

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/116045/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Qiao, Shupe, Liu, Yi, Han, Fengtong, Guo, Mian, Hou, Xiaolu, Ye, Kangruo, Deng, Shuai, Shen, Yijun, Zhao, Yufang, Wei, Haiying, Song, Bing ORCID: <https://orcid.org/0000-0001-9356-2333>, Yao, Lifan and Tian, Weiming 2018. An intelligent neural stem cell delivery system for neurodegenerative diseases treatment. *Advanced Healthcare Materials* 7 (12) , 1800080. 10.1002/adhm.201800080 file

Publishers page: <http://dx.doi.org/10.1002/adhm.201800080>
<<http://dx.doi.org/10.1002/adhm.201800080>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



An intelligent neural stem cell delivery system for neurodegenerative diseases treatment

*Shupeiqia¹, Yili¹, Fengtong Han, Mian Guo, Xiaolu Hou, Kangruo Ye, Shuai Deng,
Yijun Shen, Yufang Zhao, Haiying Wei, Bing Song, Lifen Yao^{*}, Weiming Tian^{*}*

S. Qiao, Dr. F. Tong, Dr. X. Hou, Dr. K. Ye, Dr. S. Deng, Dr. Y. Shen, Dr. Y. Zhao,
Prof. W. Tian

School of Life Science and Technology
Harbin Institute of Technology
Harbin 150080, P. R. China
Email: tianweiming@hit.edu.cn

Prof. L. Yao
Department of Neurology
The First Affiliated Hospital of Harbin Medical University
Harbin 150080, P. R. China
Email: yaolifen_2015@sina.com

Prof. B Song
Cardiff Institute of Tissue Engineering and Repair,
School of Dentistry,
College of Biomedical and Life Sciences,
Cardiff University,
Cardiff, UK

Dr. Y. Liu
Key Laboratory of Bio-Medical Diagnostics
Suzhou Institute of Biomedical Engineering and Technology
Chinese Academy of Sciences
Suzhou 215163, P. R. China

Prof. M. Guo
Department of Neurosurgery
The Second Affiliated Hospital of Harbin Medical University
Harbin 150080, P. R. China

Prof. H. Wei
Department of ophthalmology
The First Affiliated Hospital of Harbin Medical University
Harbin 150080, P. R. China

¹Equal contribution.

^{*}Corresponding author

Keywords: neural stem cell, intelligent, alginate hydrogel, neurological disorders,
Cripto-1

Transplanted stem cells constitute a new therapeutic strategy for the treatment of neurological disorders. Emerging evidence indicates that a negative microenvironment, particularly one characterized by the acute inflammation/immune response caused by physical injuries or transplanted stem cells, severely impacts the survival of transplanted stem cells. In our study, to avoid the influence of the increased inflammation following physical injuries, an intelligent, double-layer, alginate hydrogel system was designed. This system fosters the MMP secreted by transplanted stem cell reactions with MMP peptide grafted on the inner layer and destroys the structure of the inner hydrogel layer during the inflammatory storm. Meanwhile, the optimum concentration of the RGD peptide (which reportedly promotes stem cell proliferation and adhesion) is also immobilized to the inner hydrogels to obtain more stem cells before arriving to the outer hydrogel layer. We found that blocking Cripto-1, which promotes embryonic stem cell differentiation to dopamine neurons, also accelerates this process in neural stem cells. More interesting is the fact that neural stem cell differentiation can be conducted in astrocyte-differentiation medium without other treatments. In addition, the system can be adjusted according to the different parameters of transplanted stem cells and can expand on the clinical application of stem cells in the treatment of this neurological disorder.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder caused by both the

degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and reduced DA levels in the striatum [1]. PD is an age-related disease that affects 2% of those aged 65 years and older, with 7–10 million individuals affected worldwide [2]. Patients with PD usually exhibit symptoms of resting tremor, bradykinesia, rigidity, postural instability and low dopamine level in the hypothalamus. The lack of dopamine will affect cognition and with the progression of the disease they gradually lose their self-care ability [3-5]. At present, pharmacological treatments only provide symptomatic relief with no abatement or reversal of disease progression [6, 7]. Stem cell-based therapies have emerged as treatment options to repair neurological disorders, but they have been difficult to translate clinically [8, 9]. Since the efficacy of cell therapies has been limited given the low rates of cell survival, which results from the influence of the acute inflammation/immune response caused by transplantation and the lack of support from the extracellular matrix, transplanted cells usually die in the first several days after transplantation. In addition, the limited differentiation still restricts the therapeutic effect of cell therapy, as only 5% of the surviving neural stem cells (NSCs) transplanted to the damaged sites differentiate into neurons *in vivo* without any treatment [10-13]. As such, novel approaches for stem cell transplantation need to be developed.

The use of biomaterials, especially hydrogels, can create permissive microenvironments to enhance stem cell engraftment and treatment efficiency [13-15]. Several types of biomaterials have been investigated in recent years as scaffolds for the three-dimensional (3D) culture and differentiation of pluripotent cells [16-18]. Alginate

is a natural polymer that is derived from algae and composed of (1-4)-linked β -D-mannuronic acid and α -L-guluronic acid; it is commonly used as 3D scaffold biomaterials. Unmodified alginate can be molded into different mechanical properties using divalent cations such as Ca^{2+} for ionic crosslinking, which can create simple and efficient scaffold-fabrication and cell-encapsulation processes [19].

Alginate-supported cultures have been tested and can be used in the proliferation and differentiation of various pluripotent cells both *in vitro* and *in vivo* [18, 20, 21]. A recent study showed that the use of alginate hydrogels for cell transplantation and the release of neurotrophic factors can promote axonal regeneration in the injured spinal cords of rats [22]. Many studies show that unmodified alginate does not provide adequate cell adhesion. Alginate can be modified to improve cell attachment and motility. Several reports have indicated that RGD (the tripeptide motif found in several ECM proteins)-containing peptides promote NSC adhesion when coupled to polymer surfaces [23, 24]. Thus, in our study, an appropriate concentration of the RGD polypeptide was grafted to the alginate, which was used to enhance the survival of the transplanted NSCs. Furthermore, some studies showed that blocking the expression of Cripto-1, an epidermal growth factor (EGF)-CFC family member that plays a role in embryogenesis, can improve the differentiation of embryonic stem cell (ESC)-derived dopaminergic neurons *in vitro* and foster their engraftment *in vivo* [25, 26]. However, there is no evidence to suggest the roles of Cripto-1 in the differentiation of NSCs; as such, we intend to explore whether blocking Cripto-1 in NSCs achieves the same effect as is observed in ESCs in our research. Mounting evidence suggests that the properties

of hydrogels, such as their mechanical stability and elastic modulus, strongly influence the cell phenotype, except for the fact that the alginate hydrogel possesses an elastic modulus that is comparable to that of brain tissue and can promote NSC differentiation and proliferation [27-30]. Alginate hydrogels, which have suitable mechanical properties that can benefit NSC proliferation or differentiation, were designed and an intelligent hydrogel system was subsequently constructed for transplanted NSCs.

