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Review

Trends of Chitosan Based Delivery Systems in Neuroregeneration and Functional Recovery in Spinal Cord Injuries

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Abstract: Spinal cord injury (SCI) is one of the most complicated nervous system injuries with challenging treatment and recovery. Regenerative biomaterials such as chitosan are being reported for their wide use in filling the cavities, deliver curative drugs, and also provide adsorption sites for transplanted stem cells. Biomaterial scaffolds utilizing chitosan have shown certain therapeutic effects on spinal cord injury repair with some limitations. Chitosan-based delivery in stem cell transplantation is another strategy that has shown decent success. Stem cells can be directed to differentiate into neurons or glia in vitro. Stem cell-based therapy, biopolymer chitosan delivery strategies, and scaffold-based therapeutic strategies have been advancing as a combinatorial approach for spinal cord injury repair. In this review, we summarize the recent progress in the treatment strategies of SCI due to the use of bioactivity of chitosan-based drug delivery systems. An emphasis on the role of chitosan in neural regeneration has also been highlighted.

Keywords: chitosan; spinal cord injury; electrospun scaffolds; nerve regeneration; stem cell-based transplantation

1. Introduction

The spinal cord lies within a bony (skeletal) canal formed by adjacent vertebrae and soft tissue elements (the vertebral canal). The anterior wall is formed by the vertebral bodies of the vertebrae, intervertebral discs, and associated ligaments. The lateral walls and roof are formed by the vertebral arches and ligaments [1]. Within the vertebral canal, the spinal cord is surrounded by a series of three connective tissue membranes (the meninges) namely; (a) the pia mater is the innermost membrane and is intimately associated with the surface of the spinal cord, (b) the second membrane, the arachnoid mater is separated from the pia by the subarachnoid space which contains cerebrospinal fluid, and (c) the thickest and most external of the membranes, the dura mater which lies directly against but is not attached to the arachnoid mater [1]. In the vertebral canal, the dura mater is separated from the surrounding bone by an extradural (epidural) space containing loose connective tissue, fat, and a venous plexus. The primary curvature of the vertebral column is anteriorly concave, reflecting the original shape of the embryo, and is retained in the thoracic and sacral regions in adults. Secondary curvatures, which are posteriorly concave,

are formed in the cervical and lumbar regions and bring the center of gravity into a vertical line that allows the body's weight to be balanced on the vertebral column in a way that expends the least amount of muscular energy to maintain an upright bipedal stance [2]. The human spine and cross-sectional of the spinal cord are depicted in Figure 1.

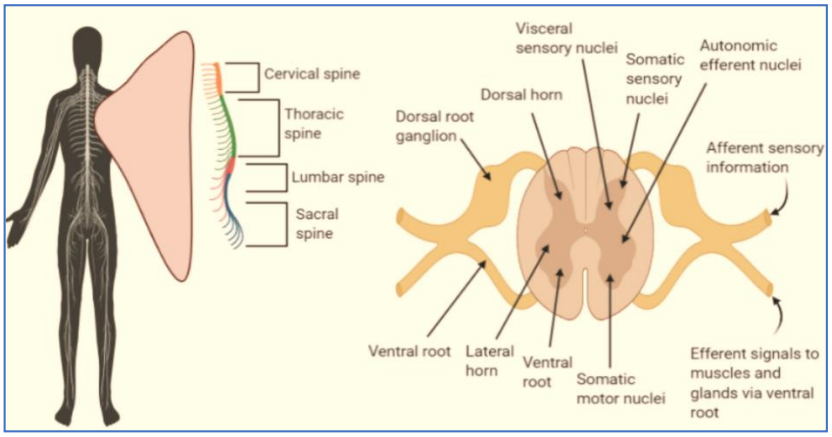

Figure 1. Human spine and the cross-section of the spinal cord [1].

Spinal cord injury (SCI) is a debilitating disease with a high rate of disability involving paralysis, sensorimotor dysfunction, urinary incontinence, and gastrointestinal dysfunction [3]. Subsequently, SCI patients and their families suffer a low quality of life with the burden of long-term medical care and disability [4]. As a result of SCI, neuronal cells die in the span of the first 12 h to a few weeks and further leads to substantial neuronal and glial cell loss, demyelination, cavitation, and glial scarring which in turn results in loss of sensory perception, distal motor paralysis, and severe functional limitations [5,6]. Albeit, recovery is more often difficult due to the limited capability of the central nervous system to regenerate the lost cells, restore disrupted myelin, and re-establish functional neural connections [7], but the recent developments and impulses in molecular and regenerative medicine had paved the way for inducing the biological active cells such as stem cells, bioactive materials and growth factors towards the healing and tissue regenerative process. In this connotation, mesenchymal stem cells (MSCs) serve as the perfect cell-based tissue regenerative modality for treating disorders under a minimally invasive environment without any significant morbidity, which further induces cellular proliferation, differentiation, characterization, regeneration, and rejuvenation of degenerated tissue to attain native homeostasis [8,9]. The efficacy of such cell therapies in animal models has been widely recognized [10]. Several preclinical studies and clinical trials have revealed that neuronal progenitor and stem cells can be used to repair SCI because of their self-renewal property and capacity for neuronal differentiation into the functional neural cells to form new synapses, release various neurotrophic factors, and provide an appropriate conducive microenvironment to promote neuronal repair [11,12]. Although the reliability of such treatment methodology for SCI is being tested in human subjects by a few clinical trials, they provide us with conflicting results and thereby clouding this only ray of hope for SCI patients [13,14]. In this review, we summarize the recent progress in the treatment strategies of SCI with an emphasis on biomaterial/chitosan scaffolds.

