Particle Size of Drug Nanocarriers Defines the Fate of Spinal Cord Injury’s Recovery

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Abstract

Spinal cord injury (SCI) is a debilitating condition for which no definitive treatment has yet been identified. Noteworthy, it influences other tissues through inflammatory reactions and metabolic disturbance. Therefore, fingolimod (FTY-720) as an FDA-approved inflammatory modulator would be promising. In the present study, nanocarriers at two distinct monodisperse particle sizes of 60 (nF60) and 190 (nF190) nm were prepared. The neural stem cell (NSC) viability and LDH release were studied in the face of the nanocarriers and free FTY-720. Results indicated that nanocarriers and free FTY-720 enhanced NSC viability than the control group. However, nF190 significantly induced less cell membrane damage than nF60. Nanocarriers and free FTY-720 enhanced motor neuron recovery in SCI rats, while body weight and return to bladder reflex by nF190 was significantly higher than nF60 groups. Return to bladder reflex might be due to the role of FTY-720 in regulation of detrusor muscle tone and preservation of the integrity of vessels by acting on endothelial cells. Moreover, nF190 gained higher soleus muscle weight than the free drugs; probably decreasing pro-inflammatory cytokines in soleus diminish muscular atrophy in SCI rats. To sum thing up, larger nanacarriers with less cell membrane damage seems to be more efficient than smaller ones to manage SCI.

Introduction

Spinal cord injury (SCI) as a severe and debilitating condition leads to a variety of complications from a transient neurologic impairment to permanent motor and sensory function loss. According to WHO, the annual incidence of SCI is between 250000 and 500000 cases around the world. The advancement of technology and the convergence of research disciplines has overcome many medical obstacles. However, the development of an efficient therapeutic approach for SCI is complicated and problematic due to the extensive axonal loss at the moment of injury and neuronal regeneration restrictions of the human central nervous system. However, it seems that hydrogel-based materials such as self-assembling peptide nanofibers are of interest. The pathology of SCI is classified into primary and secondary injuries. Primary injury refers to mechanical insult to axons, blood vessels, and the cell membrane at the moment of impact, and secondary injury is the subsequent neurodegenerative processes lead to ischemia, inflammation, necrotic, apoptotic cell death, and glial scar formation. Mainly focus of SCI treatment is to counteract the cascade of secondary injury mechanisms.

Methylprednisolone, a synthetic anti-inflammatory and immunosuppressive glucocorticoid is the current gold standard for SCI patients. However, the safety and efficacy of this therapeutic agent have been subject to debate. We proposed fingolimod (FTY-720), an immunosuppressant and anti-inflammatory small molecule, to recover SCI in the present study. FTY-720, as a nonselective sphingosine-1-phosphate (S1P) receptor modulator, is the first oral approved medication for relapsing-remitting multiple sclerosis (RRMS). It decreases the rate of relapse in RRMS patients by one-half. There is increasing evidence that FTY-720 may be helpful for neuronal reconstruction and attenuating SCI. Noteworthy, it has been disclosed that traumatic damage resulting in release of neural self-antigens, therefore,
immunomodulatory drugs to modulate immunological reaction following trauma is of interest. In one study, Efstathopoulos et al. showed FTY-720 capability of inducing adult mouse hippocampal neurogenesis. In another study, PLGA/FTY-720 microfibers stimulated NSCs differentiation into neurons and oligodendrocytes and suppressed astrocytosis. A number of studies have reported that FTY-720 decreases T-cell infiltration and gliosis and enhances motor function recovery in SCI rat model. Since S1P receptors express in various kinds of cells, this S1P analog could affect different aspects of the secondary injury process. Norimatsu et al. proposed the immune-independent potential capability of this medicine on tissue preservation and functional recovery after SCI. Likewise, Colomb et al. suggest that FTY-720 inhibits inflammation and glial scar formation through the blockade of NF-kB translocation. However, implantation of electrospun PLGA/FTY720 microfibers to a transaction rat model manifested increased functional recovery outcomes.

