BIO-CELLULOSE BASED COMPOSITE PROTEIN DELIVERY SYSTEM FOR SPINAL CORD REGENERATION

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Abstract

Background: Spinal cord injury (SCI) is a devastating condition for which current treatment strategies provide no cure. Delivery of growth factors at the injury site may stimulate endogenous stem cells for nerve regeneration. Biocellulose (BC) was reported to be biocompatible, abundant and have adjustable mechanical properties. However, BC has not been tested for the treatment of SCI.

Hypothesis: Composite microsphere loaded BC tubes can have a sustained protein release profile with high encapsulation efficiency and low initial burst rendering it suitable for spinal cord regeneration.

Methods: Bovine serum albumin loaded poly (lactic-co-glycolic acid) microspheres were fabricated and characterized while studying the effect of different process parameters on encapsulation efficiency, release profile and morphology. Microspheres were loaded to BC tubes and were characterized morphologically and mechanically.

Results: Inner phase volume and the drug:polymer ratio are the main factors impacting microsphere protein encapsulation. Furthermore, presence of different osmotic agent concentrations in the aqueous phase produced a smooth morphology while eliminating the initial burst. Finally, the composite BC tubes were fabricated, and mechanical properties were suitable for SCI applications.

Résumé

Contexte : Les lésions de la moelle épinière sont une maladie dévastatrice que les stratégies de traitement actuelles ne permettent pas de guérir. L'administration de facteurs de croissance sur le site de la lésion peut stimuler les cellules souches endogènes pour la régénération des nerfs. La biocellulose est biocompatible, abondante et possède des propriétés mécaniques ajustables. Cependant, la biocellulose n'a pas été testée pour le traitement des lésions de la moelle épinière.

Hypothèse : Les microsphères en composite situées dans les tubes de biocellulose peuvent avoir un profil de libération soutenue de protéines avec une grande efficacité d'encapsulation ainsi qu'un faible taux de libération initial, ce qui les rend appropriés pour la régénération de la moelle épinière.

Méthodes : Des microsphères de poly (acide lactique-co-glycolique) chargées d'albumine de sérum bovin ont été fabriquées et caractérisées tout en étudiant l'effet de différents paramètres du processus sur l'efficacité de l'encapsulation, le profil de libération et la morphologie. Les microsphères ont été mises dans des tubes de biocellulose et ont été entièrement caractérisées.

Résultats : Le volume de la phase interne et le ratio médicament : polymère sont les principaux facteurs qui influent sur l'encapsulation des protéines en microsphères. De plus, la présence de différentes concentrations de sel dans la phase aqueuse a produit une morphologie lisse tout en éliminant la libération initiale. Enfin, les tubes de biocellulose en composite ont été fabriqués et les propriétés mécaniques étaient adaptées pour l'application sur des lésions de la moelle épinière.

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List of Abbreviations

BC	Biocellulose, Biosynthesized cellulose
BCA	Micro bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CNS	Central nervous system
CSF	Cerebral spinal fluid
ECM	Extracellular matrix
EE	Encapsulation efficiency
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ES	Epidural stimulation
FDA	US Food and Drug Administration
FGF-2	Fibroblast growth factor-2
GDNF	Glial Derived Neutrophic Factor
GF/s	Growth Factor/s
HAMC	Hyaluronan and methylcellulose
IGF	Insulin growth factor
MPSS	Methyl prednisolone succinate
MS	Microspheres
NGF	Nerve growth factor
NPC	Neural progenitor cells
NSC	Neural stem cells
NT-3	Neutrophic factor 3
DPBS	Dulbecco’s phosphate-buffered solution
PDGF	Platelet-derived growth factor

PLGA	Poly (lactic-co-glycolic acid)
PVA	Poly (vinyl alcohol)
SEM	Scanning electron microscope
SR	Swelling ratio
SCI	Spinal Cord Injury
VEGF	Vascular endothelial growth factor

Preface

I would like to thank my supervisors Dr. Eve Tsai and Dr. Xudong Cao for their consistent support, guidance and patience throughout this journey. They provided me with the needed knowledge, skills and most importantly the questions that pushed me through to the end of this work.