2. Stem Cell-Based Interventions of Spinal Cord Injury

2.1. MSCs: Immunomodulation and Trophic Support for SCI

MSCs are particularly appealing for SCI repair and currently constitute the most promising type of stem cells in preclinical and clinical research on account of their relative ease of access and efficient in vitro expansion. Compared to other stem cells, they rouse no ethical concerns. They can be used in autologous transplants and are presumably safe when inserted into the CNS. MSCs can be collected from different sources such as

bone marrow, umbilical cord, amniotic liquid, and adipose tissue. MSCs have recently shown desirable properties for therapeutic use in CNS pathologies (Alzheimer's disease, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis) including anti-inflammatory, immunomodulatory, trophic, and anti-apoptotic effects in different animal models of CNS disorders. We shall discuss the various types of MSCs that are applied for the treatment of SCI.

2.2. Bone Marrow Stem Cells (BM-MSCs)

Bone marrow (BM)-MSCs may protect the injured spinal cord from further cellular damage via trophic support and neuroprotective activities among the trophic factors. The best-studied are vascular endothelial growth factor (VEGF), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) that are known to support neural protection and fiber regeneration [15]. Umbilical cord (UC)-MSCs are easily obtained by treating umbilical cord or cord blood from the newborn, can be stored at cryogenic temperatures until use. The key advantages of UC-MSCs are hypoimmunogenic and cause less graft rejection than other stem cells. Preclinical studies have shown their broad therapeutic capacity with multifaceted efficacy in several rats and mouse SCI animal models, including neurotrophic, anti-inflammatory anti-apoptotic, and angiogenic actions [16]. Similarly, amniotic fetal (AF)-MSCs can be derived from amniotic fluid or amniotic membrane and are considered as an alternative source of MSCs for regenerative medicine in SCI. They offer several advantages such as minimal invasive isolation and no ethical issues. They also show multipotency, efficient proliferative activity, non- tumorigenicity, and low immunogenicity [17]. The final, adipose tissue (A)-MSCs can be obtained from adipose tissue in large amounts and are characterized by the secretion of trophic growth factors (BDNF and GDNF), modulation of activated immune cells, neuroregeneration, anti-apoptotic action, and multilineage differentiation capacity which may confer potential regenerative effects in SCI [18].

2.3. Embryonic Stem Cells (ESCs)

The pluripotent nature of ESCs may allow them to generate new cells in human or animal CNS tissue, including neurons and glial cells. One of the major strategies for treating the injured spinal cord is to induce ESCs to differentiate into specific phenotypes to replace the desired cell (neurons or glia) or to produce factors that could limit the damage and sustain regeneration of the tissue [19,20]. For instance, differentiation of ESCs into motor neurons using a combination of retinoic acid and sonic hedgehog protein was demonstrated in vitro, as well as the following transplantation in vivo into the spinal cord of a paralyzed adult rat. ESCs differentiated into oligodendrocytes have been used to treat SCI, achieving some improvements in motor activity after reconstitution of part of the white matter in transection or contusion SCI rat models [21].

2.4. Neural Stem Cells (NSCs)

NSCs are isolated from the subventricular zone of the hippocampus of the brain and a region of the central canal of the spinal cord and can differentiate into specific neuronal or glial phenotypes to replace lost tissue or produce pro-regenerative factors. Transplantation of NSCs into the lesioned spinal cord leads to functional recovery, sustained through neuronal cell replacement that was able to reconstitute lost neuronal and glial tissue with trophic support (BDNF, CNTF, GDNF, NGF, and IGF-1) preserving damaged cells and axons [22]. Studies have shown the immunomodulatory activities of NSCs that can be helpful in the treatment of SCI [23].

2.5. Induced Pluripotent Stem Cells (iPSCs)

iPSCs are generated by reprogramming somatic cells in the presence of the necessary transcription factors (Yamanaka factors), as well as various other methods including viral transfection, microRNA delivery, targeted insertion, transposon-based insertion, and

protein transfection. iPSCs circumvent ethical concerns regarding the use of embryos and allow autologous transplantation of pluripotent cells which should reduce the risk of rejection. In another study, chitosan membranes for sustained proliferation and pluripotency of human induced pluripotent stem cells (hiPSCs) in long-term culture (up to 365 days) were prepared and reported. On the chitosan membranes, hiPSCs self-assembled into 3D spheroids with an average diameter of ~100 μm. These hiPSC spheroids could be directly differentiated into lineage-specific cells from the three germ layers with 3D structures. Collectively, chitosan membranes not only promoted the naïve pluripotent features of hiPSCs but also provided a novel 3D differentiation platform. [24]

2.6. Olfactory Ensheathing Cells (OECs)

OECs are glial cell types that play an important role in the neural regeneration of olfactory neurons by supporting and guiding their constant replacement and axon growth from the peripheral nervous system into the CNS. OECs can be obtained through nasal biopsies from the olfactory mucosa (OM) located in the nasal cavity or from the olfactory bulb (OB). OECs hold great promise for SCI regenerative treatment because after implantation in the damaged spinal cord they can create a permissive environment for axonal regeneration that can cross the injured site in several rodent SCI models [25].

2.7. Schwann Cells

Schwann cells in peripheral nerves support axonal regeneration after damage, and this has suggested their potential application in spinal cord injury. Schwann cells could contribute to regeneration after injury by sustaining axonal regrowth and myelination which is necessary for appropriate axonal functioning. Schwann cells offer several properties that could enhance recovery after SCI, such as the production of a variety of growth factors (including NGF, BDNF, and CNTF), cell adhesion molecules (N-CAM, N-cadherin, and integrins), and extracellular matrix proteins (collagen and laminin) [26–28].