To achieve better therapeutic effects, the acute inflammatory/immune response caused by transplantation should be considered in addition to improving the survival and differentiation rate of transplanted NSCs. In our study, to avoid the influence of high levels of inflammation following NSC transplantation to the damaged site, intelligent, double-layer, alginate hydrogels were designed. High-concentration (1.5%) alginate can inhibit NSC differentiation; here, it was immobilized with RGD and MMP substrate peptides to form the core layer of the alginate hydrogel. The outer layer of the alginate hydrogel was constituted by low-concentration alginate (1%), which can form a dense network and is immobilized with the Cripto-1 antibody to promote the differentiation of transplanted NSCs. During acute inflammatory period after physical injuries, the double layer of alginate can protect the NSCs from injury and the NSCs which surrounded by the core layer alginate hydrogel subsequently secrete MMP, which interacts with the MMP substrate to destroy the structure of the inner hydrogel. When the peak inflammation ends, the NSCs are released into the outer hydrogel layer and the Cripto-1 antibody is immobilized on the outer layer to enhance the NSCs, which are then differentiated into dopaminergic neurons, thus promoting the recovery of

injured nerves.

2. Materials and methods

2.1. Cell lines and materials

The strain C57BL/6 mouse NSCs which were purchased from Cyagen Biosciences Inc. (Guangzhou, People's Republic of China) are derived from the hippocampi and subventricular zones (SVZ) of day 12.5 post coitus C57BL/6 embryos and cultured as neurospheres in Strain C57BL/6 Mouse Neural Stem Cell Growth Medium (which contains B-27 supplement, basic fibroblast growth factor [bFGF], epidermal growth factor [EGF], GlutaMAXTM supplement, and heparin sodium) also were purchased from Cyagen Biosciences Inc. (Guangzhou, People's Republic of China). The 2B11 hybridoma cells, which can secrete the Cripto-1 antibody, were purchased from the Biosynthesis Biotechnology Company (Beijing, People's Republic of China). Dulbecco's Modified Eagle's Medium, Nutrient Mixture F-12 (DMEM/F-12), N-2 supplement, and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Propidium iodide (PI), calcein, phalloidin, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Mouse monoclonal β III-tubulin, PITX3, GIRK2, and GFAP primary antibodies, as well as FITC-conjugated goat polyclonal secondary antibodies to rabbit immunoglobulin (Ig)G, and rhodamine-conjugated rat polyclonal secondary antibodies to rabbit IgG were purchased from Abcam plc (Cambridge, MA, USA).

2.2. Experimental animals

Sprague–Dawley rats, 6–8 weeks of age, were provided by the Laboratory Animal Center of Harbin Medical University. All animals were fed ad libitum and kept under a normal 12-hour light/12-hour dark cycle. All procedures were approved by the University Ethics Committee of the Harbin Institute of Technology.

2.3. Synthesis of the inner and cover alginate hydrogel-grafted RGD and MMP polypeptide or immobilized Cripto-1-blocking antibody and its physicochemical characterization

The oxidized alginate was obtained by mixing sodium peroxide and sodium alginate (in distilled water) with a mass ratio of 1:2. The reaction was conducted at 4°C for 2 hours and was terminated by the addition of ethylene glycol; the degree of oxidation was evaluated by measuring the concentration of sodium peroxide, which was maintained after 2 hours. Then, the oxidized alginate was lyophilized at –20°C and dissolved in distilled water to achieve a concentration of 1.5% for inner alginate and 1% for cover alginate. The RGD- and MMP-grafted inner alginate and Cripto-1 antibody-immobilized cover alginate were prepared by dissolving different concentrations of RGD or MMP polypeptide and Cripto-1 antibody in an oxidized alginate solution while stirring. The RGD and MMP polypeptide-grafted inner alginate and Cripto-1 antibody-immobilized cover alginate were collected and lyophilized at –20°C. The alginate hydrogel was prepared by crosslinking with calcium ions. The gelation process and the mechanical properties of the alginate hydrogels were evaluated by examining the time of gelation onset and the evolution of elasticity at 37°C in constant strain mode by

means of a Bohlin Gemini II rheometer (Malvern Instruments, Malvern, UK) using parallel plate geometry (40 mm in diameter), as in our previous study [31].

2.4 Preparation of the intelligent double-layer alginate hydrogel system

Briefly, the 1.5% alginate solution that grafted the RGD and MMP polypeptide was added to 100 mM of CaCl₂ solution to obtain calcium alginate gel beads. After hardening for 10 minutes, the beads were dipped into 1% (w/v) chitosan solution to form an alginate–chitosan membrane around the surface. Then, the microcapsules were rinsed by saline to remove the excess chitosan and were transferred to the 1% alginate solution, which immobilized the Cripto-1 antibody. Finally, the microcapsules were cleaned with saline a second time and added to the 100 mM CaCl₂ solution for 10 minutes to form the alginate/chitosan/alginate microcapsules.

2.5. Cell culture and seeding on hydrogels

The NSCs of mice were grown in NSC growth medium and incubated in a humidified atmosphere of 5% CO₂ at 37°C, with media changes every other day. The NSCs were digested after centrifugation and seeded onto the cover alginate hydrogel, which immobilized the Cripto-1 antibody; the cells were then incubated in a humidified atmosphere of 5% CO₂ at 37°C and grown in astrocyte-favorite media supplemented with 1% FBS, 1% GlutaMAX™, 1% penicillin–streptomycin, and 1% N-2. For the inner alginate grafted with the RGD and MMP polypeptides, the NSCs were seeded on and grown in the NSC growth medium which were prepared with neural stem cell basal

medium supplement with 2% B27, 20ng/ml bFGF, 20ng/ml EGF and 50ng/ml heparin sodium and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

2.6. Scanning electron microscopy

Cell clones were released from our developed screening platform and they were fixed with 2.5% glutaraldehyde for 30 minutes at 37°C, followed by overnight incubation in 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde at 4°C. After dehydration by serial washing twice in each increasing ethanol concentration (0%, 30%, 50%, 70%, 85%, 95%, and 100%), the samples were critical point dried, sectioned, mounted, and sputter coated with platinum before imaging with a JSM-7000 scanning electron microscope (SEM) (JEOL, Tokyo, Japan).

