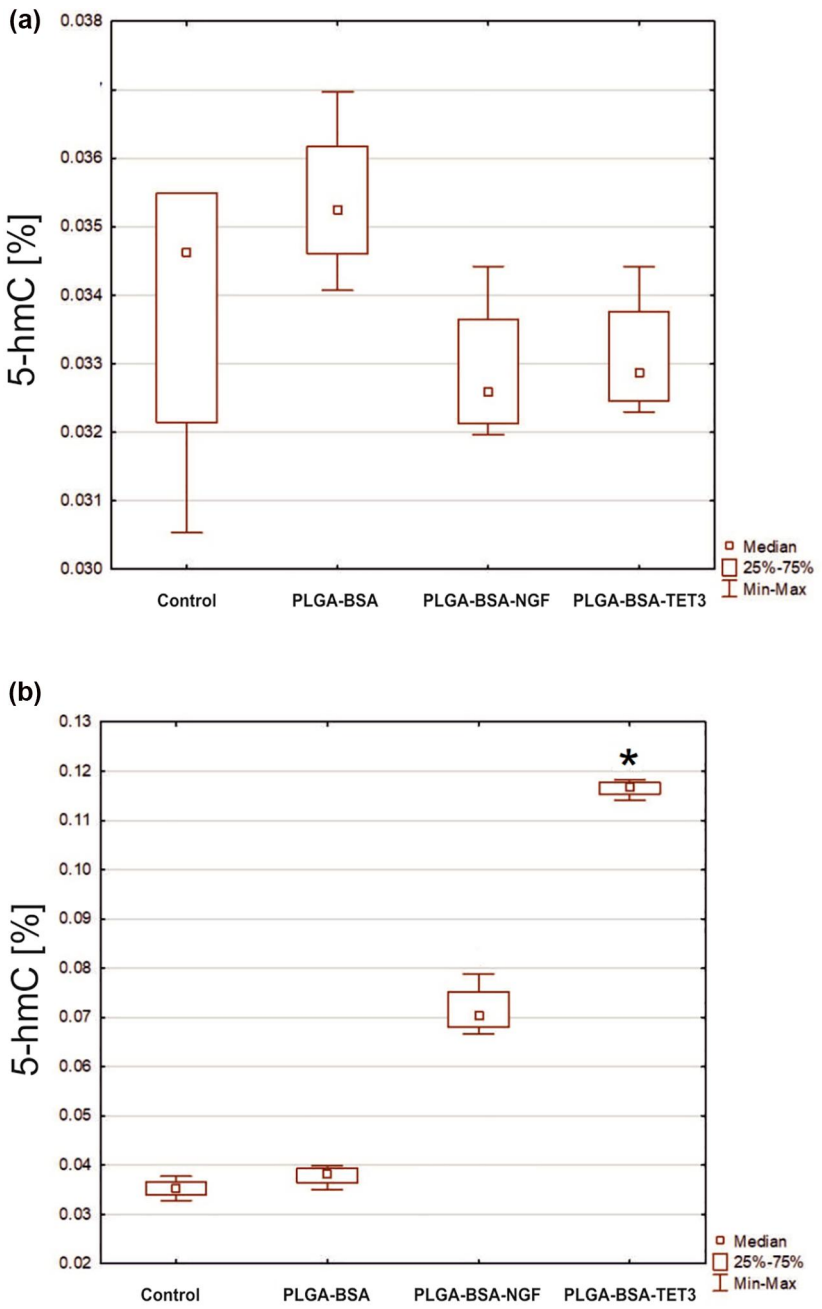

FIGURE 5 Percentage of hydroxymethylated DNA for the following cultures: Control, treated with PLGA-BSA, treated with PLGA-BSA-NGF and treated with PLGA-BSA-Tet3. (a) Uninjured and (b) cultures with scrape injury. * indicates significant difference $p < 0.05$ between PLGA-BSA-NGF and PLGA-BSA-Tet3. BSA, bovine serum albumin; NGF, nerve growth factor; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation methylcytosine dioxygenase 3 [Colour figure can be viewed at wileyonlinelibrary.com]