2.8. Adult Endogenous Stem Cells (AESCs)

AESCs such as ependymal cells, located in proximity to the central canal in the spinal cord, have stem cell properties that proliferate and constitute mostly new glial cells in the injured spinal cord. The regenerative response of these cells after an injury has been shown in different mouse or rat SCI models [29]. In another study, chitosan loaded with neurotrophin-3 (NT3) was prepared and injected into lesion of traumatic brain injury (TBI) and the results proved NT3 to effectively engaged endogenous NSCs to proliferate and migrate to the injury area. Three main actions of NT3-chitosan, i.e., pro-neurogenesis, anti-inflammation, and pro-revascularization, elicited significant regeneration after TBI [30].

3. Factors for the Regeneration of Cells in Spinal Cord Injury

A spinal cord injury is a very grave incident with varying effects on sensory and motion depending on the point of injury in the spinal cord and its severity. Receiving the sensory input signals and establishing the spinal network by the neurons attached to the spinal network is challenging and, is very crucial for the treatment. Drug delivery to the CNS has its challenges due to the presence of a blood–brain barrier (BBB) along with the blood spinal cord barrier and the blood–retinal barrier. These barriers make the delivery of the drug to the site of injury complicated, and the endothelial cells of the blood–brain barrier limit the paracellular and also the transcellular transport due to lack of fenestrae, the activity of endocytic vesicles, as well as high metabolic activity [31,32]. The small molecule pharmacological agents fail to pass through the barriers which lead to poor vascular drug delivery to the CNS [33,34], which is the key limitation of using the traditional drug delivery strategies in this case. To overcomes this issue, targeted methods are used which allow the drug to pass or move through the blood–brain barrier. There are certain ideal properties of a drug delivery system as illustrated in Figure 2 and it is a challenging task to combine all these properties in a single drug delivery system.

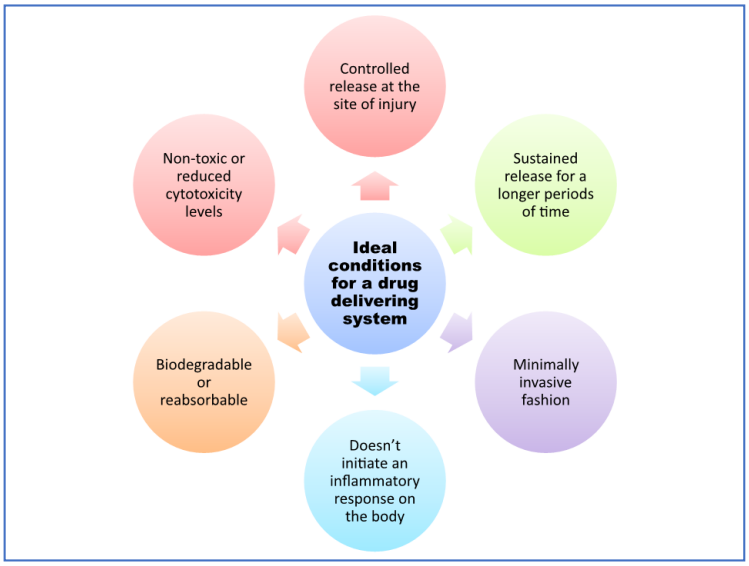

Figure 2. Ideal properties of drug delivery system.

Current applications of direct delivery system to the site of injury have many limitations and are as follows.

3.1. Bolus Injection

The bolus injection is injected into the intrathecal space for the transfer of drugs to the CNS but the route to the spinal cord is disturbed by the continuous cerebrospinal fluid (CSF) flow which moves at a rate of 0.35 mL.min^{-1} [33]. The continuous flow of CSF does not allow any accumulation of the drugs which are injected into the CNS. The speed of CSF flow in addition to the clearance and regeneration of the entire volume of CSF every five hours are the key challenges that require higher dose injections as well as increased frequency [33].

3.2. Continuous Infusion Using a Catheter or a Minipump System

This device implantation has increased risks and shortcomings. The implantation is invasive and sometimes causes cell death at the insertion site [35], and increases the risk of infection [36]. Similarly, catheters have several complications as they are susceptible to dislodgement, kinking, get torn, and disconnected. Besides, studies have shown that 40% of people who use catheter gets infected [37]. The intravenous infusion is disturbed by the low diffusion rate from the ventricular system to the brain parenchyma cells. The efficiency of the diffusion rate of drugs usually gets lowered with the square of the distance; hence, a usual small molecule has a diffusion coefficient of 5×10^{-6} cm^2 s^{-1} which takes around eight hours to diffuse through 1 mm [38]. Since the diffusion rate is of eight hours, it means that the drug gets cleared off from the CNS even before it has time to enter the tissue [33]. Recently, biodegradable polymeric implants are used as drug delivery agents for controlled delivery [39,40]. A major limitation of these pre-formed polymeric implants is that they need invasive surgery for the implantation of the polymer. Another alternative to the physical implants is the injectable in situ gelling hydrogels which is less invasive than surgery-based implantation. The polymers now used are biodegradable which even removes the need for surgical removal of polymers and these biodegradable polymers do no persist in the bloodstream after drug release.

4. Role of Chitosan in Neuroregeneration

Chitosan, which is derived from Chitin, is a renewable natural polymer (cationic polysaccharide) and is the most abundant natural polymer after cellulose. It acts as a significant biomaterial for developing drug delivery vehicles, tissue engineering scaffolds,

and wound dressings due to its hemostatic and mucoadhesive properties. Chitosan's versatility lies in its chemical reactivity, which allows the development of several chitosan derivatives with varied chemical, physical and biological properties.