2.7. qRT-PCR assay

Total RNA was extracted by Trizol, cDNA was prepared according to RNA, and FastStart Universal SYBR Green Master (Rox) and the ABI Prism 7500 sequence detection system were used to complete the RT-PCR: 50°C, 2 minutes; 95°C predegeneration, 10 minutes; 95°C, 15 seconds; 60°C, 60 seconds. This was done for a total of 40 cycles and each sample had three repeats; the $2^{-\Delta\Delta Ct}$ method was used for conversion. The following table shows the primer sequences used in the study.

| Gene | 5' primer | 3' primer |
|---------------------|-----------------------|----------------------|
| Mus GAPDH | TGGCCTTCCGTTCTAC | GAGTTGCTGTTGAAGTCGCA |
| Mus GFAP | GGAGAGGGACAACCTTTGCAC | CCAGCGATTCAACCTTTCTC |
| β III-tubulin | TTCTGGTGGACTTGGAACCT | ACTCTTTCCGCACGACATCT |

2.8. Immunofluorescence analysis

Following 3D culture in AbCripto-1 hydrogel microbeads for 14 days, 4% paraformaldehyde was used to fix the samples; then, 1% TritonX-100 0.9% NaCl was used to permeabilize the cytomembrane when the intracellular protein was tested. After fixation for 1 hour or permeabilization for 30 minutes, 0.9% NaCl was used to wash the sample three times, at 5 minutes each. Cell markers were used to identify the different cell types: β III-Tubulin was used for neurons, GFAP for astrocytes, Nurr 1 and TH for dopaminergic neurons, and PITX-3 and GIRK-2 for A9 dopaminergic neurons. NSCs were transfected with green fluorescent protein (GFP) to emit green; these markers were used in combination with the red fluorescent antibody. Following the staining reaction, 0.9% NaCl was used to wash the sample three times, 5 minutes each. Every step was carefully treated to not break the hydrogel structure.

2.9. Statistical Analysis

The results are presented as the mean \pm standard deviation. Student's two-tailed *t*-test was performed to compare the differences between the experimental and control groups. A *P*-value <0.05 (*) was considered significant, while a *P*-value <0.01 (**) was considered very significant.

3. Results

3.1 Exploration of the optimum conditions used to build the intelligent double-layer alginate hydrogel system

One of the key issues with stem cell transplantation is ensuring cell survival, as only a small percentage of transplanted cells can survive several days after transplantation. Recent work has revealed that the acute inflammatory/immune response, oxidative stress, and other processes that result from injury or transplantation are likely to result in cell death [32-34]. In our study, we aimed to establish an intelligent, double-layer, alginate hydrogel system where NSCs can proliferate in the inner layer, while the outer material was used to block the inflammatory storm. **By the end of the inflammatory storm, the NSCs arrived at the outer layer through secreting MMP to slice the MMP polypeptide grafted on the inner layer.** We also designed the intelligent, double-layer, alginate hydrogel system for PD treatment, so we immobilized the Cripto-1 antibody to the outer alginate, which reportedly improves the differentiation of ESC-derived dopaminergic neurons *in vitro* and their engraftment *in vivo*. In this way, the optimal conditions for the development of an intelligent, double-layer, alginate hydrogel system were explored in our research (Fig. 1).

3.2 Effect of the hydrogel-immobilized Cripto-1 antibody on the neuronal differentiation of NSCs

Considerable evidence indicates that the blocking of Cripto-1 can promote the differentiation of ESCs to DA neurons *in vitro* and *in vivo* [25]. however, no research has reported on the differentiation of NSCs to DA neurons by blocking Cripto-1. In our study, different concentrations of the Cripto-1-blocking antibody (0 $\mu\text{g}/\text{mL}$, 20 $\mu\text{g}/\text{mL}$, and 100 $\mu\text{g}/\text{mL}$) were immobilized on the partial oxidation of sodium alginate. The calcium crosslinked, alginate-composite hydrogel was developed by mixing the alginate-immobilized Cripto-1 antibody and cell suspension at a volume ratio of 1:1;

the NSCs were then wrapped inside and cultured in astrocyte-differentiation medium, which mimics the microenvironment for NSC differentiation *in vivo*. This was done to investigate whether blocking Cripto-1 can promote NSC differentiation to DA neurons. Normally, 90% of NSCs will differentiate into astrocytes in astrocyte-differentiation medium without any other treatment. Interestingly, after 14 days of culture in our alginate-composite hydrogel-immobilized Cripto-1 antibody with different concentrations, we observed significant changes in the cell morphology of cultured NSCs. As shown in Fig. 2, the typical morphology of astrocytes can only be seen in the encapsulated cells in 0 $\mu\text{g/mL}$ of the AbCripto-1 hydrogel. Further, the slender cell-shaped component network can be observed in the hydrogel with both low and high concentrations of immobilized Cripto-1 antibody. SEM was used to further study the cell morphology changes. AbCripto-1-immobilized, hydrogel-cultured NSCs longed out of the synapse and presented typical neuron morphology (Fig. 2). Although the cell morphology exhibited typical changes, additional verification and support are needed through numerous experiments to demonstrate how blocking Cripto-1 can promote NSC differentiation to neurons.

To further verify that the neural differentiation of NSCs occurred after blocking Cripto-1, we performed molecular analyses at day 14 of culture (D14); at this time, cells were released from the alginate microcapsules by sodium citrate, and RNA was collected for quantitative real-time reverse transcription (qRT-PCR) analysis. Moreover, some alginate microcapsules were fixed directly for immunocytochemistry. As shown in Fig. 3, after 14 days of being cultured in astrocyte-differentiation medium, the cells were negative for pluripotent markers OCT4 and NANOG (data not shown), but in the AbCripto-1-immobilized hydrogel, neural differentiation occurred, as evidenced by the

significantly reduced expression of GFAP – a marker of astrocyte differentiation. In contrast, the neuron marker (β III-tubulin) and dopaminergic neuron markers (Nurr1 and TH) were significantly higher in the AbCripto-1-immobilized, hydrogel-cultured NSCs when compared with controls. In addition, β III-tubulin and Nurr1/DAT1 expression was determined by immunofluorescence, and the results were consistent with those found for qRT-PCR; the NSCs cultured in the hydrogel-immobilized, Cripto-1 antibody showed a faster progression toward neuronal differentiation with positive expression when compared to the control, where none of the Cripto-1 antibodies were immobilized (Fig. 4 and Supplementary Fig. 1). The progressive degeneration of A9 dopaminergic neurons is regarded as the major cause of PD. To determine whether blocking Cripto-1 in the NSCs can induce A9 dopaminergic differentiation, the PITX-3/GIRK2 protein – which is responsible for the characteristics of A9 dopaminergic neurons – was detected by immunofluorescence analysis (Supplementary Fig. 1). When blocking Cripto-1 in the NSCs by culturing these cells in the AbCripto-1-immobilized hydrogels, it was possible to enable NSC differentiation into A9 dopaminergic neurons, and the efficiency of NSC differentiation tended to increase with increasing concentrations of the grafted Cripto-1 antibody. As shown in Supplementary Fig. 2, approximately 56% of cells were Nurr1 positive, as they were cultured in the highest concentration of the AbCripto-1 grafted hydrogel; this rate was significantly higher than that of the control group, as analyzed by flow cytometry. To further characterize the extent to which the cells had differentiated, and to identify the morphology of the differentiated cells, immunofluorescence staining with Nurr1 and GIRK2 antibodies was used to observe

the clear neural network in a 3D reconstruction, as viewed by confocal microscopy (Supplementary Fig. 3). Taken together, these data suggest that blocking the Cripto-1 antibody in NSCs cultured in hydrogel not only prevented NSC differentiation in astrocytes in astrocyte-favorite medium, but it also promoted the differentiation of most stem cells into dopaminergic neurons.

