Preparation and *in vitro* Evaluation of Injectable Alginate/Thiolated Chitosan Hydrogel Scaffold for Neural Tissue Engineering

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Introduction: Spinal cord injuries are one of the main causes of disability with devastating neurological consequences and secondary conflicts in other organs. Tissue engineering and regenerative medicine have been recognized as novel, promising methods in the treatment of tissue injuries, especially in neurological damage in recent decades. Hydrogels have the advantage of compatibility with damaged tissue, and injectable hydrogels can be applied in minimally invasive surgeries. This study aimed to evaluate an injectable hydrogel-based scaffold consisting of thiolated chitosan and alginate for neural tissue regeneration. **Materials and Methods:** In the present study, an injectable hydrogel-based containing thiolated chitosan and alginate was prepared. Microbiology and pH tests were performed. Microstructural properties and porosity of scaffold were evaluated by scanning electron microscope (SEM). The swelling /shrinkage ratio and rates of biodegradation were also conducted. Finally, the viability of L929 cells on the scaffold was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **Results:** Thiolated chitosan/ alginate hydrogel had low pH with no contamination. SEM showed hydrogel had a porous microstructure with a mean pore diameter of 21.89 ± 0.32 µm which is suitable for cell culture. Furthermore, according to MTT test results, this hydrogel was biocompatible. **Conclusion:** Thiolated chitosan/ alginate hydrogel is convenient for application in neural tissue engineering based on its structural properties and its ability to support cell proliferation. According to the in vitro analysis, it can also be used as a scaffold to create a suitable environment for increasing cell viability.

Keywords: Alginate; Hydrogel; Injectable; Neural Tissue Engineering; Thiolated Chitosan

Introduction

Spinal cord injuries often lead to permanent functional defects due to the lack of axonal regeneration at the lesion site (1). To provide axon guidance and synapse development in nerve repair after injury, axonal communication is critical for healing (2). Extensive studies have been shown the inability of the central nervous system to repair itself (3, 4). Nowadays, the use of regenerative medicine with the aim of replacing or preserving the anatomical structure and function of damaged or lost tissue, in combination with other methods, has been considered (5). Producing a suitable scaffold with good biocompatibility and mechanical properties that can simulate natural microenvironments for cells is one of the key principles in tissue engineering (6). Conventional methods of tissue repair using pre-formed scaffolds were associated with increased risk of infection, and improper adaptation to the lesion site which leads to regeneration failure. To tackle such problems, injectable hydrogels have been proposed. Their ease of application, good permeability, access to the very deep tissue lesions with minimal invasion, better compliance with the lesion boundaries, and complete filling of the lesion site, and ultimately, their low risk of infection, scarring, and pain made them attractive biomaterials for neural regeneration (6).

Among biomaterials, hydrogels are swollen and have porous three-dimensional polymer networks with elastic properties; hence, can store and release various minerals and

nutrients (7). Alginate, consists of repeating units of β-D-mannuronic acid and α-L-gluronic acid, is a biocompatible hydrogel, extracted from brown algae (8). It has been shown that is suitable for transferring the required materials to the nervous tissue due to its capability to slow release in the target tissue. This anionic polymer also has properties including low toxicity and slow gelling rate under the influence of divalent cations such as calcium (9).

Chitosan is a versatile polymer used in tissue engineering. Topical application of chitosan showed the ability to repair damaged nerve membranes and thus dramatically improves the conduction of nerve impulses after complete nerve severance (10). Studies have shown that the intermediate product of chitosan degradation, known as chitooligosaccharide (COS), has a regenerative stimulation effect on the nerve in a rat model (11).

So far, different natural and synthetic materials, with different degrees of success, have been used to repair nerve tissue and create a connection between two ends of the damaged spinal cord. Based on the properties of chitosan and alginate and their role in improving the repair of spinal cord injuries, the aim of the present study was to produce a composite and injectable scaffold and evaluate its structural properties with neural tissue engineering approach.