Medical nanotechnology strategies have provided significant breakthroughs over the past several decades. Nanoparticle formulations have been successfully employed to treat and ameliorate many diseases and disorders, including advanced cancers, diabetes, HIV, cardiovascular disease, etc. Large surface area to mass ratio, enhanced bioavailability, prolonged retention, reduced toxicity, increased therapeutic efficacy, and biocompatibility is some benefits of nano-drug systems as an alternative to conventional drugs. Nanoemulsions are heterogeneous colloidal dispersions consisted of a mixture of two immiscible liquids stabilized by amphiphilic surfactant. These lipid-based formulations are considered a kinetically stable system and can be prepared by employing low and high-energy methods. Drug delivery with nanoemulsions is a simple yet effective method for encapsulating hydrophobic drugs.

Despite several investigations on the potential of nanocarriers for SCI recovery, we do not know which particle size would be effective to recover spinal cord injury. These data help pharmaceutics companies to develop more efficient drug formulation to recover SCI. The present study responds to whether FTY-720 nanoemulsions enhance therapeutic efficacy compared to its bulk counterpart in a contusion SCI rat model. Meanwhile, it defines which particle size is more efficient for SCI recovery.

Results And Discussion Section

Nanocarrier characteristics

One of the most challenging issues in pharmaceutics is defining an efficient particle size to recover SCI. Recent decades have witnessed an explosive growth in the design and delivery of nanomedicines. In this regard, FTY-nanocarriers at two distinct particle sizes were prepared and compared, and their therapeutic efficacy was investigated in an SCI rat model. We assumed that encapsulation of FTY-720 into a nanoemulsion drug delivery system probably promotes neuronal survival and functional reconstruction of this powerful immunomodulatory medicine.
DLS was performed to study particle size and Zeta potential of stable nanocarriers. DLS results showed that nanocarriers synthesized by stirring and ultrasound methods had particle sizes of 60 and 195.5 ±7.78 nm, respectively (P <0.001). Meanwhile, they showed that monodisperse nF60 and nF190 nanocarriers had the PDI value of 0.1 and 0.2, respectively (Fig. 1a and b). In other words, low energy emulsification method produced larger nanocarriers compared to high-energy methods of ultrasound. However, both methods at an optimized oil, surfactant, and co-surfactant ratio make monodisperse nanoemulsions.

ζ-potential data related to nF60 and nF190 showed a slightly negative surface charge. ζ-potential of nF60 and nF190 nanocarriers were -9.98 ± 0.15 mV and -10.58 ± 0.35 mV, respectively. Their ζ-potential had significantly difference together (p = 0.0198). Smaller nanocarriers showed less negative ζ-potential than larger nanocarriers. However, both showed ζ-potential around -10 mV.

SEM micrographs revealed that both nF60 and nF190 nanocarriers had spherical morphology with uniform size and homogeneous distribution without agglomeration (Fig. 1c and d). Environmental conditions such as temperature, ionic strength influence the curvature of apolar spontaneous nanoemulsion. It might be said that the heating produced by the ultrasound method reduces the particle size of nanocarriers with more homogeneous particles (PDI: 0.1) through decreasing viscosity and interfacial tension between oil and water phases. However, cooling following the high energy method reduces particles' velocity and enhances oil phase viscosity.

NSC viability and LDH release

NSCs viability was evaluated in the face to the nanocarriers and FTY-720 at 10 ng/ml concentration. Results indicated that there was no significant difference between cell viability of nanocarriers together and compared to the bulk drug (P > 0.05). However, the control group had significantly less cell viability than the nanocarriers and bulk FTY-720 (P< 0.001). Nevertheless, both nanoemulsions manifested higher cell viability as compared to the control group. It seems that FTY-720 can significantly enhance NSCs viability and may be considered as a promising drug for neurogenesis (Fig.2a).