3.3 Exploring the optimal concentration of grafted RGD and MMP polypeptides for NSC proliferation and migration in core layer hydrogel materials

The importance of time-dependent changes in the microenvironment of injured or transplanted areas in regulating transplanted stem cell survival and differentiation is becoming increasingly evident. As is well known, the inflammatory storm that results from injury or transplantation is one of the primary factors that induce transplanted stem cell death. Several studies have shown that MMPs, which are secreted by NSCs and the RGD peptide, play important roles in the proliferation, migration, and adhesion of NSCs. The oxidation of sodium alginate, which is used to synthesize the inner hydrogel layer in our study, can produce aldehyde groups that form unstable amines found within the free amino peptide groups; thus, we covalently linked the MMP and RGD peptide to the alginate polymer chain. The inner alginate hydrogel was prepared by the gelation of oxidation alginate grafted different concentration RGD peptide with Ca^{2+} . The Live/Dead assay was used to detect the influence of the different concentrations of RGD on the proliferation and survival of NSCs. As shown in Fig. 5, 50 $\mu\text{g}/\text{mL}$ of grafted RGD promoted the survival and proliferation of NSCs cultured in the alginate hydrogel. Further, to culture the NSCs in the inner hydrogel layer, so as to escape the

inflammatory storm, we decided to choose the optimal concentration of MMP peptide to graft to the hydrogel. We prepared alginate hydrogels grafted with different concentrations of MMP (10 $\mu\text{g}/\text{mL}$, 60 $\mu\text{g}/\text{mL}$, and 100 $\mu\text{g}/\text{mL}$) and cultured the NSCs in the hydrogel. As shown in Fig. 6, we found that the 60 $\mu\text{g}/\text{mL}$ concentration yielded the best results, insofar as the highest number of NSCs migrated out the hydrogel after 12 days of culture.

3.4 A tentative study of the differentiation effectiveness of this intelligent double-layer alginate hydrogel system for NSCs transplantation *in vivo*

While the NSCs cultured in our alginate hydrogel system can yield better differentiation effects by mimicking the intracorporeal environment through cultured the NSCs in astrocyte-differentiation medium, further validation is required *in vivo* due to the complicated microenvironment of brain internal. Therefore, in our study, a rat brain trauma model was prepared by creating two symmetrical, 5 mm \times 5 mm wounds on the left and right side of the rat brain. The left wound was transplanted with NSCs containing 0 $\mu\text{g}/\text{mL}$ of AbCripto-1 hydrogel, while the right wound was transplanted with NSCs containing 50 $\mu\text{g}/\text{mL}$ of AbCripto-1 hydrogel. Fourteen days after transplantation, the rats were sacrificed and their brains were submitted for section and immunofluorescence staining. As shown in Fig. 7, our alginate hydrogel system-immobilized Cripto-1 antibody can promote the survival and differentiation of NSCs transplanted *in vivo*.

4. Discussion

Numerous studies have demonstrated that stem cell-based therapies have therapeutic efficacy against neurological disorders [10, 11, 35-37]. Due to the low survival rates

and reduced differentiation efficiency following stem cell transplantation, the therapeutic effects toward neurological disorders is minimal [37-39]. Recent work has revealed that the use of hydrogels (a class of biomaterials), which are composed of water-swollen polymeric networks, can improve stem cell engraftment and function [15, 40]. It is known that hydrogels provide support to the extracellular matrix during stem cell transplantation, offering benefits to cell retention and survival [21]. As such, in our study, a non-toxic and low-immunogenicity alginate hydrogel was used. Several studies have shown that alginate creates excellent, flexible scaffolds for stem cells transplanted in different animal species [41-43]. Although lots of alginate hydrogel systems have been developed for stem cell transplantation and have achieved good results *in vitro* and *in vivo*, it is generally difficult to use them in the clinical treatment of neurological disorders [22, 24, 40]. There is now overwhelming evidence to support the idea that changes in the microenvironments over time affect stem cell survival and the therapeutic potential of stem cell transplantation in the treatment of central nervous system trauma and neurodegenerative diseases [32]. The acute inflammatory/immune response, as well as some negative factors caused by transplant surgery or transplanted stem cells, also severely impact the survival of transplanted stem cells [33, 34]. To address this clinical issue, the establishment of an efficient and available hydrogel system used for stem cell transplantation remains a challenge in the treatment of neurological disorders. In our study, we developed an intelligent, double-layer, alginate hydrogel system that contained an inner layer comprised of alginate hydrogel-grafted MMP and RGD polypeptides, and an outer layer of alginate hydrogel-grafted Cripto-1

antibodies. The transplanted stem cells that proliferated in the inner hydrogel can secrete MMP enzymes while slicing the grafted MMP polypeptides and breaking through the inner materials; the cells then moved to the outer hydrogel layer following the inflammatory storm that resulted from damage or surgery. In the present study, it was observed that the optimal concentration of the MMP peptide enabled the stem cells to move to the outer hydrogel layers, while avoiding the inflammatory storm. At the same time, the optimal concentration of the RGD peptide, which was shown to be good for the adhesion and proliferation of NSCs, was grafted to the inner hydrogel to promote the proliferation of stem cells encapsulated in the inner hydrogel [44].

When examining stem cell-based therapies for neurological disorders, researchers tend to choose ESCs or induced pluripotent stem cells (iPSCs), and these cells can usually obtain good outcomes when treating neurological disorders *in vitro* and *in vivo* [39]. However, the application of ESC or iPSC transplantation in the treatment of neurological diseases is also associated with the inevitable risks of teratoma development [11, 45]. Many researchers have proved that reducing the risk of teratoma development, as induced by stem cell transplantation, can be achieved by regulating the signaling pathway or engaging in molecular therapy, while other studies have successfully reduced the probability of teratoma development, but there are still some related safety concerns [46]. To avoid the generation of teratomas during treatment, we chose NSCs for this study. Furthermore, considerable evidence indicates that the material properties of hydrogels are important in the stem cell proliferation and differentiation [16, 29, 30]. **Research has shown that a 1% concentration of alginate**

hydrogel which present elastic modulus in the range of soft substrates (100-1000pa) promotes NSC differentiation, while a 1.5% concentration which more stiffer inhibits NSC differentiation and accelerates NSC proliferation [27, 43]. Thus, in our study, the inner and outer alginate hydrogel concentrations were 1.5% and 1%, respectively.

It has been reported that blocking Cripto-1 in ESCs facilitates their differentiation to dopaminergic neurons, while significantly reducing the incidence of teratoma formation; however, it was also found that teratomas were still observed in as many as 27% of the grafted rats [25]. In our study, Cripto-1 in NSCs was blocked by grafting the Cripto-1 antibody to the hydrogel. We noticed an interesting phenomenon when culturing the cells in astrocyte differentiation medium: the NSCs cultured in our hydrogel system showed progressive neuronal differentiation with extensive neurite formation. Additionally, cells cultured on our hydrogel system displayed increased expression of the dopaminergic neuron-related genes such as *PITX-3* and *GIRK2*. The degeneration of dopamine neurons has become the leading cause of PD. As such, our study provides a novel approach in the treatment of PD through NSC transplantation. Although the overall approach adopted in this study focused on studying NSCs, these intelligent, double-layer, alginate hydrogels were designed to easily adapt to other types of transplanted stem cells. Therefore, we anticipate that these double-layer alginate hydrogels can be used for stem cell-based therapies in the clinical treatment of neurological disorders.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (grant

51773050, 21407036, 81572472 and 81770923), English-language editing of this manuscript was provided by Journal Prep.