Materials and Methods

Preparation of thiolated chitosan

All materials were initially sterilized with a UV chamber. Then, 1% solution of high viscosity chitosan (Sigma- Aldrich (Cat No. = 48165-100G)) was dissolved in acetic acid at 50°C using a magnetic stirrer. Then 1g of thioglycolic acid (TGA) (Sigma-Aldrich (T3758)) was added to thiolating chitosan. Next, 1g of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) (Sigma-Aldrich (E0388)) was added and dialyzed 5 times by dialysis bag (Pore size: 12-14 kd) in three consecutive days. At each stage of dialysis, hydrochloric acid at concentrations of 5 M (once), 5 mM, 1% NaCl (twice), and 1 mM (twice) were used. The chemical structures of chitosan and thiolated chitosan are shown in Figure 1.

Preparation of hydrogel

Chitosan and alginate were dissolved in sterile distilled water at concentrations of 2% w/v and 1% w/v, respectively. Then, the solutions were mixed with a volume ratio of 50/50. For chemical cross-linking, beta glycerol phosphate and calcium chloride

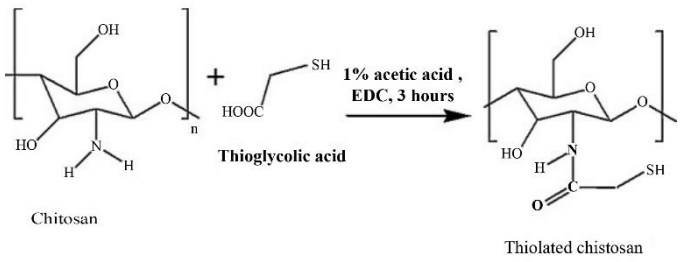

Figure 1. Chemical structure of chitosan and thiolated chitosan

were added to chitosan and alginate, respectively. Finally, the properties of this hydrogel were evaluated.

Microbial contamination and pH tests: After preparing the hydrogel, the sample was cultured in blood agar, McConkey agar, and Sabouraud dextrose agar media for 72 hours. The pH value was also measured.

Scanning electron microscopy (SEM)

The microstructural features of chitosan, alginate, and thiolated chitosan/alginate hydrogel were assessed by SEM (FESEM NOVA NanoSEM 450). The lyophilized samples were sprayed with gold nanoparticles in a thickness of 10 nm and prepared for scanning.

Swelling/shrinkage ratio

The swelling ratio was measured *in vitro* by checking the changes in scaffold weight in PBS (pH = 7.4) in an incubator at 37°C. The rinsing or dewatering processes were evaluated at different time points (0, 6, 24, 48, and 72 hours), and the swelling percentage was calculated as below:

$$\text{Swelling (\%)} = (Wt - W0)/W0 \times 100$$

In this equation, W0 and Wt are the weights of hydrogel before and after immersion in PBS solution, respectively.

Biodegradability rate

The biodegradability rate of thiolated chitosan/alginate hydrogel was measured in PBS (pH = 7.4) at 37°C by examining the changes in scaffold weight. After preparation, the hydrogel was placed in an incubator at 37°C for 1h to coagulate and form a three-dimensional network structure. Then, the obtained gel was immersed in PBS. Weight changes of samples were measured at different time intervals (days 1, 2, 3, 4, 6, 10, 14, and 21) to assess the biodegradability period. The degradation percentage was calculated as follows:

Degradation (%) = (W0 –Wt)/W0 × 100

Here, W0 and Wt are the weights of hydrogel before and after immersion in PBS solution, respectively.