4.1. Delivery Systems for Neuroregeneration

Neural regeneration is the key step to CNS injury and there have been several studies to understand neuronal regeneration. There are several nanomaterials, but this chapter discusses the potential of chitosan to aim at modifying the CNS nerve regeneration; following which several possible clinical applications have been attempted. The nanoparticles can accomplish non-toxic, non-ionic properties with their polymers. Such membrane active polymers like PEG (polyethylene glycol) have demonstrated to be capable of sealing or repairing the CNS nerve system through a series of steps [41]. The reasons behind their ability are as followed;

I. They actively interact with the lipid bilayer.
II. The rearrangement of the membrane is spontaneous.
III. They can reduce membrane defects.

To avoid the side effects as much as possible, in the past few decades the research is focused on the particulate drug delivery system [42]. The direct transport of therapeutic drugs is very challenging due to the blood–brain barrier (BBB), which is addressed by the application of nanoparticles that not only effectively cross the BBB but also extend the circulatory half-life of the drugs [43–45]. The first drug delivery using nanotechnology was done with the aid of PEG, i.e., nanoparticle-based fusogen [35–41]. The feasibility of a controlled delivery system with specificity and selectivity is due to certain properties of NP formulations such as inert, non-toxic properties with a large surface to volume ratio. The nanoparticle delivery systems also offered supplementary advantages such as the NP systems could be encapsulated with CNS-targeting drugs through conjugation to increase the drug efficacy and the target site-specificity. Besides, NP allows a long circulation time and a controlled delivery system thereby, enhancing the bioactivity. The advantages offered by the nanoparticle system are worth noting and offer a potential delivery system to treat and predict disease and also have a wide variety of biomedical applications.

4.2. Chitosan-Based Delivery in CNS Therapeutics

Chitosan has been reported for a controlled drug delivery system due to its ability to conjugate with organic material along with biomolecules. It can incorporate biologically active substances like DNA, proteins, anticancer drugs, and insulin as well [46]. A variety of applications of chitosan including drug carriers, wound-healing agents, and tissue engineering are attributed to the cationic charge on the chitosan and its mucoadhesive features make it highly reactive and thereby used as a potential biomaterial [47–50]. A study report by Cho et al., 2010 showcases the ability of chitosan to function as a membrane sealant and more importantly as a potent neuroprotector. In several different studies, the capability of chitosan was discovered to induce the sealing of neuronal membranes and could potentially renew the damaged nerve impulses along the entire length of the spinal cord, specifically targeting the site of the injury. Apart from that, chitosan could be promptly involved in the formulation of microspheres or microcapsules which function as a carrier for the restrained drug delivery system. In one of the recent studies, it has been shown that when chitosan was loaded with hydralazine it created a liberal environment that leads to the survival of cells facing the endogenous toxins [51–56]. The surface charge of chitosan varies with its functional groups, i.e., polyanions. For instance, the surface charge for phosphoric acid in tripolyphosphate is 14.51 ± 2.58 mV and for the sulfate group in dextran sulfate, the charge is -4.84 ± 1.38 mV. The surface charge is linked with the electrostatic interaction taking place in the positively charged amine molecules of hydralazine and also the active moieties of polyanions which is crucial in improving the encapsulation efficiency of hydralazine loaded nanoparticles. Hydralazine loaded chitosan nanoparticles reported an encapsulation efficiency of 15.8% for the spherical shaped solid

structure of phosphoric acid in tripolyphosphate (TPP) while the efficiency of 23.5% for the sulfate group in dextran sulfate (DS). Several factors could be associated with accounting for the drug release of hydralazine which involves the loose attachment of hydralazine to the chitosan nanoparticle surface or the lack of controllability in the dissociation of the drug from the core of the nanoparticle system [57].

5. Role of Chitosan-Based Formulations in SCI

5.1. Micro/Nanoparticles (NP)

The role of nanoparticles is increasingly being investigated with the help of experimental models for SCI. The nanoparticles which are tested for the experimental models to treat the SCI range from metals like gold, silver, oxide, iron oxide [58,59], polymers including PLGA [60,61] liposomes [62], and others. Let us briefly look at each category.

5.1.1. Iron Oxide, Gadolinium, or Cobalt Platinum Nanoparticles

Iron oxide nanoparticles are a famous tool for clinical applications in SCI, specifically for cell tracking followed by the cellular implantation in the spinal cord [63–65]. The size range could vary from 5 to 300 nm for superparamagnetic iron oxide NP. The magnetic properties of iron oxide make it a useful application in MRI (magnetic resonance imaging) [66]. Cells that are injected with the iron oxide nanoparticles can be tracked in vivo through MRI [67]. These nanoparticle formulations could be directly infused inside the animal body to visualize the infiltration of the macrophages in the CNS region which is followed by autoimmune encephalomyelitis [68] or blood–spinal cord barrier permeability following SCI [69]. Apart from the cell tracking applications iron oxide nanoparticles could be used for gene transfer in progenitor cells with a good transfection level and the results indicate a high cell viability rate in the culture models [70,71]. The iron oxide NP location could be controlled using the magnetic fields in the spinal cord. This magnetofection could be achieved using the superparamagnetic iron oxide NP formulation at precise locations in the spinal cord [72]. There are many studies which exhibit demonstrate the use of iron oxide nanoparticles. Nishida et al. used the iron oxide nanoparticles that were magnetically labeled bone marrow stromal cells along with the mesenchymal stem cells to target the spinal cord lesions through the magnetic field in the rat model [73]. Another study used the heated iron oxide NP which was heated using a high frequency of magnetic fields to destroy the tumor cells by inducing hyperthermia [74]. Similarly, Jordan et al. showed that the iron-oxide-induced hyperthermia also extends the survival rate of animals, i.e., rats following tumor implantation in CNS [75]. Despite several benefits offered by the metal nanoparticles, it also has several drawbacks, which include the use of metal nanoparticles can lead to certain undesired effects in the tissue such as toxicity in the CNS region [76], gold nanoparticle-induced DNA damage [77], silver nanoparticle-induced developmental deficits in the spinal cord flexure [58].