References:

- [1] P. P. Michel, E. C. Hirsch, S. Hunot, *NEURON* **2016**, *90*, 675.
- [2] A. Reeve, E. Simcox, D. Turnbull, *AGEING RES REV* **2014**, *14*, 19.
- [3] A. Bjornestad, O. B. Tysnes, J. P. Larsen, G. Alves, *NEUROLOGY* **2016**, *87*, 1599.
- [4] C. H. Adler, T. G. Beach, J. G. Hentz, H. A. Shill, J. N. Caviness, E. Driver-Dunckley, M. N. Sabbagh, L. I. Sue, S. A. Jacobson, C. M. Belden, B. N. Dugger, *NEUROLOGY* **2014**, *83*, 406.
- [5] A. Schapira, K. R. Chaudhuri, P. Jenner, *NAT REV NEUROSCI* **2017**, *18*, 509.
- [6] B. S. Connolly, A. E. Lang, *Jama* **2014**, *311*, 1670.
- [7] R. Balestrino, A. Schapira, *NEUROSCIENTIST* **2018**, 629631883.
- [8] P. L. Martinez-Morales, A. Revilla, I. Ocana, C. Gonzalez, P. Sainz, D. McGuire, I. Liste, *Stem Cell Rev* **2013**, *9*, 685.
- [9] P. T. Yin, E. Han, K. B. Lee, *ADV HEALTHC MATER* **2016**, *5*, 10.
- [10] L. Titomanlio, A. Kavelaars, J. Dalous, S. Mani, G. V. El, C. Heijnen, O. Baud, P. Gressens, *ANN NEUROL* **2011**, *70*, 698.
- [11] M. Ronaghi, S. Erceg, V. Moreno-Manzano, M. Stojkovic, *STEM CELLS* **2010**, *28*, 93.
- [12] P. J. Hallett, M. Deleidi, A. Astradsson, G. A. Smith, O. Cooper, T. M. Osborn, M. Sundberg, M. A. Moore, E. Perez-Torres, A. L. Brownell, J. M. Schumacher, R. D. Spealman, O. Isacson, *CELL STEM CELL* **2015**, *16*, 269.
- [13] M. M. Adil, T. Vazin, B. Ananthanarayanan, G. Rodrigues, A. T. Rao, R. U. Kulkarni, E. W. Miller, S. Kumar, D. V. Schaffer, *BIOMATERIALS* **2017**, *136*, 1.
- [14] M. Shafiq, Y. Jung, S. H. Kim, *BIOMATERIALS* **2016**, *90*, 85.
- [15] J. A. Burdick, R. L. Mauck, S. Gerecht, *CELL STEM CELL* **2016**, *18*, 13.
- [16] O. Chaudhuri, L. Gu, D. Klumpers, M. Darnell, S. A. Bencherif, J. C. Weaver, N. Huebsch, H. P. Lee, E. Lippens, G. N. Duda, D. J. Mooney, *NAT MATER* **2016**, *15*, 326.
- [17] A. M. Pasca, S. A. Sloan, L. E. Clarke, Y. Tian, C. D. Makinson, N. Huber, C. H. Kim, J. Y. Park, N. A. O'Rourke, K. D. Nguyen, S. J. Smith, J. R. Huguenard, D. H. Geschwind, B. A. Barres, S. P. Pasca, *NAT METHODS* **2015**, *12*, 671.
- [18] K. Ziv, H. Nuhn, Y. Ben-Haim, L. S. Sasportas, P. J. Kempen, T. P. Niedringhaus, M. Hrynyk, R. Sinclair, A. E. Barron, S. S. Gambhir, *BIOMATERIALS* **2014**, *35*, 3736.
- [19] K. Y. Lee, D. J. Mooney, *PROG POLYM SCI* **2012**, *37*, 106.
- [20] J. L. Wilson, M. A. Najia, R. Saeed, T. C. McDevitt, *BIOTECHNOL BIOENG* **2014**, *111*, 618.
- [21] S. S. Ho, K. C. Murphy, B. Y. Binder, C. B. Vissers, J. K. Leach, *Stem Cells Transl Med* **2016**, *5*, 773.
- [22] M. I. Gunther, N. Weidner, R. Muller, A. Blesch, *ACTA BIOMATER* **2015**, *27*, 140.
- [23] B. Ananthanarayanan, L. Little, D. V. Schaffer, K. E. Healy, M. Tirrell, *BIOMATERIALS* **2010**, *31*, 8706.
- [24] S. J. Bidarra, C. C. Barrias, K. B. Fonseca, M. A. Barbosa, R. A. Soares, P. L. Granja,

BIOMATERIALS **2011**, *32*, 7897.