There is just one report, belonged to our group, associated with the dependency of neuro-toxicity to drug nanocarriers' particle size. There are some other reports related to the effect of nanocarriers on different cell types, such as fibroblast. For example, mesoporous silica NPs with a particle size of 250 nm induced higher endothelial toxicity than 30 nm NPs in part through the mitophagy mechanism. Moreover, Tavakol et al. reported that larger curcumin nanocarriers induce higher fibroblast cell viability than smaller nanocarriers with a particle size of approximately 60 nm through the down expression of Bax and NFκB genes.

Earlier, we showed that small nanocarriers exhibited higher cellular viability than larger nanocarriers in neural BE(2)-M17 cells. This data is contrary to our recent results and is due to different cell types. Therefore, to neurotoxicity investigation, the cell type is critical, and we cannot refer an outcome to other cell types. Although nanocarriers with a particle size of 60 nm may be considered a candidate particle
size in dopaminergic cells, larger nanocarriers of 190 nm will act as the preferred particle size for SCI recovery. Besides, it has been shown that silica NPs at the range of 200 nm exhibits significantly higher neuronal viability than small NPs (50 nm) through the calcium perturbation and apoptosis mechanisms. However, in accordance with our results, Prabhu et al. demonstrated that copper NPs with particle sizes of 40, 60, and 80 nm did not induce a significant impact on the cell viability of DRG neurons. Besides, Coelho et al. reported the anti-apoptosis mechanism of FTY-720 to enhance oligodendrocytes viability. This data was similar to our study in that FTY-720 had increased neural cell viability compared to the control group.

To further study the effect of nanocarriers and bulk FTY-720 on NSCs, LDH release as a marker of necrosis and cell membrane damage was investigated. Results showed that smaller nanocarriers exhibit more serious cell membrane damage to NSCs and produce higher LDH release than the larger nanocarrier (P< 0.01). At the same time, there was no significant difference between other groups (P> 0.05). In other words, it seems that larger nanocarriers induced minor NSCs membrane damage, while there was no significant difference between cell membrane damage of smaller nanocarriers and bulk FTY-720 (P> 0.05) (Fig.2b). To sum things up, it seems that although both nanocarriers induced significantly high cell viability in NSCs, smaller nanocarriers caused higher NSCs membrane damage compared to the larger nanocarrier with 190 nm particle size. Prabhu et al. disclosed that small and large copper NPs of 40 and 80 nm did not significantly induce LDH release from DRG neurons, and notably, these NPs significantly induced higher LDH release compared to the control group. Moreover, Gillespie et al. reported that although both fine and ultrafine particles induced apoptosis in the neural cell, ultrafine particles induced more apoptosis of neural cells than fine particles. This finding was in good agreement with our results that larger nanocarriers induced less cell membrane damage of NSCs.

**BBB score**

Nanocarriers and FTY-720 were directly injected into the lesion to investigate their effects on the SCI model. In vivo SCI models were exerted on Wistar male rats employing the weight drop contusion model. Blunt injury models, including weight drop, represent human injuries and can efficiently study secondary damages. Herein we choose local administration to transport the medicine across the blood-spinal cord barrier directly to the lesion area. Local delivery significantly decreases systemic administration side-effects and effectively eliminates the risk of potential exposure and toxicities within the non-targeted organs.

To assess motor function recovery, BBB open-field locomotor rating scales were carried out in contusion rat models received local FTY- nanocarriers and bulk drug during six weeks post-injury (Fig. 2c). In the first four weeks, no significant difference was detected among different groups. However, from week four, nanocarriers and bulk FTY-720 started to show a gradual rise in BBB score compared to the control group. Finally, bulk FTY720 and nanocarriers induced an improved motor hind limb function compared to the control group (P< 0.001). There was no significant difference between the motor neuron recovery of nanocarriers and free FTY-720 (P> 0.05). These findings were in accordance with MTT assay data. It
seems that NSC viability has a direct impact on the potential of nanocarriers to enhance motor neuron recovery. To further investigate the effect of nanocarriers on SCI rats, bladder reflux, body and muscle weights were evaluated.