Viability

L929 cells were used for cell viability using 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT). To do this, the appropriate number of cells were seeded in a 96-well plate with a final volume of 200 µL complete culture medium. After 24 hours of incubation, the cells adhered to the surface. 100 µL of hydrogel was added to each test well. Each concentration was tested in triplicate. After 1, 3, 5, and 7 days, the culture medium was removed and 20 µL of MTT solution (5 mg/mL) was added to each well. After about 4 hours, when the purple crystals were formed as shown in Figure 2, the supernatant was completely removed and 200 µL of DMSO or isopropanol solution was added. After 5 minutes, the UV absorbance rate was read at 570 and 630 nm using a spectrophotometer (cytation imaging reader, BioTek, USA).

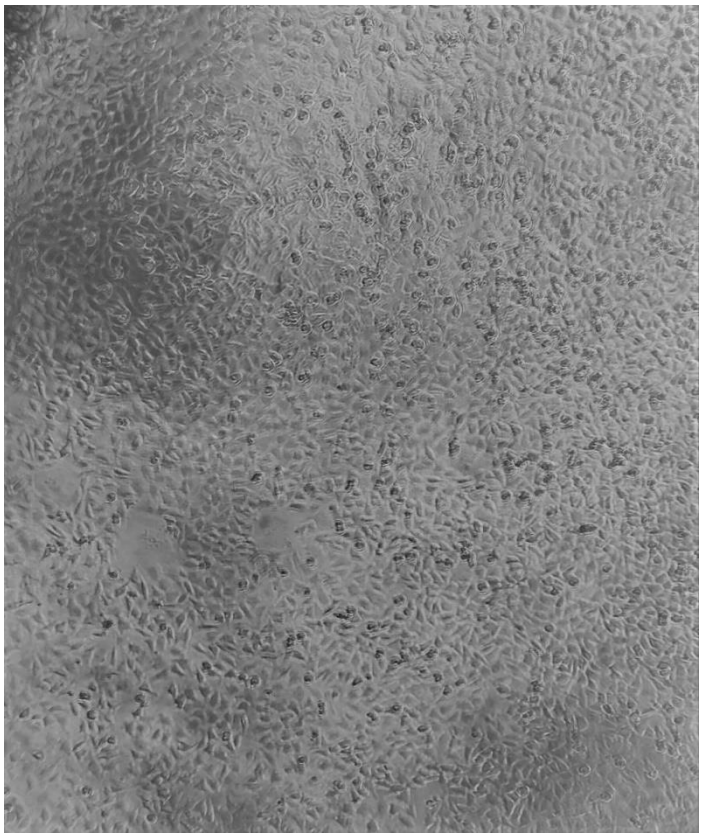

Figure 2. Formazan produced from MTT salts by mitochondria

Statistical analysis

The results were analyzed using IBM SPSS Statistics version 26.0 software (IBM, Armonk, NY, USA) and Prism version 6. For variables in this study, data were analyzed with the repeated measures and a two-way ANOVA test. $P \leq 0.05$ was considered statistically significant.

Results

Microbial contamination and pH tests

After 72 hours, no microorganisms grew in any medium, and the pH obtained was in the range of 6.5-7.

SEM

The microstructures of thiolated chitosan, alginate, and thiolated chitosan/alginate hydrogel were shown in Figure 3. The morphology of thiolated chitosan appeared compact and porous (Figure 3a). On the other hand, alginate had a rough, inhomogeneous, compact surface, and polyhedral particles, as shown in Figure 3b. Figure 3c illustrates that thiolated chitosan/alginate hydrogel had a porous microstructure which makes it suitable for cell culture and also had a looser structure than alginate. The mean pore diameter was 21.89± 0.32 µm.

Swelling/shrinkage ratio

The swelling percentage of thiolated chitosan/alginate hydrogel was evaluated for 3 days. The purpose of this test was to select the appropriate volume of hydrogel for injection into the neural tissue. Hence in case of swelling, an amount of hydrogel that would not cause pressure necrosis in neural tissue was selected to be injected. No significant difference was found between different times. According to Figure 4, the highest swelling was obtained in 3 hours; and after this time, the hydrogel gradually lost water and shrank.