- [25] E. Lonardo, C. L. Parish, S. Ponticelli, D. Marasco, D. Ribeiro, M. Ruvo, S. De Falco, E. Arenas, G. Minchiotti, *STEM CELLS* **2010**, *28*, 1326.
- [26] C. L. Parish, S. Parisi, M. G. Persico, E. Arenas, G. Minchiotti, *STEM CELLS* **2005**, *23*, 471.
- [27] A. Bozza, E. E. Coates, T. Incitti, K. M. Ferlin, A. Messina, E. Menna, Y. Bozzi, J. P. Fisher, S. Casarosa, *BIOMATERIALS* **2014**, *35*, 4636.
- [28] M. Chayosumrit, B. Tuch, K. Sidhu, *BIOMATERIALS* **2010**, *31*, 505.
- [29] A. Banerjee, M. Arha, S. Choudhary, R. S. Ashton, S. R. Bhatia, D. V. Schaffer, R. S. Kane, *BIOMATERIALS* **2009**, *30*, 4695.
- [30] F. Guilak, D. M. Cohen, B. T. Estes, J. M. Gimble, W. Liedtke, C. S. Chen, *CELL STEM CELL* **2009**, *5*, 17.
- [31] S. P. Qiao, Y. F. Zhao, C. F. Li, Y. B. Yin, Q. Y. Meng, F. H. Lin, Y. Liu, X. L. Hou, K. Guo, X. B. Chen, W. M. Tian, *ACTA BIOMATER* **2016**, *37*, 83.
- [32] S. Nishimura, A. Yasuda, H. Iwai, M. Takano, Y. Kobayashi, S. Nori, O. Tsuji, K. Fujiyoshi, H. Ebise, Y. Toyama, H. Okano, M. Nakamura, *MOL BRAIN* **2013**, *6*, 3.
- [33] M. T. Harting, F. Jimenez, S. D. Adams, D. W. Mercer, C. J. Cox, *SURGERY* **2008**, *144*, 803.
- [34] M. G. Tansey, M. S. Goldberg, *NEUROBIOL DIS* **2010**, *37*, 510.
- [35] O. Lindvall, Z. Kokaia, A. Martinez-Serrano, *NAT MED* **2004**, *10 Suppl*, S42.
- [36] C. V. Borlongan, L. E. Glover, N. Tajiri, Y. Kaneko, T. B. Freeman, *PROG NEUROBIOL* **2011**, *95*, 213.
- [37] P. L. Martinez-Morales, A. Revilla, I. Ocana, C. Gonzalez, P. Sainz, D. McGuire, I. Liste, *Stem Cell Rev* **2013**, *9*, 685.
- [38] S. P. Liu, R. H. Fu, S. J. Huang, Y. C. Huang, S. Y. Chen, C. H. Chang, C. H. Liu, C. H. Tsai, W. C. Shyu, S. Z. Lin, *CELL TRANSPLANT* **2013**, *22*, 631.
- [39] Y. Imaizumi, H. Okano, *J NEUROCHEM* **2014**, *129*, 388.
- [40] T. C. Tseng, L. Tao, F. Y. Hsieh, Y. Wei, I. M. Chiu, S. H. Hsu, *ADV MATER* **2015**, *27*, 3518.
- [41] P. Prang, R. Muller, A. Eljaouhari, K. Heckmann, W. Kunz, T. Weber, C. Faber, M. Vroemen, U. Bogdahn, N. Weidner, *BIOMATERIALS* **2006**, *27*, 3560.
- [42] K. Kataoka, Y. Suzuki, M. Kitada, T. Hashimoto, H. Chou, H. Bai, M. Ohta, S. Wu, K. Suzuki, C. Ide, *TISSUE ENG* **2004**, *10*, 493.
- [43] J. Kim, P. Sachdev, K. Sidhu, *STEM CELL RES* **2013**, *11*, 978.
- [44] B. Ananthanarayanan, L. Little, D. V. Schaffer, K. E. Healy, M. Tirrell, *BIOMATERIALS* **2010**, *31*, 8706.
- [45] T. Chen, X. Wang, L. Guo, M. Wu, Z. Duan, J. Lv, W. Tai, H. Renganathan, R. Didier, J. Li, D. Sun, X. Chen, X. He, J. Fan, W. Young, Y. Ren, *Front Immunol* **2014**, *5*, 275.
- [46] J. Yoo, H. S. Kim, D. Y. Hwang, *J CELL BIOCHEM* **2013**, *114*, 743.

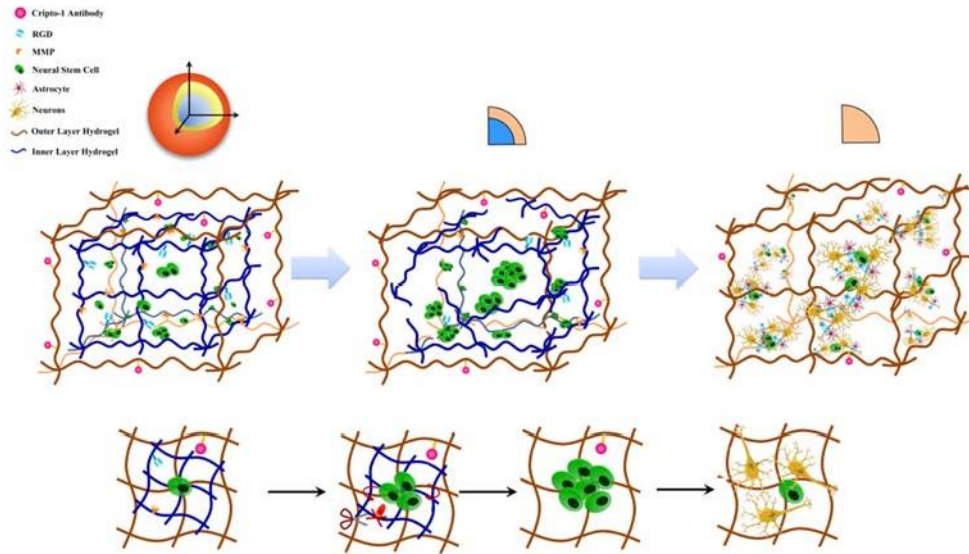


Figure 1. The scheme to show the use of our intelligent, double-layer, alginate hydrogel for NSCs transplantation to achieve better therapeutic effect for PD treatment.

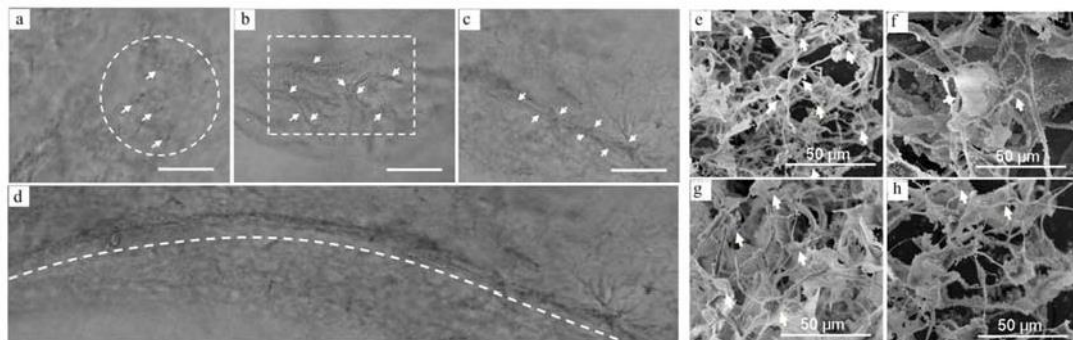


Fig. 2. Morphology observation of NSCs cultured in alginate hydrogel immobilized with different concentrations of AbCripto-1. The typical morphology of astrocytes was evident in the hydrogels, which immobilized 0 $\mu\text{g/mL}$ of AbCripto-1 (a); the slender cell-shaped component network was found in the alginate hydrogel, which immobilized 10 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ of AbCripto-1 (b, c, d). Scanning electron microscope image of differentiated cells cultured with 50 $\mu\text{g/mL}$ of AbCripto-1 hydrogel (e, f, g, h).

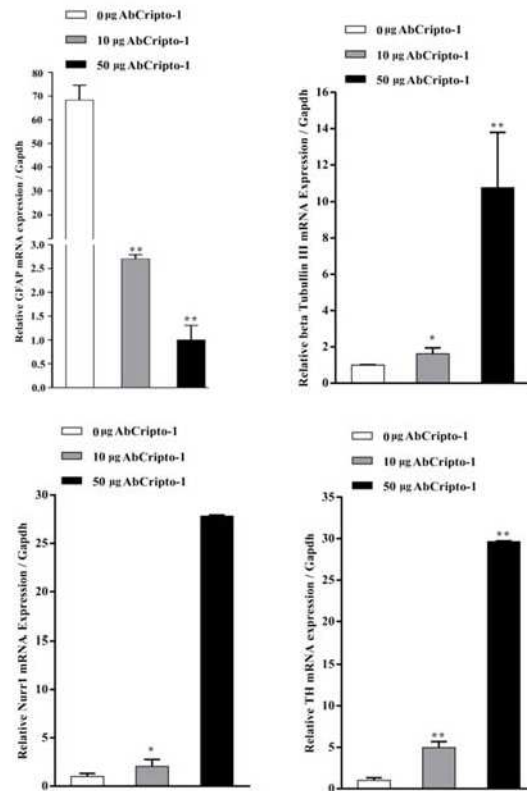


Fig. 3. RT-qPCR analyses on glial marker(GFAP), neural differentiation markers (bIII-tubulin) and dopaminergic neuron markers (Nurr1 and TH) at D14.