5.1.2. Polymer Nanoparticles

Polymer nanoparticles have diverse applications in spinal cord injury and are very crucial in therapeutic delivery. The size of the nanoparticles ranges from 50 nm to 1000 nm and most commonly are spherical [78]. Polymers are biocompatible and thus have low toxicity even in the CNS region which makes them an ideal system for the delivery of therapeutic drugs to the spinal cord. The synthetic polymers include PLGA [79], poly(methyl methacrylate) [80], poly-*L*-lactic acid (PLLA), and polycyanoacrylate [81] which are very commonly utilized in the SCI models, whereas the natural polymers include chitosan which is rather a new approach to the spinal cord therapy and applications [82]. The polymer nanoparticles have been utilized in deducting the permeability of BBB followed by experimental allergic encephalomyelitis and induce antigen-specific T cell tolerance [81,83]. Biological materials such as proteins, DNA, or even RNA could be encapsulated in the polymeric nanoparticles for extended drug release duration. The research on the abilities of polymeric nanoparticles in spinal cord therapy is ongoing and looks promising. Chitosan

nanoparticles have been recognized as a novel type of biomaterial for the treatment of SCI which helped in targeted drug delivery to the injured spinal cord. A recent study revealed that the valproic acid-labeled chitosan nanoparticles (VA-CN) promote the recovery of neuronal injury after spinal cord injury in rat models [84]. The evaluation of valproic acid labeled chitosan nanoparticles demonstrated significant recovery of the function and tissue repair after SCI. Figure 3 shows the data depicting the decreased lesion cavity volume by the application of VA-CN compared to sham (negative control), SCI (positive control), CN, and VA.

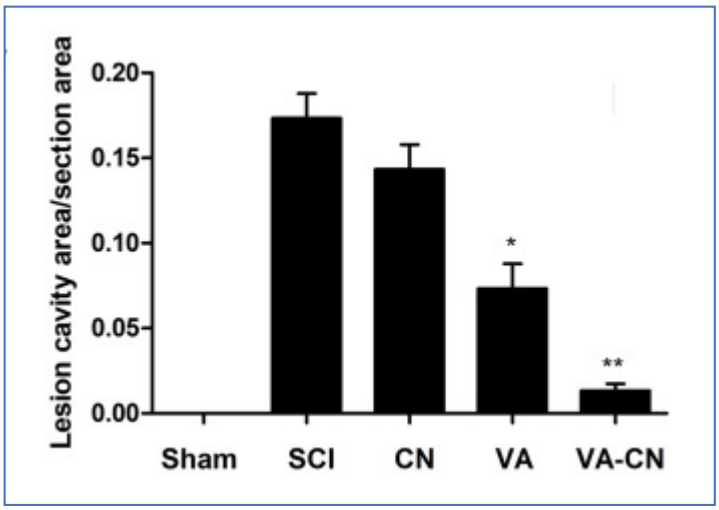

Figure 3. The lesion cavity was quantified in the injured spinal cords, i.e., n = 10 for the sham group, n = 12 per experimental group. * $p < 0.05$, ** $p < 0.01$ versus SCI group. Adapted with permission [84].

5.2. Nanoscaffolds

Nanoscaffolds are physical structures that are used as delivery agents in the cell to create an artificial permissive atmosphere for the regeneration in the CNS region. These implants are mainly restricted for use in a transaction injury of the spinal cord. In cases where the injury on the cord is severe, the scaffolds or stumps can be placed in nerve guidance channels corresponding to systems utilized in the periphery nerve fixing system. The scaffolds are prepared considering certain physical factors like the design of the material of scaffolds, tube dimension, the thickness of the wall, the porosity of the scaffolds, and the strength of the material of scaffolds. To avoid necrosis, the specific modulus and the characteristics of the material must be similar to that of the injured tissue specifically at the implant tissue junction [85,86]. Poly(2-hydroxyethyl methacrylateco-methyl methacrylate) has been used to construct the nerve guidance channels [87]. The nerve guidance channel must be biocompatible, but not biodegradable. There are certain biodegradable materials like, poly(*L*-lactide) [88], poly(hydroxybutyrate) [89], chitosan [90–92], and collagen [93] which are used by the researchers in vivo experiments. The scaffolds are used as a delivery agent for delivering various cells including the Schwann cells [88,89,94], astrocytes [94], and neural stem cells [90,91,95]. The drug is loaded in the inner lumen of the tube which is either adherent to the inner surface of the tube or suspended in a hydrogel; for instance, dilute collagen [96], laminin functionalized agarose [97], or even fibrin [95], within the tube of the scaffold. If the spinal cord is partly transected implantable hydro in-situ gelling system could be used to fill the tissue defects hereby speeding up the tissue growth to fill the gaps [98–100]. The implantable scaffolds are prepared from materials like; PLGA (poly(*L*-lactic acid)acid) [101], PMMA (laminin coated poly(methyl methacrylate)) [102], PGS (poly(glycerol-sebacate)) [103,104], PCL (polycaprolactone) [105] and electrospun PLGA moieties [106]. These scaffolds have some limitations such as scaffolds remain dissimilar to tissue modulus, lack of flexibility in the sub-retinal delivery system and the delicate tissue are vulnerable to get damaged from the implant [101]. The similarity between the

scaffolds and the tissue is very critical, in particular to the delivery in CNS regions which requires a simultaneous application for drug and cell delivery. A recent study reported that the scaffold prepared from merging the chitosan nanoparticles into polypyrrole/alginate composite showed minimal cytotoxicity and improved proliferation when evaluated with OLN-93 neural cells and fibroblasts [107]. The viability and proliferation of OLN-93 neural and fibroblast cells confirmed cytocompatibility of Nanochitosan/PPy-Alg scaffold depicting as an ideal candidate for neural tissue engineering. Figure 4 shows the increased number of OLN-93 cells from Day 7 to Day 14 on nano chitosan/PPy-Alg indicating the synergistic effect on the proliferation of neural cells.