Biodegradability rate The biodegradability rate of thiolated chitosan/alginate hydrogel was examined by immersion in PBS for 3 weeks. The results are indicated in Figure 5, with the highest rate of degradation observed on day 21. No significant difference was found between different times.

Viability

According to Figure 6, although cell viability slightly decreased in the first and second days in the hydrogel group compared to

the control, the cell population increased on the 5th day. These findings indicated that the hydrogel was not toxic to L929 cells.

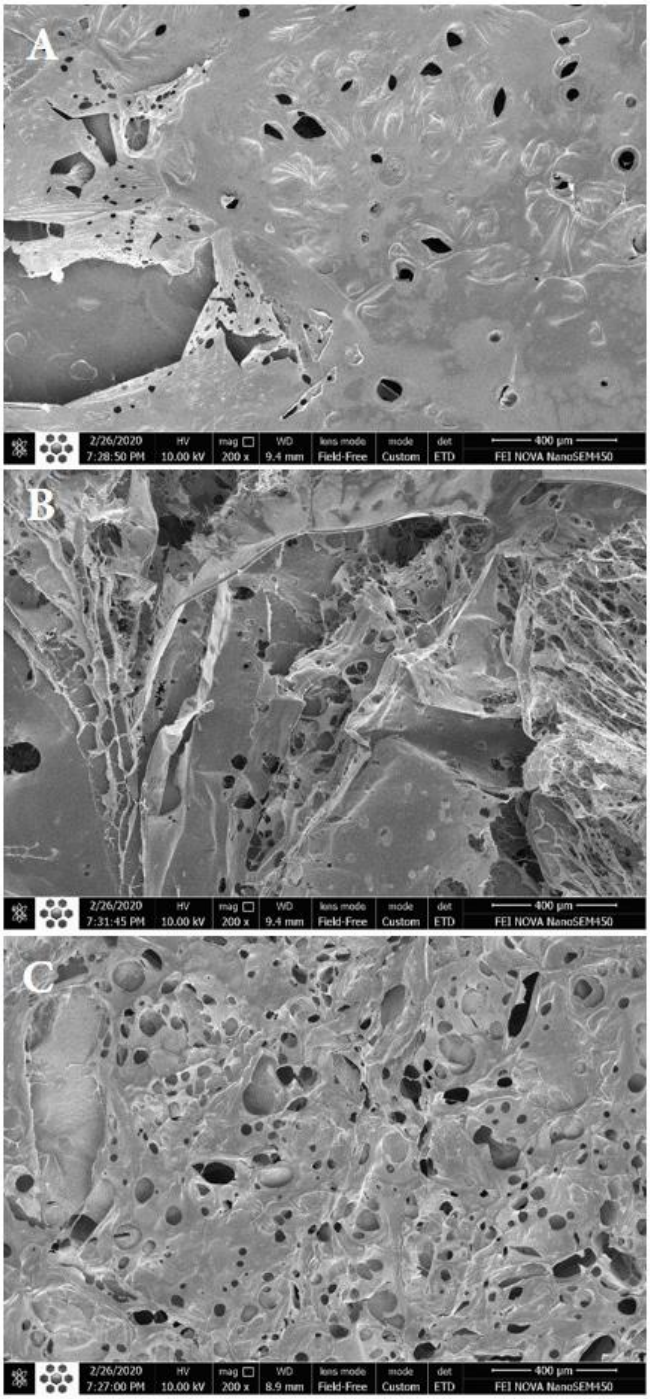

Figure 3. A) Scanning Electron micrographs of thiolated chitosan tube. B) Alginate. C) Thiolated chitosan/alginate hydrogel Magnification (×200)

Besides, this hydrogel was suitable for the growth and proliferation of these cells.