for PLGA-BSA-NGF and 54.80 μm for PLGA-BSA-Tet3 (Figure 4a). It was shown that the co-expression of p75NTR is essential for TrkA-mediated NGF survival signaling in cultured hippocampal neurons (Culmsee et al., 2002). However, in a study by Palmer et al. (1997), the authors demonstrate that BDNF and NT-3 increase survival of hippocampal progenitors in culture, but they did not find an effect of NGF (Palmer et al., 1997). Furthermore, delivery of neurotrophic factors does not take into account the transcriptional silencing of pro-generative gene of mature neurons. The recent discoveries in this field are focused on epigenetic processes, mainly DNA demethylation, which can be employed in reversing the reduced intrinsic growth capacity of neurons in the mature nervous system (Weng et al., 2017). Weng et al. (2017) proposed that removal of methyl groups from DNA is crucial for restoring of pro-generative gene activity. As a consequence of axonal injury, active DNA demethylation is likely activated by a retrograde calcium signaling within the nuclei of injured cells. In their study, They demonstrated that peripheral sciatic nerve lesion in adult mice leads to elevated levels of Tet3 and 5-hydroxylmethylcytosine in DRG neurons. However, DNA demethylation is not performed by Tet3 alone. Thymine-DNA glycosylase (TDG) makes use of the base excision repair pathway downstream of 5hmC to generate an unmodified cytosine, and thus completing the DNA demethylation process.

As Tet3 and TDG demethylate regeneration-associated genes ultimately increasing their expression and allowing mature neurons to regain the capacity for growth in cases of injury, we examined a

strategy to enhance outgrowth potential of nerve cells with the use of a demethylating agent (Tet3) encapsulated in polymeric microspheres. We showed that Tet3 protein entrapped in microsphere system can be taken up by cells. After their internalization, it is present either in cytoplasm and cell nucleus. We observed that Tet3 protein released from PLGA microspheres appeared to be translocated into the nucleus via nuclear pores. PLGA microspheres are known to slowly disintegrate upon contact with water-based solutes, causing gradual release of the cargo molecules. Importantly, it has previously been measured that the highest doses (around 20% for particle size 20–50 μm and 40% for smaller sized spheres, respectively) of macromolecules are being released during the initial 24 h following contact with water. However, the remaining load is freed into the medium at much slower rates, due to particle erosion. According to studies on the mouse Tet3 protein, the functional Nuclear Localization Signal (NLS) "KKRK," driving the transport of Tet3 protein to the nucleus is present in the carboxyterminal extremity of Tet3 (Xiao et al., 2013). The same study suggests that the nuclear import of Tet3 proteins from the cytoplasm is likely routed via the classical importin-α and importin-β pathway. Interestingly, the presence of NLS sequences does not only support nuclear transport but was also found to aid cargo internalization across the plasma membrane in other studies. NLS-derived peptides are exploited in cell-penetrating transport systems for macromolecule delivery (Ragin et al., 2002; Ruseska & Zimmer, 2020). While it is believed that the presence of short stretches of basic amino acids, like the NLSs, induces protein endocytosis or triggers the formation of an inverted micelle, the exact nature of this phenomenon remains elusive (Ragin et al., 2002) (Figure 6). We showed that treatment of cells with Tet3 allowed neurons to regain the capacity for growth upon injury. Thus, our data suggest that treatment of damaged nerve cells with a demethylating agent may represent a potentially promising approach to improve the therapeutic effects of protein-loaded microspheres.

The process of DNA demethylation leads to oxidation of 5mC to 5hmC. The level of 5hmC is approximately 10 times higher in various brain tissues, such as Purkinje neurons and DG in hippocampus, as well as ESCs than in other tissues. Research findings have indicated that 5hmC is acquired during embryonic and postnatal brain development and highly distributed in neuronal cells (Sun et al., 2014). During neuronal development, 5hmC not only displayed cell- and tissue-specific features but also displayed dynamic features in the genome (Hahn et al., 2013). 5hmC, 5fC, and 5caC were shown to be