**Return of bladder reflex**

Impaired bladder function is another incapacitating consequence of SCI in humans and animals. The degree of SCI severity is correlated with bladder dysfunction. In other words, the return of bladder reflex was considered as a sign of improvement and recovery. As shown in Fig. 3a, nF190 nanocarriers exhibited a noticeable faster regain of spontaneous bladder function compared to nF60 (P<0.01), free drug (P< 0.05), and the control group (P<0.001). There was no significant difference between the return of bladder reflex in nF60 and free drug (P> 0.05). Based on these results, although larger nanocarriers could enhance motor neuron recovery at the score of nF60 and free drug, they positively impacted bladder reflex, which is very important in SCI patients. Kangmin et al. demonstrated that daily IP injections of FTY-720 for four weeks following a contusion model in a Long-Evans hooded rat model significantly enhanced functional outcomes and bladder recovery. Moreover, it has been shown that FTY-720 regulates detrusor muscle tone and preserves the integrity of vessels by acting on endothelial cells.

**Bodyweight changes**

Bodyweight changes were recorded weekly as an indicator of general health and recovery. The weight of animals was recorded each week until the sacrifice (Fig. 3b). There was a marked decline in all groups' body weight in the first week after surgery. All animals' weight was increased upon the time, as expected. However, this increment trend was more significant in nanocarrier treated groups (P< 0.001). By the time, the body weight of rats treated by nanocarriers was significantly enhanced compared to the control group and free drug on four weeks. nF190 induced significantly higher body weight than the nF60 on 42 days post-treatment (p< 0.001).

Meanwhile, nF60 significantly influenced higher bodyweight than the free drug (P< 0.001). Based on these findings, it seems that although there was no significant difference on motor neuron recovery of nanocarriers and free drugs, gained bodyweight and bladder reflux as the markers of health and recovery in rats have been enhanced using nF190 and nF60. Eventually, the return of bladder reflex in SCI rats has positively impacted bodyweight and general recovery.

**Gastrocnemius and soleus muscle mass**

Skeletal muscle as an endocrine organ is associated with inflammation. Therefore, inflammation is leading to muscle atrophy and reduced satellite cells. Muscle atrophy leads to metabolic disorders in SCI patients. In this study, gastrocnemius and soleus muscle were dissected and weighed after perfusion on the 42nd day. Results showed no significant difference between the gastrocnemius weights of the groups (P= 0.0982). There is some difference between the two types of gastrocnemius and soleus muscles in which gastrocnemius is predominantly glycolytic muscle while soleus is more largely slow-
twitch muscle. At the same time, soleus muscle mass was higher in nF190 (p<0.01) and nF60 (p<0.05) compared to the control group. nF190 showed significantly higher soleus weight compared to the free drug (p<0.05) (Fig.3c). Based on these findings, it might be said that larger nanocarriers can diminish the soleus muscle atrophy. Ormond et al. showed greater soleus muscle weight is correlated with better hind limb functional recovery. Waterson et al. showed a regulatory effect of FTY720 on detrusor muscle tone in a rabbit model. Graham et al. showed that SCI induces the up-regulation of IL-6, TNFα, and p53 in soleus muscle while IL-6 and TNFα return to baseline in a short period. However, the return of inflammatory cytokine to baseline happens in rodent, and they cannot undergo senescence and may not translate to humans. In other words, in human may inflammatory cytokines following SCI promotes muscle to cellular senescence. Since FTY-720 reduces the up-regulation of pro-inflammatory cytokines such as IL-17A, IL-1, IL-6, and TNFα, therefore, nF190 has diminished soleus muscular atrophy in SCI rat.