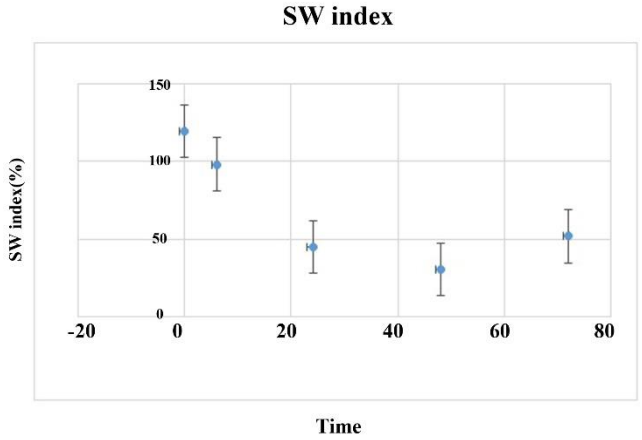

Figure 4. Swelling ratio of thiolated chitosan/alginate hydrogel during 3 days of immersion in PBS at 37 ° C as a function of incubation time

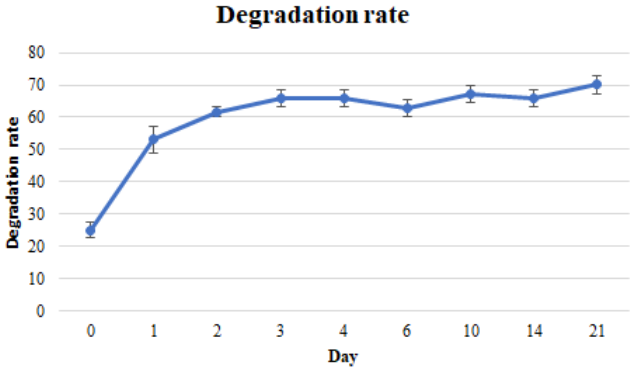

Figure 5. Biodegradability rate of thiolated chitosan/alginate hydrogel during 21 days of immersion in PBS at 37 ° C as a function of incubation time

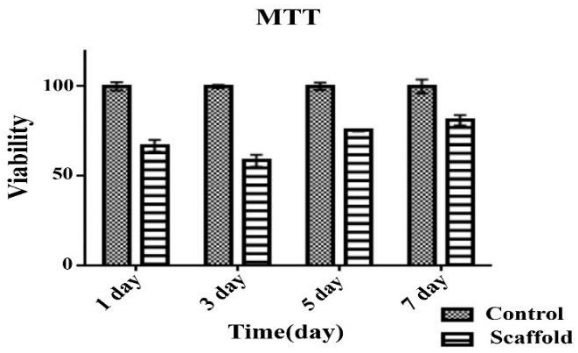

Figure 6. Evaluation of cell viability using MTT assay. The graph shows MTT results in L929 cell culture after 1, 3, 5 and 7 days of culture

Discussion

Spinal cord injuries are one of the principal causes of disability, with neurological destructive consequences in medicine and veterinary medicine (5). Recent advances in the pathophysiology of spinal cord injuries have led to the development of promising treatments. In this context, tissue engineering plays a key role in bridging the structural lesions of spinal cord injury and providing facilitation for functional improvement (3-5). Most studies have been carried out on transected spinal cord models, while approximately 60% of clinical spinal cord injuries are due to contusions, which form a cavity containing growth and repair inhibitory factors in the spinal cord (1). In these cases, accelerating the healing process and preventing the formation of glial scars require the use of cell therapy, injectable scaffolds, or a combination of them with the aim of replacing them with the contents of the vacuole formed due to injury (1). Limited research has addressed the use of injectable scaffolds in spinal cord contusion models (1). Adaptation of scaffold to the damaged tissue is the principal goal of tissue engineering. Regarding the fact that hydrogels have good porosity and three-dimensional structure that enables them to absorb a significant volume of water, they are accounted a good candidate for this purpose (6).