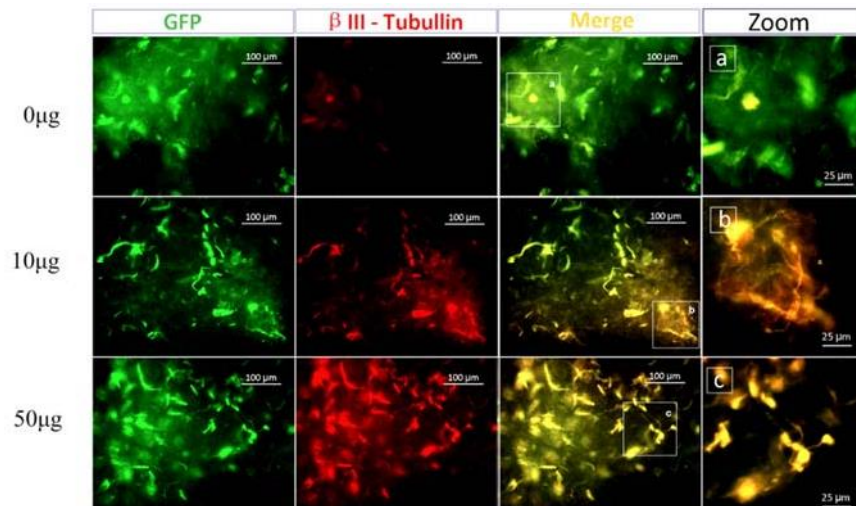


Fig. 4. Immunocytochemistry analyses at D14 for the early neural differentiation marker, β III-tubulin, in red.

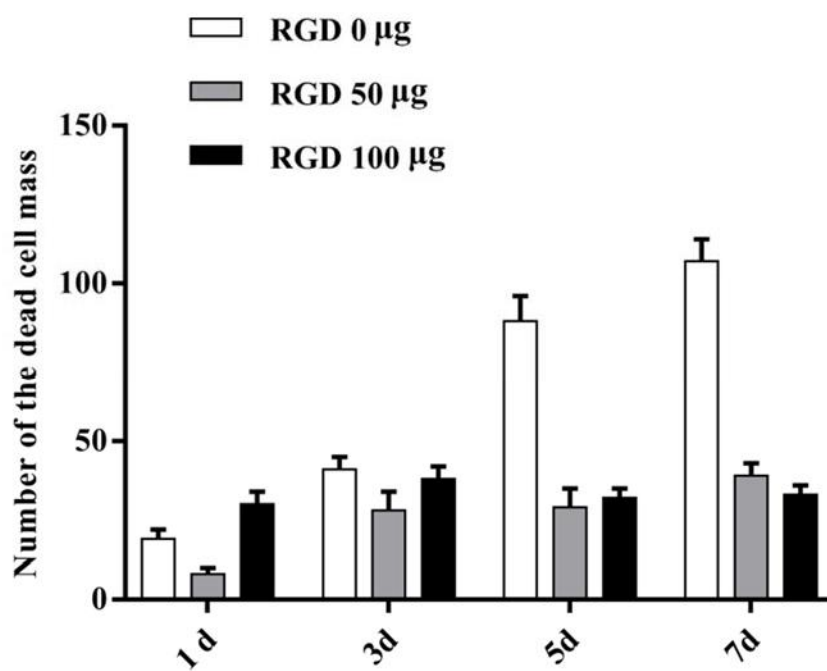
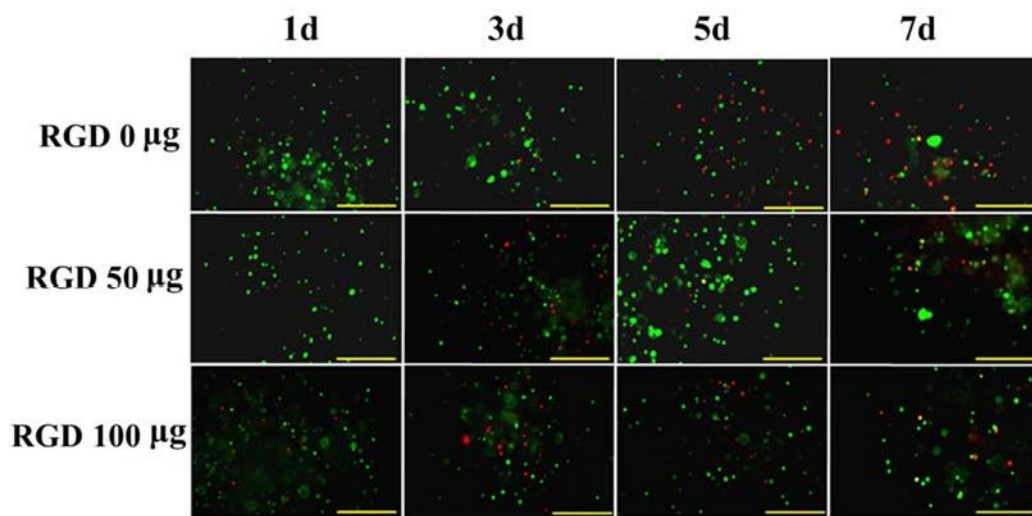


Fig. 5. Effect of different concentration of RGD immobilization on NSC proliferation.