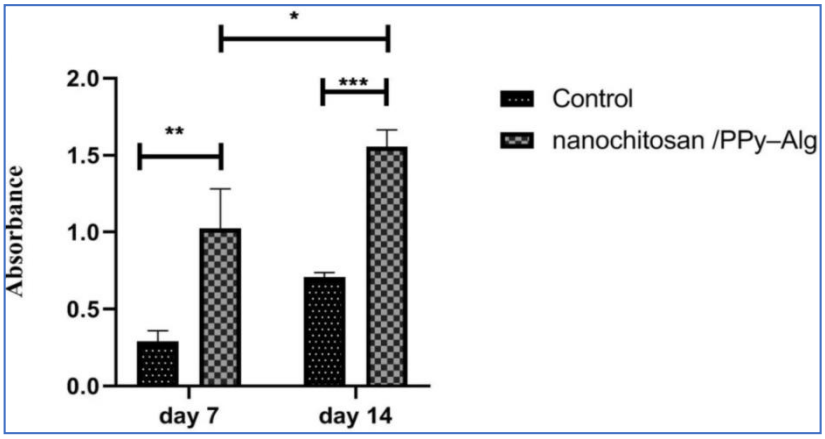

Figure 4. Proliferation of OLN-93 cells cultured on nanochitosan/PPy-Alg scaffold compared to control after 7 and 14 days. *: $p < 0.05$, **: $p < 0.001$, and *** $p < 0.0001$. Adapted with permission [107].

5.3. Nanoemulsions

Albumin conjugated multilayered nanoemulsions also commonly known as albumin-MNE of methylprednisolone were constructed to aim specifically at the spinal cord injury site and the drug delivery at the site of injury [108]. The study showed that albumin-MNE showed sustained release, improved systemic circulation, and site-specificity of methylprednisolone in SCI rats. Besides, decreased toxicities of higher concentrations of methylprednisolone was observed on astrocytes. Another study reported that albumin functionalized, cholesterol stabilized, phospholipid nanoemulsion showed controlled release of rapamycin resulting in improved cytokine inhibition and in-vivo efficacy to offer effective treatment for post-SCI-like conditions [109]. Figure 5 shows the in vitro rapamycin drug release profile in nanoemulsion (RN7) and nanoemulsion conjugated with albumin (ARN7) against rapamycin solution (RS) for 12 h. The cumulative drug release was plotted against time which showed that the cumulative percentage drug release from RS in 2 h was 89 ± 6 whereas RN7 and ARN7 released 57 ± 9 and 62 ± 11%, respectively, in 12 h. The sustained release of rapamycin in RN7 and ARN7 helped to keep the astrocytes viable when compared to RS, which was confirmed in another set of experiments (data not shown).

5.4. Hydro/Nano/In-Situ Gelling Systems

Hydrogels, as the name suggests are polymeric material whose main component is water, i.e., 90% and are physically or chemically cross-linked in a manner that they become highly bio-compatible and a popular choice, mainly for the tissue regeneration strategy [110]. Hydrogels possess a porous structure allowing them to be a suitable candidate for drug delivery with the drug discharge depending rate of diffusion of the drug into the hydrogel matrix. The rate of diffusion could be maneuvered by changing the crosslink density of the hydrogel which creates a space in the matrix for the drug and a controlled drug release [111]. Hydrogels are not all perfect and have some limitations which include their high percentage of water due to which the hydrophilic drugs like

proteins get easily solubilized and diffuse out of the gel in a period of several hours to days. To solve this issue, hydrogels are covalently bound to hydrophilic drugs through a cleavable linker which in turn increases the delivery time. The rate of linker cleavage is also directly linked to the rate of drug release [112,113]. Another technique that is also very well-known is the combination of hydrogel with other drug carriers like liposomes and a lipid microtubule [114,115] or the use of polymeric microspheres [116–119]. The hydrogel limits the burst release which resembles the properties of the microsphere system and keeps the particles at the site of injury. The hydrophobic liposome or microsphere gives extended discharge duration for hydrophilic molecules [120]. Injectable hydrogels also have in-situ gel formation application. The formation of gels in polymers differs in the case of polymer the formation of gel takes place when the temperature increases than the lower critical solution temperature below body temperature while for the other polymers like chitosan and alginate the formation of gels takes place through ionic interactions by the addition of salt or changing the pH values [121]. The light-induced hydrogel formation takes place through the addition of a photo-initiator to the monomer [122]. The biodegradability of the hydrogel is the reason for its removal from the body; since they are biodegradable the delivery system is easily eliminated. The hydrogels, therefore, come as a promising material that fulfills the entire criteria for an ideal drug delivery system. Studies have shown that the chitosan-based hydrogels contribute to inflammatory response modulation, tissue repair, gain in locomotor function recovery, and induces neural tissue repair to treat SCI [123,124]. Chitosan and water as fragmented physical hydrogel suspension (Chitosan-FPHS) promoted reconstitution of spinal tissue and vasculature while diminishing the fibrous glial scarring in post SCI rat models [123].