In this study, high-viscous chitosan was used as a natural biomaterial in the composite hydrogel. Chitosan-based hydrogels are used in tissue engineering, regenerative medicine, drug delivery, and wound healing (12). Chitosan is composed of a partial deacetylated product of chitin and includes N-acetyl-glycosamine, which is a biodegradable, biocompatible, non-antigenic, and non-toxic natural substance (13, 14). Chitosan encapsulates Schwann cells, differentiates neural stem cells, and creates bridge-like ducts inside and outside the tissue (15). Chitosan also has antibacterial and hemostatic activities (4, 13). It has properties such as minimal immune response, appropriate processing conditions, i.e., synthetic polymers often need to be dissolved in harsh chemicals but chitosan is soluble based on pH in water, mechanical properties, and manageable biodegradability (7). In 2008, Nomura *et al.,* used a chitosan scaffold seeded with neural stem cells to repair rat transected spinal cord. The results showed the formation of a tissue bridge containing the implanted cells and the survival of the host axons though no motor progress was seen (16). In 2017, Chedly *et al.,* used a chitosan scaffold to repair the transected spinal cord and they showed the regeneration of spinal tissue and its blood vessels, reduced glial scarring, growth of myelinated axon fibers, migration of Schwann cells to the lesion site, and reduced inflammatory response (17). In 2015, Jian *et al.,* implanted chitosan with an extracellular matrix of glioma to the transected spinal cord and observed the differentiation of a large number of neural stem cells into neurons, astrocytes, and oligodendrocytes (18).

Alginate was another biomaterial used in this study. Sodium alginate is a natural polysaccharide extracted from brown algae that have good stability, solubility, viscosity, and biodegradability (3, 4). Alginate is soluble in aqueous solvents and forms a stable gel at room temperature in the presence of divalent cations (e.g., Ba2 +, Ca2 +) (14). In 2006, Prang *et al.,* found that alginate-based hydrogel has an axonal repair effect on spinal cord injuries (19). Grulova *et al.,* (2015) reported a significant functional improvement in the use of injectable alginate scaffolds together with growth factors in repairing spinal cord injuries resulting in contusions (20).

In 2018, Yao *et al.,* studied the effect of sodium alginate and chitosan composite on the transected spinal cord. They reported the repair of neural fibers and the formation of astrocyte scar tissue(4). Cell proliferation on the chitosan/alginate scaffold occurs faster than that on pure chitosan. Another advantage is the ability to accommodate drugs or proteins uniformly in their matrix without changes in their nature (14). Unlike pure chitosan and alginate, chitosan/alginate scaffolds can be made from neutral pH solutions. In the present study, the hydrogel was prepared in a neutral range of pH, which is suitable for use in neural tissue.

The swelling ratio is another key feature of hydrogels for material exchange in tissue engineering. Considering that scaffold must be destroyed after implantation to permit cell growth, proliferation, and extracellular matrix production, the function of biodegradability rate, as another crucial determinant in tissue engineering, reveals. In the comparison of the swelling ratio and biodegradability rate graphs, the first chitosan/alginate hydrogel had the highest swelling rate after 3 hours, which was considered a zero-time point, and then it shrank. The highest rate of shrinkage was reported on the second day. Accordingly, the highest rate of degradation was observed on the 21st day. In 2020, Rahmati *et al.,* used chitosan/alginate hydrogel to regenerate the sciatic nerve. In their study, the highest rates of swelling and biodegradability were observed at 4 and 21 hours, respectively (21). In another study conducted by Ehterami *et al.,*

the highest swelling rate of chitosan/alginate hydrogel was reported after 4 hours, but the highest biodegradation was seen at the end of the second week (13). Archana *et al.,* investigated three types of scaffolds and they reported the highest swelling and biodegradability rates of chitosan/alginate scaffolds at the fifth hour and in the fourth week, respectively (22). In 2007, Shao and Hunter reported the highest degradation rate of chitosan/alginate scaffold on the fourth day (23).