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Dedication

This work is dedicated to my beloved family

Mrs. Zeinab Zidan

Ahmed, Khaled, Bassant, Aya, Mohamed, Nour, Fannie

And

My Loving Father

Mr. Mamdouh A. El-Khadem (1944-2014)

1. Introduction and literature review

Definitions and impacts

Spinal cord injury (SCI) is a devastating condition and can result in the impairment of communication between the brain and the body below the level of the injury. It can impact sensory, motor and autonomic functions and can cause physical, mental and financial burdens. The injury can lead to irreversible functional impairment associated with inefficient regenerative capabilities of the central nervous system (1). The United States has the highest incidence and prevalence of SCI with about 40 and 906 cases per million, respectively (2). In Canada, the Rick Hansen Spinal Cord Injury Registry reported 86,000 patients living with a form of SCI. Traumatic injuries constituted 51% of these cases with a higher incidence in young adult males (3,4). In addition, 66% of the cases under the age of 65 suffer from tetraplegia and 34% suffer from paraplegia (5). These numbers are higher in cases above the age of 65, with 87% of the cases developing tetraplegia. The causes of SCI in Canada varied from falling (37%), accidents (38%), sports related injury (15%) and injuries caused by assault (3%) (3). Financially, it was estimated that the annual cost for traumatic SCI patient was about 1.9 million dollars in the form of direct and indirect costs. In addition, 42% of patients lost their jobs after the injury, creating a higher financial burden compared to other chronic diseases (3,6).

SCI can be classified into complete and incomplete injuries depending on the sensory or motor function preserved. It was reported that 69% of the cases below 65 years of age suffer from incomplete injury where varying degrees of sensory or motor function below the level of injury are spared such sacral sensation or voluntary anal contractions. On the other hand, 31% of the

cases have complete SCI where they lost all sensory and motor function below the level of injury.

Pathophysiology of SCI

The first case reports of SCI were found in the Edwin Smith papyrus that dates to the seventeenth century B.C. However, our understanding of the pathology of SCI substantially increased recently (7). Extensive research was conducted utilizing different models and aimed to identify the detailed pathology of SCI.

1.2.1 Stages of SCI

SCI has been divided into two stages of injury: primary and secondary. Primary injury is caused by the initial traumatic event and secondary injury is due to the biological and functional changes that occur after the primary injury. The primary injury starts immediately with the initial mechanical trauma such as due to a fracture and/or dislocation of spinal vertebrae. The initial trauma can cause laceration and tissue distortion leading to secondary injury mechanisms such as hemorrhage, oxidative cellular and axonal damage (8). Figure 1 is a schematic of an injured spinal cord. Secondary complications start to develop due to the primary injury. The formation of edematous cysts can occur at the lesion site due to the infiltration of blood or plasma fluids due to vascular injury. Ischemia occurs due to torn blood vessels and is followed by an increased level of glutamate and free radicals due to axonal tearing accompanied by elevated levels of inflammatory mediators from activated neutrophils (9,10,11,12). In addition, degenerative macrophages release inflammatory cytokines that can result in increased neurotoxicity and neuronal cell death (13). These secondary complications not only cause neurotoxicity, but can also create a growth inhibiting niche at the injury site

that hinders healing mechanisms later on (11,14). Scar formation can occur that isolates the injury site from the surrounding tissue. This occurs through the formation of astrocytic and fibrotic scars. Astrocytic scar is formed through the accumulation of astrocytes at the lesion periphery followed by their proliferation, accumulation of filament proteins and the formation of the mesh structure of the scar wall (15). This forms a barrier that confines the lesion and prevents its expansion and averts the leakage of inflammatory mediators to the uninjured surrounding tissue (15,16,17). It was reported that when astrocytic scar was removed, SCI was more catastrophic in terms of an inability to repair the blood-spinal cord barrier with more severe inflammation and increased degeneration of oligodendrocytes, neurons and myelin (17). Afterwards, fibrotic scar is formed at the core of the injury where fibroblasts accumulate at the lesion site and start forming different extracellular matrix proteins. This scar is believed to be an impediment to significant axonal regeneration due to the increased expression of inhibitory factors such as chondroitin sulfate proteoglycans (CSPGs) and myelin associated inhibitors (16).