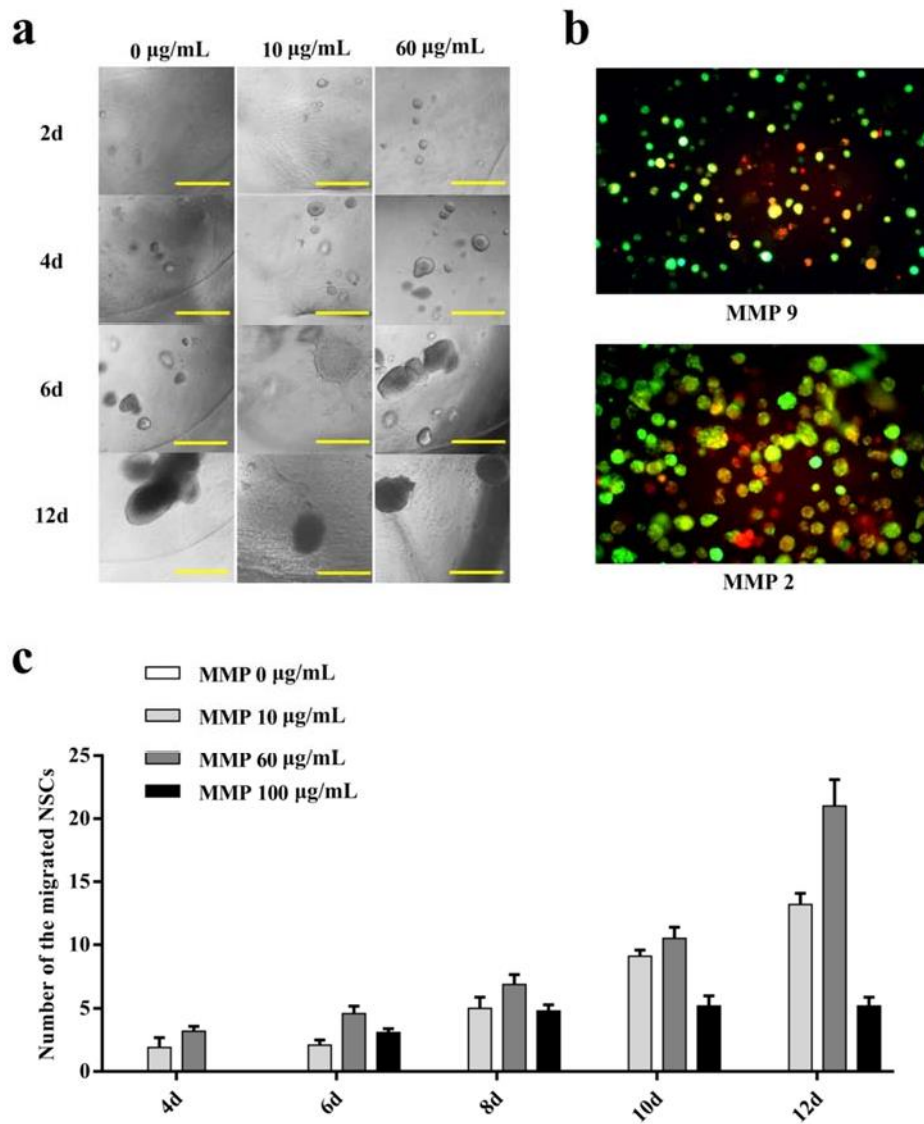


Fig. 6. Effect of MMP immobilization on NSC migration. The number of NSCs migrated beyond the inner layer hydrogel with the extending of incubation time (a, c). The expression of MMP protein in NSCs (b).

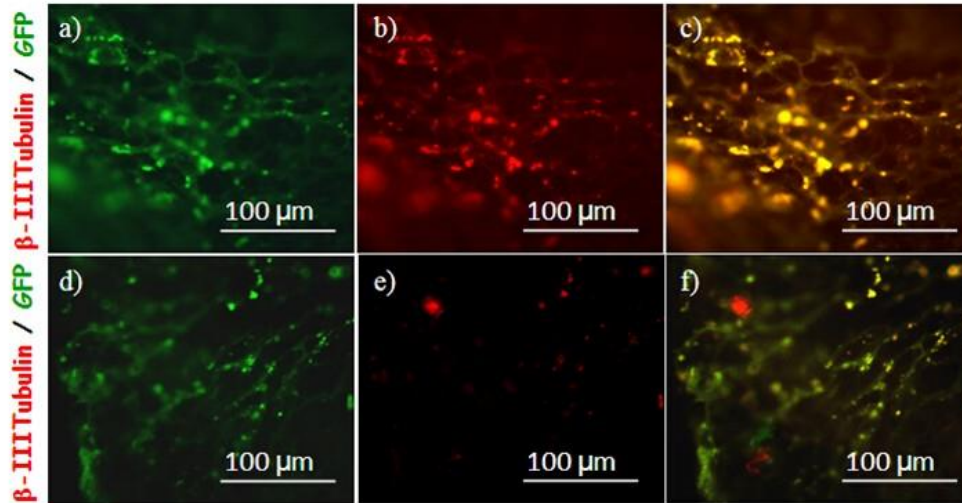
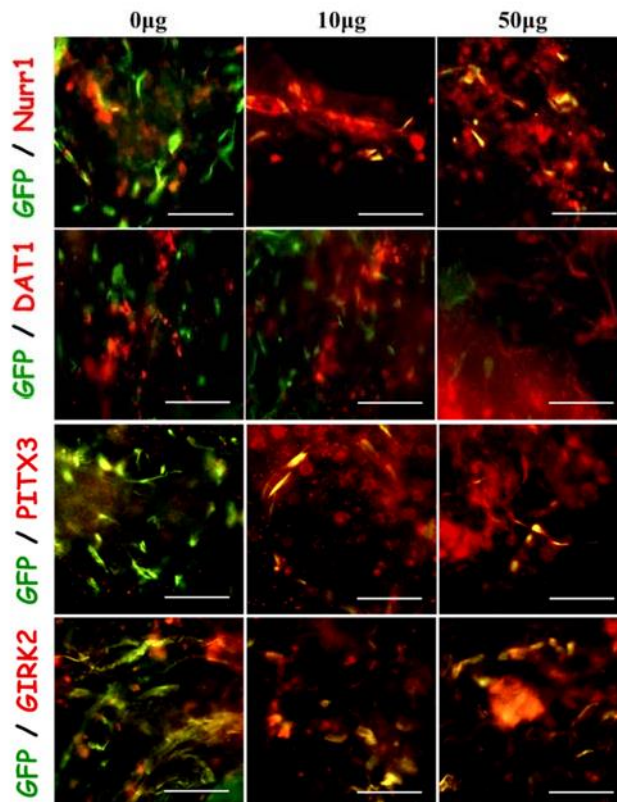
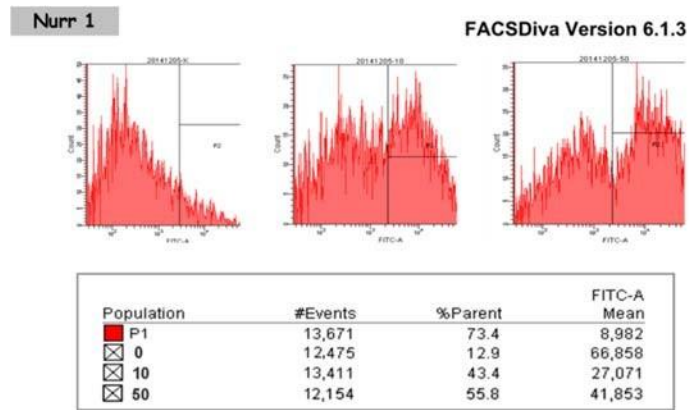


Fig. 7. The differentiation of transplanted NSCs through our intelligent, double-layer, alginate hydrogel system *in vivo*.



Supplementary Fig. 1 Immunocytochemistry analyses at D14 for the A9 dopaminergic differentiation marker, the PITX-3/GIRK2, in red .



Supplementary Fig. 2 Flow cytometric examination the differentiation rate of the NSCs cultured in alginate hydrogel with different concentration Cripto-1 antibody immobilized.



Supplementary Fig. 3 The 3D reconstruction of the neural network formed by the differentiation of NSCs cultured in our alginate hydrogel with Cripto-1 antibody immobilized.