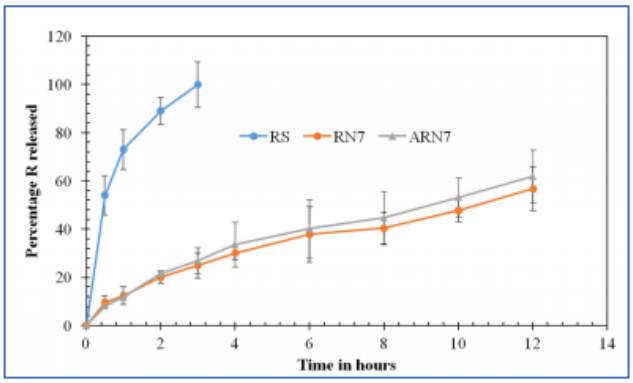

Figure 5. In-vitro rapamycin drug release profile in rapamycin solution (RS), nanoemulsion (RN7), and nanoemulsion conjugated with albumin (ARN7). Adapted with permission [109].

5.4.1. Natural Polymers

Hyaluronan, fibrin, and collagen are certain natural polymers that are used in clinical applications; for instance, filling of the dermal layer, lubricants, wound sealants, and also surgical sponges which are very advantageous [125,126]. Other popular natural polymers include agarose and chitosan which possess active functional groups allowing a wide range of chemical modification and make them suitable for diverse clinical applications. Natural polymers are available in the form of gels which could be controlled by several factors like temperature and pH; for instance, in agarose, the gel formation takes place with the decreasing temperature while on the contrary the methylcellulose and collagen show gel formation with increasing temperature. The chitosan is not temperature-dependent for gel formation but instead pH-dependent, and it forms a gel with increasing pH. Agarose, a derivative of red algae is a polysaccharide of *D*-galactose and 3,6-anhydro-*L*-galactopyranose. Agarose is also a suitable candidate for drug delivery due to the presence of its certain properties like; soft tissue likes properties and forms a porous gel at low temperature [127]. Agarose forms hydrogen bonds to form a gel structure when the powder is mixed with liquid media and heated. The agarose has the drawback that, it cools down in an unmodified form very

slowly at body temperature [128]. To overcome this problem, researchers suggested the use of an external liquid nitrogen cooling system to fasten up the process [127].

5.4.2. Synthetic Polymers

Synthetic polymers are another type of polymers that are frequently used in drug delivery systems as they can be altered in both their composition and molar mass. The synthetic polymers could be modified for the active functional groups which make the crosslinks or react with biomolecules to form modified structures. PNIPAAm (poly(*N*-isopropyl acrylamide) is widely studied and investigated for its temperature-dependent drug delivery system [129]. The unique property of poly(*N*-isopropyl acrylamide) lies in its low critical solution temperature (LCST), due to which it is a liquid or soluble form at room temperature while it solidifies to a gel form at the body temperature. The unmodified PNIPAAm has a poor elastic recovery and has less water holding capacity which is overcome by combining it with polyethylene glycol (PEG), thus, altering its physical and mechanical characteristics [130]. The PNIPAAm-PEG was experimented with by Lowman, which showed that the delivery of BDNF for repairing the partial hemisected SCI was feasible by the addition of the CNS targeting the drug to the PNIPAAmm-PEG at the room temperature in the aqueous form [131].

6. Recent Advancements in Chitosan-Based Stem Cell Therapy

The current therapeutic approach for the treatment of SCI mainly aims at eliminating further damage to the spinal cord. The ultimate goal for the management of SCI is to reduce cell death and minimize the extent of the injury, as well as to facilitate the process of neuroregeneration to repair the damaged tissue. Stem cell therapy offers great potential for neural repair. There have been several strategies to improve the survival and function of the grafted stem cells, i.e., stem cells seeding on various biomaterials and scaffolds. Scaffold-based strategies have been established as a very efficient alternative for the neuroregeneration after SCI. By definition, scaffolds are temporary supporting structures for growing cells and tissues [132,133]. Up to date, electrospun guidance channels, scaffolds, and hydrogels have been the most promising for neural engineering in SCI. Electrospinning is a simple and rapid technique used for the fabrication of the nanofibrous scaffolds where a high-voltage electric field is applied to polymer solution coming out from the tip of a needle to be deposited on the ground collector to form the ultrafine fibers [134,135]. In neural tissue engineering applications, the electrospun scaffolds mimic the neural extracellular matrix by altering their fibrous structures. Besides, another key advantage to these scaffolds is the neurotrophic factors can be incorporated into the scaffolds during the electrospinning process to be delivered at the site of SCI. Studies reported a variety of electrospun scaffolds have been used to treat injuries in the peripheral nervous system and SCI. Electrospun scaffolds with aligned structures have been used in several studies to direct regenerating nerve fibers and thus promote axonal regeneration and functional recovery. A study reported the functional recovery of the hemisected thoracic spinal cord due to the sustained delivery of chondroitinase ABC (ChABC) using polypropylene carbonate (PPC) electrospun fibers with chitosan (CS) microspheres as the vehicle [136]. The study reported that PPC-CS supported a stable release of ChABC for over 10 days in PBS at 37 °C and 5% CO_2. The quantity of ChABC release accounted for 89.10 ± 1.41% of the total amount whereas the active ChABC accounted for 26.29 ± 0.46% of the total amount as shown in Figure 6. The sustained delivery of ChABC promoted axon sprouting and reduced glial scarring suggesting PPC-CS micron fibers containing ChABC as a feasible treatment for SCI.

The most recent comparative study evaluated the extent of nerve regeneration by chitosan scaffolds prepared by using electrospinning and lyophilization [137]. The experimental data showed that the proliferation rate and the adhesion rate of Schwann cells in the electrospinning group were higher than that of the lyophilization group. Figure 7

shows the increased proliferation rate of the Schwann cells on the electrospun chitosan scaffold than that of the chitosan lyophilization scaffold.