In conclusion, endogenous axonal regeneration in SCI is minimal due to the higher levels of growth inhibitory molecules together with the formation of scar tissue. In addition, axonal sprouting aids in the restoration of a part of the affected axons but is not enough and not sustained for a full functional recovery (18). Therefore, an ideal treatment approach should be introduced as early as possible to target the inflammatory cascade at its beginning, minimizing the secondary complications and to should target one or several stages in the cascade of events following spinal cord injury. One strategy is to stimulate endogenous regeneration and improve the growth promoting environment at the injury site using targeted and strategic delivery systems of growth promoting factors (19).

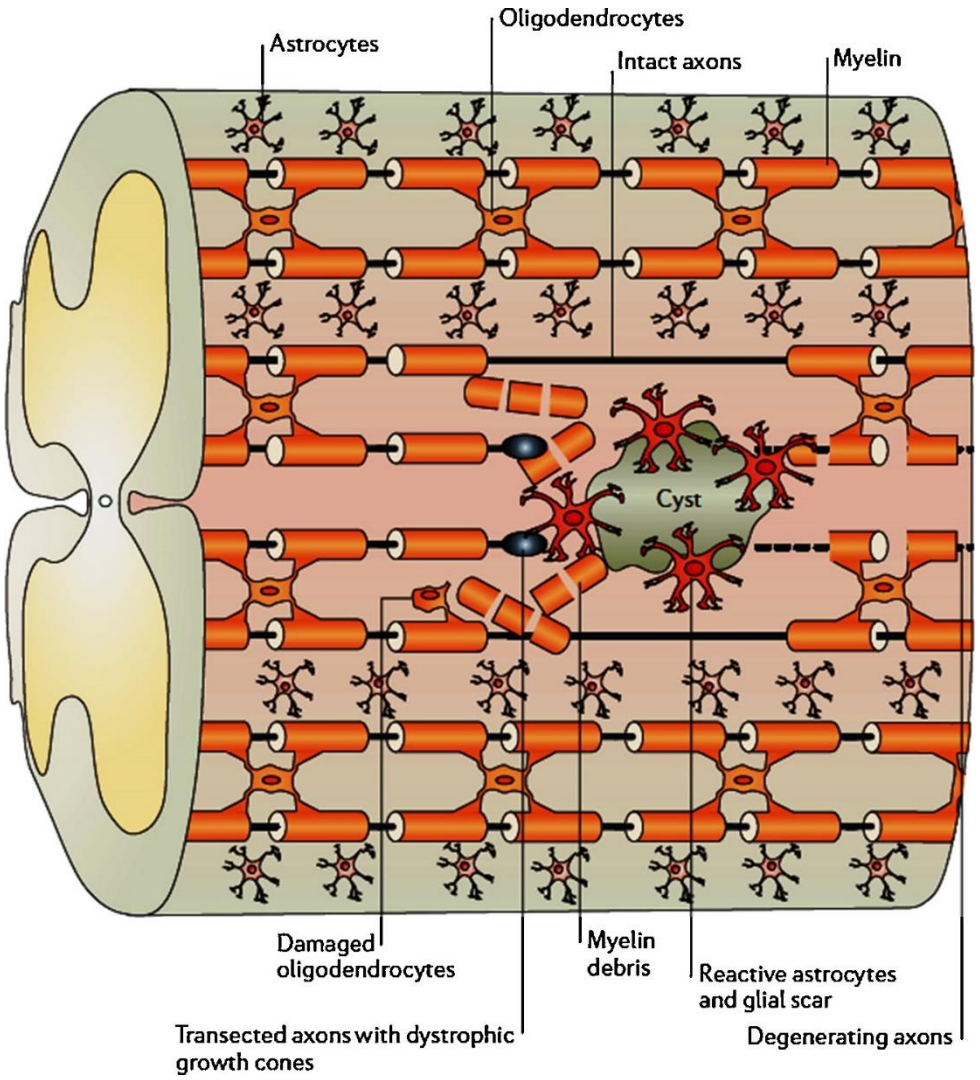


Figure 1 Schematic representation of the injured spinal cord showing different cell types involved in primary and secondary stages. Figure obtained from (20) with permission from Elsevier.