Histological evaluation

Histological evaluation was performed using H&E staining. As demonstrated in Figure 4, a scar was made in the spinal cord adjacent to T9 spin in this model. In the control group, the cavity was significantly larger and more extended outside than other groups on 42nd-day post-treatment. At the same time, the cavity had a smaller size in nF190. Since a severe model was induced in the spinal cord, the astroglial scar could not completely recover in all groups, and fibroconnective tissue is observed in the cavity of all groups. However, nanocarriers showed less cavity size compared to others. It seems that nanocarriers help the astroglial scare to be reconstructed.

Analyzing functional recovery of SCI rats treated with free FTY-720, nF60, and nF190 indicated that nF190 has remarkably improved outcomes faster regain of bladder reflux, a gain of body weight, and soleus muscles compared to the free FTY-720, and in some parts more than smaller nanocarriers. Kong et al. discovered that PLGA microfibers containing FTY-720 and NSCs reduce the glial scar cavity size and suppress astrocyte differentiation and induce NSCs differentiation into neurons oligodendrocyte, which is critical for axonal reconstruction. In vivo results of that study pointed out the remarkable efficacy of FTY-720 loaded into electrospun fibers on motor recovery in a spinal cord transection rat model. Moreover, Norimatsu et al. demonstrated that FTY-720 has a more extensive activity of FTY-720 than being S1P1 receptor antagonist. They declared that permanent internalization of the S1P1 receptor in astrocytes through functional antagonism is probably a primary function for FTY-720 efficacy. Although FTY-720 declined the migration of lymphocytes to the site of SCI, it cannot diminish the infiltrated granulocytes and glial cells. Cytokines and other biomolecules released by these cells are in part responsible for the neurotoxicity of SCI. However, microglial scavenger debris and inhibitory biomolecules in SCI help recover injured neurons through axon regeneration signaling. Therefore, it seems that SCI recovery following SCI is in part through T cell attenuating.
In conclusion, the high energy method using the ultrasonicator and the low energy method using the magnetic stirrer was employed to synthesize nanoemulsions of 60 and 190 nm particle size, respectively. Particle size was analyzed using SEM and DLS. Nanocarriers were biocompatible and exhibited higher cell viability compared to the control group. Local delivery of FTY-720 nanoemulsion after contusion model in an SCI rat model significantly improved hind limb motor function recovery. Collectively, our data demonstrated that nF190 provides us with neuroprotective and neuroregenerative properties. This synthetic nanocarrier not only could impede further damages but also helps to improve motor dysfunction and recovery. Nanocarriers at the particle size of 190 nm through enhanced cellular uptake positively impact bladder reflex, bodyweight, and muscular weight. Eventually, smaller nanocarriers through enhanced cell membrane damage than the nF190 exhibit less beneficiary effect than nF60 in SCI rats. We suggest that the nanocarriers are studied in a moderate SCI model to significantly shown the impact of particle size on motor neuron recovery.

Methods

Preparation of fingolimod-loaded nanoemulsion

FTY720 nanocarriers at two different particle sizes with an equal concentration of FTY-720, oil, surfactant, and co-surfactant were prepared via a low (stirring) and high-energy (probe ultrasound) emulsion oil in water (O/W) methods. In brief, the mixture of 1 mg/ml fingolimod, 2% (w/w) mineral oil, 6% (w/w) Span 80 -Tween 80 (surfactant), 1% (w/w) ethanol (co-surfactant), and distilled water were exposed to either magnetic stirrer (800 rpm, 10 min), and or ultrasound probe (400 W s⁻¹, 4 min) to obtain 190 nm and 65 nm Oil-in-water (o/w) fingolimod nanoemulsion. Nanoemulsions were then stored in sealed glass vials covered with aluminum foil for further investigation. In this paper, NF60 and NF190 will be referred to as fingolimod nanoemulsion with 65 nm and 190 nm particle sizes, respectively.