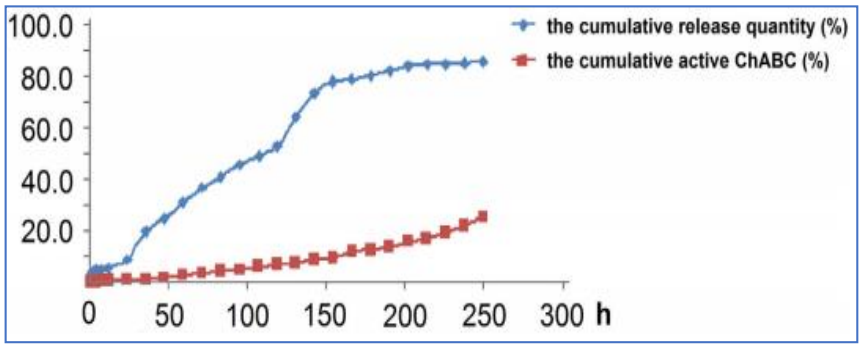

Figure 6. Release curve of chondroitinase ABC (ChABC) with the number of hours on the *x*-axis and total ChABC on the *y*-axis. Adapted with permission [136].

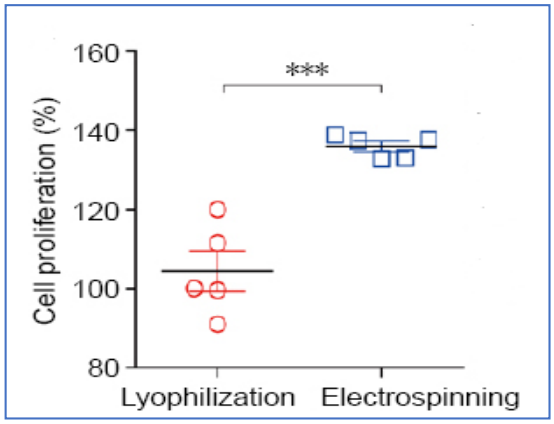

Figure 7. Comparison of the proliferation rate of Schwann cells on the electrospun chitosan scaffold vs. lyophilized chitosan scaffold. Adapted with permission [137].

Stem cells have played a key role in the SCI treatment due to their inherent property of self-replication along with the multi-directional differentiation. The secretion of ant-inflammatory cytokines inhibiting the inflammatory response of the lesion microenvironment plays a key role in the tissue regeneration at the SCI site. The major limitation of stem cells, i.e., survival and limited repair effect on SCI is overcome by the three-dimensional scaffolds. Stem cell survival and differentiation can be improved at the SCI site by using stem cell-bearing biological scaffolds [138]. A study showed that the scaffold consisting of the adult neural stem cells contained within a methacrylamide chitosan (MAC) hydrogel protected by a chitosan conduit resulted in a significant reduction in the lesion area and macrophage infiltration at the SCI site contributing to its repair [139]. In another study, the role of chitosan scaffolds in the neural differentiation of DPSCs in vitro and to assess the supportive effects of chitosan scaffolds in an animal model of spinal cord injury was investigated. Human dental pulp stem cells (DPSCs) were incubated with chitosan scaffolds treated with neural differentiation medium for 14 days. The studies found that in comparison with the control group, the levels of BDNF, GDNF, b-NGF, and NT-3 were significantly increased in the DPSC/chitosan-scaffold group indicating the key role of Wnt/β-catenin signaling pathway in the neural differentiation of DPSCs when combined with chitosan scaffolds. Apart from being non cytotoxic in in-vitro studies, there was a marked recovery of hind limb locomotor functions observation when the DPSC/chitosan-scaffold was transplanted in in-vivo spinal cord injury rat model [140].

7. Future Developments

There has been decent progress in the SCI therapy by tissue engineering techniques, however, the work so far can be deemed as the tip of the iceberg. There exist several challenging aspects to be addressed such as build an ideal regenerative microenvironment at the lesion site which is specifically difficult owing to the dynamic pathology of SCI. There is a need for the harmonization of the safety evaluation criteria of scaffold materials across different regions and SCI treatment applications. Further, the most important repair mechanism of SCI by tissue engineering needs to be fully understood and elucidated. The most effective and optimized composition of the biomaterials for neural tissue engineering is yet to be evaluated and harmonized. Future research should focus on developing such optimized structures for SCI therapy which also offer better feasibility to deliver the drugs needed for recovery. With advances in the mechanical engineering, biopolymer chemistry, and regenerative medicine utilization of chitosan alone or to formulate drug delivery systems for treating neurological problems can be advanced.

8. Conclusions and Outlook

Spinal cord injury (SCI) is a severely traumatic event in the central nervous system, which usually leads to motor and sensory loss, leaving the patients without the ability to regenerate lost tissues. Treatment of SCI remains a significant challenge for both clinicians and scientists. Though there have been several attempts to apply stem cell therapy the efficacy is limited to date. The strategy of combining the three-dimensional scaffolds with bioactive molecules or stem cells along with a drug delivery system at the site of injury has proven to be most effective. Chitosan has demonstrated immense potential for the widespread application for SCI repair in the form of scaffolds and micro-particles. Electrospun scaffolds have proven to be the most effective and a comparative study was reported to depict the advantages of electrospun chitosan scaffolds over that of the one prepared from lyophilization. Electrospun scaffolds have revealed promising results both in-vivo and in-vitro as they can actively mimic the extracellular matrix of neural cells and thus influence the growth, differentiation, and proliferation at the injured site. The new advancements in the design of the scaffolds with the integration of the new techniques to improve the chemical, directional and structural aspects are expected to contribute to the development of treatment of injured spinal cord. In short, all characteristics of the spinal cord tissue could be effectively modeled and used for the development of a more effective biomaterial composition to propel the clinical research and application for the treatment of SCI.

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