1.2.2 Endogenous stem cell fate after SCI

The main neural cell populations playing a role in SCI endogenous repair are: oligodendrocytes, astrocytes, ependymal cells and neurons. Although cells with regenerative capabilities are found in the central nervous system, no evidence of adult neurogenesis was previously detected. In a study conducted on a T8 hemi-section rat SCI model, a significant number of active progenitors was lost 24 hours post injury (21). These cells were replaced with another dormant population of proliferating, spinal cord derived, BrdU positive cells composed of oligodendrocytes and astrocyte progenitors and ependymal cells. The level of these cells was elevated 24 hours post injury followed by decline after 9 days; this was significantly higher at both time points compared to the uninjured control. Further analysis revealed that despite the elevated number of these cells across the spinal cord after injury, no proof of neurogenesis was detected (21). Out of the four cell types mentioned, only ependymal cells demonstrated neural stem cell capabilities in vitro by forming neurospheres (10, 21). Unfortunately, those cells were only capable of forming new ependymal cells while lacking multipotency in vivo and hence, no neuronal differentiation (22). Similar findings were reported following less severe SCI. In a study, it was found that ependymal cells experienced no apoptosis following mild injury that did not breach the ependymal tissue in a rat model. In addition, progenitor cell migration and proliferation from the central canal and towards the injury site was observed. However, these cells showed differentiation capabilities into astrocytes only while no neurons or oligodendrocytes were formed (23). Interestingly, ependymal cell proliferation and differentiation was reported to be responsive towards exogenous growth factors. Hence, a promising treatment strategy would be through the

introduction of growth factors to stimulate endogenous stem cell proliferation and optimal differentiation.

Current approved SCI treatment strategies

Current treatment guidelines of SCI involve targeting both primary and secondary. Primary injury management involves surgical decompression of the spinal cord and/or realignment of the dislocated vertebrae leading to the stabilization of any spinal instability (24). However, studies have shown that many of the pathological changes and complications of SCI occur due to the secondary injury and that if minimized, can lead to less catastrophic complications (14).

Recent guidelines involve different strategies focusing on minimizing secondary damage. One management option involves a 24-hour infusion of the anti-inflammatory drug methyl prednisolone succinate (MPSS). This was reported to have several positive effects such as facilitating impulse transmission, lowering the levels of lipid peroxidation and enhancing blood flow to the lesion which prevents ischemic tissue damage in animal models(25). In addition, anti-coagulation therapy is recommended to prevent any thromboembolic events secondary to the injury. Finally, rehabilitation and physiotherapy are recommended after stabilization of the patient. However, these guidelines were reported differently in several studies; for instance, the timing of decompression surgery, the dose and interval for administration of MPSS, and the type of anti-coagulation therapy and rehabilitation are controversial and were subject to case by case variation (26). Consequently, we can conclude that current treatment strategies aim to minimize further complications. Research is still ongoing to find an optimal novel regenerative treatment strategy that can restore the lost spinal cord tissue and function and hence, achieve complete structure and functional recovery.

Secondary injury treatment under investigation

1.4.1 Strategies in clinical trials

Recent studies have utilized different strategies to target different phases of SCI and were tested *in vitro* and *in vivo* in murine and rodent models. Transgenic mice were used to evaluate genetic alterations and their impact on SCI as its easier to produce transgenic mice than rats. On the other hand, rats were a preferable SCI model due to their pathophysiology which is more similar to humans (16).

Treatment strategies under investigation include pharmacological agents, epidural stimulation (ES), biomaterial scaffolds and regenerative stem cell-based therapies. Drugs targeting inflammatory and scarring cascades such as such as riluzole (27), imatinib (28) and minocycline (29) are being investigated in clinical trials. Yet, none of them have been established as effecting significant functional recovery in humans. Epidural stimulation combined with physiotherapy was reported to connect lesioned neurons by amplifying weak existing axon pathways in all human participants of one trial. Moreover, one of those patients could gain leg control only via training while the ES device was off (30).