In tissue engineering, scaffolds with high porosity are used as a support for growing tissue. The interconnected pores provide a large surface area for cell attachment, proliferation, and migration (24). In the present study, chitosan/alginate composite caused structural loosening and increased porosity compared to alginate; hence it was more suitable for tissue engineering. Based on the SEM findings of the study of Archana *et al.,* chitosan/alginate scaffold had a porous surface structure with aggregation of fibers (22). In the morphological study of the hydrogel in Ehterami *et al.,* study, the alginate and chitosan hydrogels had high-porous structures with interconnected pores (13). Shao and Hunter (2007) also stated that the combination of alginate and chitosan provided a looser and softer structure than that of alginate (23).

The cytotoxicity of the scaffold is another important feature that should be considered before implantation. One of the differences between this study and the others was the use of chemical cross-linkers to prepare chitosan/alginate hydrogel, so we could convert an acid-soluble compound to an aqueous solution. Nowadays, water-soluble hydrogels and soluble compounds in environments without the presence of organic solvents have been considered. In 2007, Shao and Hunter reported no cytotoxicity of alginate/chitosan scaffold on 3T3 fibroblast cells (23). In the present study, it was observed that the prepared scaffold had no toxic effect on cell growth in L929 cells. In 2017, Wang *et al.,* studied chitosan/alginate hydrogel and its enhancing effect on neural cell proliferation (3).

Chitosan/alginate hydrogel is easy and fast compared to other hydrogels in terms of preparation method and is very cost-effective. On the other hand, the injectability of this hydrogel is also very important. Considering the sensitivity and vulnerability of spinal cord tissue, applied treatment methods for scaffold placement should be as non-invasive as possible. Therefore, very fine needles should be used, and the possibility of injectable hydrogels with suitable viscosity would be very important.

Conclusion

In this study, the structural properties and injectability of thiolated chitosan/alginate hydrogel on one hand, and the easy and cost-effective method of preparation, on the other hand, suggested it as a suitable option for being used in spinal cord injuries resulting in contusions.

Conflict of Interest: 'None declared'.

References

1. Cornelison RC. An injectable acellular nerve graft as a platform for treating spinal cord injury 2015.
2. Han Q, Jin W, Xiao Z, Ni H, Wang J, Kong J, et al. The promotion of neural regeneration in an extreme rat spinal cord injury model using a collagen scaffold containing a collagen binding neuroprotective protein and an EGFR neutralizing antibody. 2010;31(35):9212-20.
3. Wang G, Wang X, Huang LJB, Equipment B. Feasibility of chitosan-alginate (Chi-Alg) hydrogel used as scaffold for neural tissue engineering: a pilot study in vitro. 2017;31(4):766-73.
4. Yao Z CF, Cui H, Lin T, Guo N, Wu H. Efficacy of chitosan and sodium alginate scaffolds for repair of spinal cord injury in rats. Neural Regen Res. 2018;13(3):502-9.
5. Volpato FZ FT, Migliaresi C, Hutmacher DW, Dalton PD. Using extracellular matrix for regenerative medicine in the spinal cord. Biomaterials. 2013;34(21):4945-55.
6. Sivashanmugam A, Kumar RA, Priya MV, Nair SV, Jayakumar RJEPJ. An overview of injectable polymeric hydrogels for tissue engineering. 2015;72:543-65.
7. Li J, Chen G, Xu X, Abdou P, Jiang Q, Shi D, et al. Advances of injectable hydrogel-based scaffolds for cartilage regeneration. 2019;6(3):129-40.
8. Hashimoto T, Suzuki Y, Suzuki K, Nakashima T, Tanihara M, Ide CJJoMSMiM. Review Peripheral nerve regeneration using non-tubular alginate gel crosslinked with covalent bonds. 2005;16(6):503-9.
9. Lee KY, Mooney DJJPips. Alginate: properties and biomedical applications. 2012;37(1):106-26.
10. Cho Y, Shi R, Borgens RBJJoeb. Chitosan produces potent neuroprotection and physiological recovery following traumatic spinal cord injury. 2010;213(9):1513-20.
11. Wang Y, Zhao Y, Sun C, Hu W, Zhao J, Li G, et al. Chitosan degradation products promote nerve regeneration by stimulating schwann cell proliferation via miR-27a/FOXO1 axis. 2016;53(1):28-39.