Nanoemulsion characterization

Particle size and Zeta potential

The hydrodynamic particle size and Zeta potential of nanocarriers were studied using dynamic light scattering (DLS) apparatus (Scatteroscope I, Qudix, South Korea and Malvern Instrument Ltd, UK) at the refractive index of 1.531 and neutral pH to avoid the impact of the false charge on Zeta potential. The assay was triplicate performed, and mean ± SD was reported.

Morphology analysis using a scanning electron microscope (SEM)

Surface morphology, shape, and size of nanoparticles were evaluated using SEM (KYKY-EM3200, China). Before imaging, one droplet of nanocarriers was mounted on a glass slide and dried. Then samples were coated with gold, and SEM was recorded at 20 kV.

Cell viability assay
Neural stem cells (NSCs) were isolated from the subventricular zone (SVZ) of the lateral ventricles and seeded in NPBM as a cell culture medium. To evaluate cell viability of nanocarriers and bulk drug, an MTT assay was performed. NSC was seeded in a 96-well plate (10⁴ cells/well) for 24 h, and then FTY-720 and FTY-nanocarriers were added to NCSs at the concentration of 10 ng/ml for 48 h. Cell media was removed, and 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium Bromide (Sigma, USA) (0.5 mg ml⁻¹ PBS) was added to the triplicate wells at 37 °C in 5% CO₂ and 95% moisture for 4 h. DMSO was added, and after 20 min, absorbance was read using Elisa reader (Bio-Teck) at 570 nm. The assay was triplicate repeated three times, and mean ± SD was reported.

**Lactate dehydrogenase release (LDH)**

Isolated NSCs were treated with FTY-720, nF190, nF60, and the control group. After 24 h, 100 µl of cell supernatant was transferred to a 96-well plate and mixed equally with an LDH solution kit (Roche). After 20 min, absorbance was read using Elisa reader (Bio-Teck) at 490 nm. The assay was triplicate repeated three times, and mean ± SD was reported.

**Severe SCI contusion model**

Iranian Ethical Committee of Iran University of Medical Sciences approved all of the experiment protocols and animals used in this study (IR.IUMS.REC.1399.087). All procedures were performed in accordance with the Iranian Ethical Committee for animal care and use. The study was carried out in compliance with the ARRIVE guidelines. All experimental procedures were performed in accordance with the relevant guidelines and regulations. Acute severe SCI was induced in 24 male Wistar rats (220–250 g) using the weight compression method, 6 rats in each group [31]. Rats were randomly and equally divided into four groups, including FTY-720, nF190, nF60 and the control (PBS) groups. Animals were intramuscularly (IM) anesthetized by ketamine/xylazine (80 mg/kg and 10 mg/kg). Complete thoracic laminectomy was performed at T9 level, and exposed spinal cord was subjected to contusion impactor, and a severe SCI model was induced. Following the injury, 10 µl of the nanocarriers, FTY-720, and PBS at the concentration of 10 ng/ml were injected using a 26 gage Hamilton syringe at the speed of 1µl/min at the lesion epicenter. The muscles and skin were sutured, and animals were kept on a heating pat until they awoke. Animals were monitored constantly and maintained on free access to water and food. They daily received antibiotic (5% Gentamicin) for five days post-surgery.

**Behavioral Analysis**

Basso Beattie Bresnahan (BBB) locomotor open-field locomotor test was carried out to examine hind limb stepping movements at days 1, 7, 14, 21, 28, 35, and 42 post-injury. The rat was placed in a Plexiglas circular apparatus (107 cm diameter, 60 cm wall height) with a nonslip floor. BBB Score assessment was performed by a portable camera for 4 min and evaluated by two blinded independent observers, and averaged from both hind limbs. The scores were ranging from 0 to complete paralysis and 21 to normal locomotion.
**Return of bladder reflex**

After SCI, the sacral micturition center may send signals to the bladder to squeeze. Bladder emptying was done twice a day manually until regaining the reflex function. Regain of spontaneous bladder function was regarded as a measure of recovery. Mean ± SD was reported for four groups.