Several biomaterial scaffolds have reached phase 3 clinical trials demonstrating various degrees of functional recovery (31,32). For instance, a combination of poly(lactic-co-glycolic acid) (PLGA) and poly-l-lysin (PLL) was used to synthesize a porous scaffold to be used for SCI (31,32). This combination takes the advantages of both PLGA, a biocompatible polymer used for resorbable sutures and PLL, a polymer used in cell culture plates to improve cell adhesion. The neural stem cell loaded scaffold was applied in a rat hemisection SCI model that showed an enhancement in functional recovery and tissue sparing (31). The same group

used the blank scaffold in a monkey model after partial and complete thoracic SCI (31). Significant increase in muscle activity and tissue remodeling was observed twelve weeks following the injury compared to untreated controls. Moreover, histological analysis revealed the possibility of neuronal sprouting within the remodeled tissue (31). Data from rodent and primate models provided the preclinical data to take this scaffold into clinical trials (33). In one trial, 16 patients with complete lack of motor and sensory function below the level of injury were treated with this scaffold. Seven patients showed an improvement after 6 months with one case showing some muscle movement below the level of injury (33). This approach focusses on appositional healing processes where the scaffold acts as a glue connecting the tissue at the injury site resulting in tissue remodeling and sprouting. Although the study is still active, the preliminary results pave the way to use such promising biomaterials as delivery systems for stimulating tissue regeneration, neuroprotection and decreasing apoptosis.

Regenerative stem cell-based strategies have also been studied. These studies utilized cells from different sources either directly to the spine or by the aid of an engraftment material. For instance, autologous grafts with Schwann cells, stem cells from human umbilical cord, bone marrow and adipose tissue were all reported to be used for stem cell transplantation to treat SCI (34,35,36). In addition, olfactory ensheathing cells were also reported as a promising approach for regeneration in SCI (35). These approaches were tested on animal models and some of them have made it to clinical trials as shown in Table 1. However, some of these strategies proceeded quickly to clinical trials with insufficient positive rodent results (37,38). In addition, in many human trials, minor improvements were reported in the patients where most studies were conducted on chronic SCI patients rather than acute ones. Moreover, the direct injection of stem cells is still controversial as there is no control over the cell fate post

injection where some of these cells can migrate away from the injury site and cause cancer (39,40). Other reports demonstrated that positive results in studies utilizing stem cells were attributed to the ability of these cells to provide a constant supply of growth factors (GFs) at the injury site (41,42). Several studies were conducted to confirm the role of stem cells in releasing active factors that enhance the growth promoting environment at the injury site as well as stimulating endogenous stem cell proliferation and differentiation (43,44). In another study, modified stem cells overexpressing certain GFs in animal SCI models demonstrated axonal regeneration across the scar (45). From this we can conclude that the promising approach of direct introduction of growth factors may improve the niche at the injury site while avoiding the cell fate concerns associated with stem cells.

Growth factors as a treatment for SCI

Neurotrophic factors are being researched extensively to be used to reduce SCI complications and stimulate regeneration and hence, promote functional recovery. Unfortunately, they have poor oral bioavailability and short half-life (46). To date, more than 50 factors were reported, each of which belongs to a family that performs a specific function. During SCI, the lack of these factors at the injury site can leads to regeneration failure. Consequently, a constant supply of GFs at the injury site in therapeutic doses can stimulate long distance axonal regeneration, synaptogenesis and neurogenesis. Table 1 is a summary of some of the factors that have been reported previously with a positive impact in the case of SCI. These factors were studied independently or in combinations aiming for synergistic and more accelerated effects (47).

Many GFs useful for SCI work via different tyrosine kinase (Trk) receptors as shown in Table 1. Interestingly, they have similar intracellular cascades and pathways demonstrated by elevation of cyclic adenosine monophosphate. Consequently, the combination of GFs can lead to amplified favourable or unfavorable effects (20). For instance, the combination of epidermal growth factor (EGF)/ basic fibroblast growth factor (bFGF) demonstrated a synergistic effect in terms of functional recovery, ependymal cell proliferation, survival of motor neurons, lesion size reduction, angiogenesis, and inflammation reduction in rats only when co-administrated (48,49). Intrathecal administration of brain derived neutrophic factor (BDNF) and NT-3 rescued half of the neuron population with significant functional recovery after 1 week (36).