12. Stefanov I, Hinojosa-Caballero D, Maspoch S, Hoyo J, Tzanov T. Enzymatic synthesis of a thiolated chitosan-based wound dressing crosslinked with chicoric acid. J Mater Chem B. 2018;6(47):7943-53.
13. Ehterami A, Salehi M, Farzamfar S, Samadian H, Vaez A, Ghorbani S, et al. Chitosan/alginate hydrogels containing Alpha-tocopherol for wound healing in rat model. 2019;51:204-13.
14. Li Z, Zhang MJJoBMRPAAOJoTSfB, The Japanese Society for Biomaterials,, Biomaterials TASf, Biomaterials tKSf. Chitosan–alginate as scaffolding material for cartilage tissue engineering. 2005;75(2):485-93.
15. Madigan NN MS, O'Brien T, Yaszemski MJ, Windebank AJ. Current tissue engineering and novel therapeutic approaches to axonal regeneration following spinal cord injury using polymer scaffolds. Respir Physiol Neurobiol. 2009;169(2):183-99.
16. Nomura H, Zahir T, Kim H, Katayama Y, Kulbatski I, Morshead CM, et al. Extramedullary chitosan channels promote survival of transplanted neural stem and progenitor cells and create a tissue bridge after complete spinal cord transection. 2008;14(5):649-65.
17. Chedly J SS, Montembault A, Von BY, Veron-Ravaille M, Mouffle C, Benassy MN, Taxi J, David L, Nothias F. Physical chitosan microhydrogels as scaffolds for spinal cord injury restoration and axon regeneration. Biomaterials. 2017;138(91):107.
18. Jian R, Yixu Y, Sheyu L, Jianhong S, Yaohua Y, Xing S, et al. Repair of spinal cord injury by chitosan scaffold with glioma ECM and SB 216763 implantation in adult rats. 2015;103(10):3259-72.
19. Prang P, Muller R, Eljaouhari A, Heckmann K, Kunz W, Weber T, et al. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials. 2006;27(19):3560-9.
20. Grulova I, Slovinska L, Blasko J, Devaux S, Wisztorski M, Salzet M, et al. Delivery of Alginate Scaffold Releasing Two Trophic Factors for Spinal Cord Injury Repair. Sci Rep. 2015;5(1):13702.
21. Rahmati M EA, Saberani R, Abbaszadeh-Goudarzi G, Rezaei Kolarijani N, Khastar H, Garmabi B, Salehi M. Improving sciatic nerve regeneration by using alginate/chitosan hydrogel containing berberine. Drug Deliv Transl Res. 2020.
22. Archana D, Upadhyay L, Tewari R, Dutta J, Huang Y, Dutta P. Chitosan-pectin-alginate as a novel scaffold for tissue engineering applications. 2013.
23. Shao X, Hunter CJJJoBMRPAAOJoTSfB, The Japanese Society for Biomaterials,, Biomaterials TASf, Biomaterials tKSf. Developing an alginate/chitosan hybrid fiber scaffold for annulus fibrosus cells. 2007;82(3):701-10.
24. Aksoy EA, Sezer UA, Kara F, Hasirci NJJoB, Engineering T. Heparin/chitosan/alginate complex scaffolds as wound dressings: characterization and antibacterial study against Staphylococcus epidermidis. 2015;5(2):104-13.

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