**Weight gain evaluation**

Weight gain is a marker of recovery in SCI. Animals was weighed before surgery and at days 1, 7, 14, 21, 28, 35, and 42 post-injury. Then, weight gain was compared to the first day. Mean ± SD was reported for four groups.

**The weighting of gastrocnemius and soleus muscle**

Soleus and gastrocnemius muscles are essential muscles in walking and running. Muscle atrophy is adjacent to less movement. Therefore, in this study, the weight of soleus and gastrocnemius muscles was measured in rats on 42nd post-surgery. In order to dissect the soleus muscle, the skin was detached, and the Achilles’ tendon was removed. After lifting the gastrocnemius muscle, the underlying soleus muscle was exposed, and then both muscles were sectioned for weighting. Mean ± SD was reported for four groups.

**Histological assessments**

On day 42 post-injury, rats were perfused transcardially with PBS and fixed by 4% paraformaldehyde in 0.1 M PBS. Spinal cords were isolated and transverse sections with a thickness of 5 μm were obtained from the paraffin-embedded spinal cords. Sample slides were stained with Hematoxylin and Eosin (H&E) and covered with coverslips. A camera captured images at the resolution of 40X.

**Statistical Analysis**

Graphpad Instat software version 3 was applied to analyze particle mean size, zeta potentials, cell viability, body weighting, muscle weighting, and BBB scores. Data were reported as the mean ±SD. One-Way ANOVA (analysis of variance) was used for statistical analysis. The significance level was set at a p-value less than 0.05.

**Declarations**

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**Author Information.**
S. T designed and supervised the research and performed in-vitro investigation. D. P and B. R S performed in-vivo investigation. F. A performed NSCs isolation. Hani Tavakol performed statistical analysis. S M. R helps to write a discussion.

**Competing interests**

The authors declare no competing interests.

**Data availability**

The data will be available upon request.

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Figures
Characterization of nanocarriers using (a, b) DLS and (c, d) SEM. DLS graph of (a) ~190 nm and (b) 60 nm particles, and SEM micrographs of (c) ~190 nm and (d) 60 nm particles showed that the nanocarriers with uniform size were dispersed in water.
Figure 2

a) Cell viability percentage of NSCs treated by nF60, nF190, bulk FTY-720 at 10 ng/ml concentrations by 48 h. The control group showed significantly less NSCs viability compared to other groups. b) LDH release from NSCs treated by nF60, nF190, bulk FTY-720 at 10 ng/ml concentrations by 24 h. nF190 showed significantly less LDH release from NSCs compared to nF60. c) BBB rating scale in severe SCI model received NF60, NF190, bulk fingolimod, and PBS (control) at days 1, 7, 14, 28, 35, and 42 post-injury by two blinded observers. NF190 showed a significantly higher functional recovery score compared to other groups (*P<0.05, **P<0.01, ***P<0.001). * means P<0.05, ** means P<0.01 and *** means P<0.001.
Figure 3

a) The first day of regaining normal bladder function at SCI rats received nF60, nF190, free FTY-720, and PBS to the lesion area. All animals restored normal bladder expression within 10 days. nF190 treated rats were the first group to regain spontaneous bladder function. 3b) Monitoring bodyweight of SCI rats for 42 days post-injury. In the first week following SCI, all groups experienced a considerable weight loss. Animals received nF190 showed promoted body weight gain on day 42. 3c) Soleus and gastrocnemius
muscles have been weighted in severe model SCI rats treated with nF60, nF190, free FTY-720, and PBS after perfusion. nF190 showed improved soleus muscle weight compared with free drug and the control groups. (* means P<0.05, ** means P<0.01, *** means P<0.001).

Figure 4

Histological H&E staining of injured spinal cords in rats on 42 days. As shown in the captured image, the cavity could not completely recover in all groups, and nF190 has shown a stronger recovery effect than the other groups (X40).