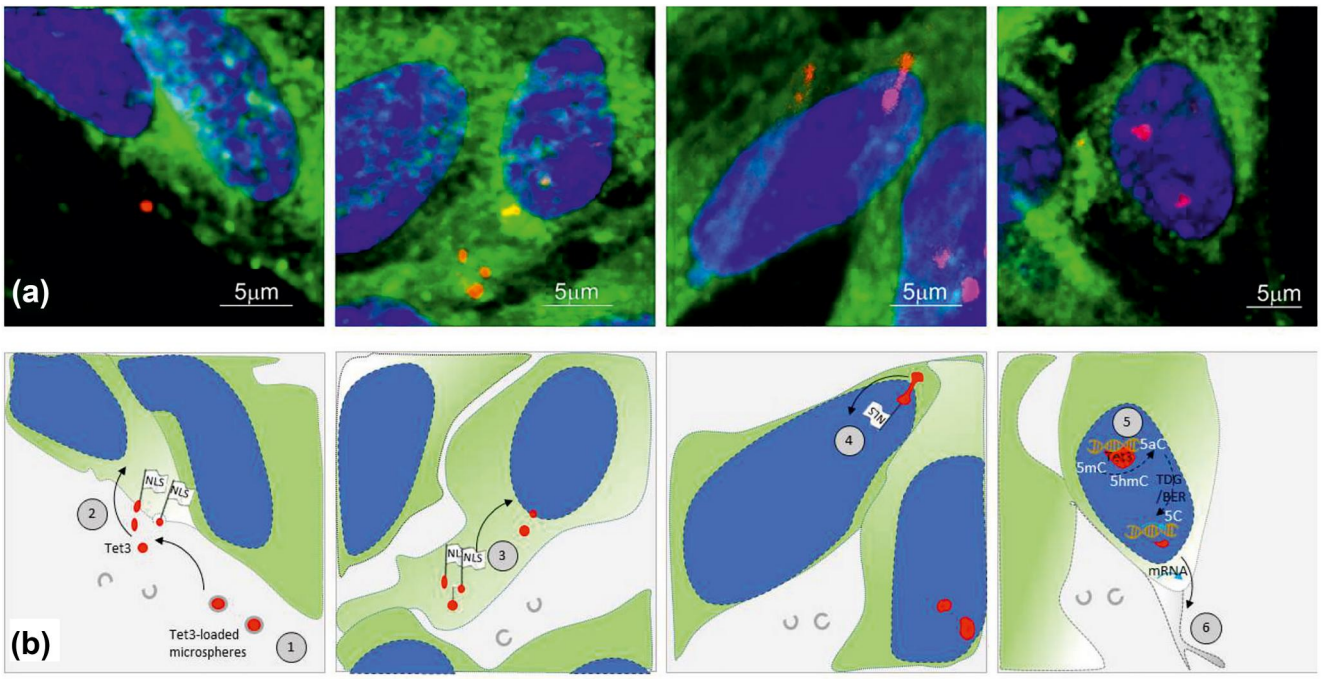

FIGURE 6 Confocal microscopy images (a) and proposed model representing possible stages of Tet3 translocation (b) for Tet3-loaded microspheres in mHippoE-18 mouse hippocampal cells after 24 h culture. Merged images showing: cytoplasm (green—DiOC), nuclei (Blue—DAPI), and Tet3 (red—CF™594 conjugated secondary antirabbit IgG (H + L) F(ab')2 fragment antibody). The release of Tet3 from the PLGA microspheres into cell culture milieu (1); short stretches of basic amino acids, like the NLSs, induce protein endocytosis or trigger the formation of an inverted micelle (2); the functional NLS (carboxyterminal extremity of Tet3) drives the transport of Tet3 protein to the nucleus (3); importin-α and importin-β pathway is likely to be involved in the nuclear import of Tet3 proteins from the cytoplasm (4); when delivered into nucleus, Tet3 facilitates DNA (5-methylcysteine) demethylation followed by generation of unmodified cytosine by TDG (5); enhanced expression of regeneration-associated genes allowing the injured neurons to restore their morphology and regenerate (6). BSA, bovine serum albumin; NGF, nerve growth factor; NLS, Nuclear Localization Signal; PLGA, poly (D, L-lactide-co-glycolide); Tet3, ten-eleven translocation methylcytosine dioxygenase 3 [Colour figure can be viewed at wileyonlinelibrary.com]

enriched in distinct genomic regions including promoters and enhancers (Szulwach et al., 2011) and to display dynamic alteration during lineage specification (Wheldon et al., 2014). Thus, dynamic and specific distribution of active DNA demethylation could contribute to the proper gene expression during neuronal development (Tao et al., 2018). In accordance with these findings, we can assume that different cells with different age may require different dosages of demethylating agents. Moreover, it was shown that Tet3 expression in brain neurons decreases significantly with age (Langie et al., 2017). Therefore, it will be interesting to test the action of the developed microspheres on different cell lineages with different ages, profile their gene transcriptional influences, and evaluate their neurogenerative capabilities in vivo.

5 | CONCLUSIONS

We have demonstrated a novel strategy of leveraging a recombinant demethylating protein, Tet3, encapsulated in polymer-based microspheres, to improve the outgrowth potential of damaged nerve cells. For clinical translation, it will be important to further characterize the optimal dose of Tet3 in order to generate the best therapeutic effects. Furthermore, our results warrant the exploration of other demethylating agents to enact of proregenerative gene transcription programs. For instance, Thymine-DNA glycosylase represents a potential candidate. Ongoing and future research should focus on agents capable of recruiting DNA demethylation mechanisms in clinical translation.

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CONFLICT OF INTERESTS

The authors have declared that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Conceptualization: Katarzyna Nawrotek, Brandon L. Pearson, and Marek Wieczorek. Investigation: Katarzyna Nawrotek, Karolina Rudnicka, Justyna Gatkowska, and Sylwia Michlewska. Methodology: Katarzyna Nawrotek, Karolina Rudnicka, Justyna Gatkowska, and Sylwia Michlewska. Supervision: Marek Wieczorek. Visualization: Katarzyna Nawrotek. Writing—original draft: Katarzyna Nawrotek. Writing—review and editing: All authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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