On the other hand, BDNF was reported to have low penetration in the spinal cord tissue with increased pain sensation when exogenously introduced (50). In addition, the combination of insulin growth factor-1 (IGF-1) and PDGF promoted myelination but inhibited axonal regeneration in a rat complete transection model (51,52). However, PDGF exogenous introduction is questionable as it was reported to induce malignancies, atherosclerosis and neuropathic pain (50). Interestingly, it was reported that the presence of certain GFs at the lesion site can induce or inhibit the endogenous production of other factors. For instance, NT-3 over expression lead to an increase in the production of nerve growth factor (NGF) and BDNF endogenously, which promoted neural repair (53).

Table1 A summary of the growth factors most commonly utilized in the treatment of SCI in animal models and the pathways involved.

Factor	Role in SCI	Pathway involved	References
Nerve growth factor (NGF)	-Sensory axonal sprouting -Increased pain sensation -Induce neuritic growth -Neuropathic pain	-TrkA -P75 NTR	(47,54,55)
Brain derived neutrophic factor (BDNF)	-Highly expressed in spinal cord -Anti-apoptotic, enhance axonal survival -Neuroprotective for motor neurons (in rubrospinal tract, corticospinal motor system, motor cortex) -Stimulated axonal regeneration and sprouting -Enhanced re-myelination -Low penetration in spinal cord tissue (high molecular weight) -Increased pain sensation in continuous doses or in uninjured state	-TrkB	(36,47)
Neutrophin-3 (NT-3)	-Regeneration of cerebrospinal tract axons -Enhanced functional recovery -Enhance endogenous neural stem cell migration to lesion site and neurogenesis -Enhanced axonal regeneration -Anti-inflammatory and neovascularization stimulation	-TrkC (high affinity) -TrkA, TrkB (lower affinity)	(47,56,57,58)
Basic fibroblast growth factor (b-FGF)	-Sensory axon regeneration -Mitogen for stem cell renewal/ enhance endogenous NSC and NPC recruitment in SCI -Angiogenesis, anti-apoptotic and neuroprotective -Enhances functional recovery (unknown mechanism) and reduction of lesion size -Stimulate proliferation of ependymal cells - Recovery of blood spinal cord barrier at the injury site	-FGF-receptors (FGFR) -Stabilizing neuronal Ca^{2+} homeostasis and regulation of NMDA receptor protein -Reduce endoplasmic reticulum stress / Activate PI3K-Akt and ERK1,2	(48,59,60,61,62,63,64)

Table. 1 continued. A summary of the growth factors most commonly utilized in the treatment of SCI in animal models and the pathways involved.

Factor	Role	Pathway involved	References
Epidermal growth factor (EGF)	-Stimulate proliferation and differentiation of endogenous stem cells. -Combined with FGF-2, reduce lesion size, anti-inflammatory, neovascularization, anti-apoptotic for motor neurons. -Preserve Blood spinal cord barrier permeability post injury/ enhance functional recovery. -Inhibition of EGFR enhances re-myelination, inhibits the activation of astrocytes.	-EGFR pathway -PI3K-Akt-Rac1 -EGFR inhibitor	(49,65,66)
Insulin growth factor (IGF-1)	-Promote mitosis of oligodendrocytes, astrocytes and stem cells. -Promote regeneration of motor axons, induce myelination. -Neuroprotective.	-IGF-1 receptor (Trk)	(52,67)
Hepatocyte growth factor (HGF)	-Angiogenesis and vascularization. -Neuroprotective. -Axonal motor regeneration enhanced recovery of motor function. -Reduction of astrocytic scar. -Promote axonal regeneration beyond the scar tissue.	HGF receptor	(45,68)
Platelet derived growth factor (PDGF)	-Over activity leads to malignancies, atherosclerosis, neuropathic pain. -Promote survival. -Enhance recruitment of endogenous stem cells. Proliferation of astrocytes and oligodendrocytes	-PDGFα/ PDGFβ (Trk) receptor pathway	(50,69,70)

Our current study aims to fabricate a sustained release GF delivery system to be used for SCI by stimulating endogenous regeneration and recovery mechanisms. Future studies will include two of the GFs that will potentially be used as a proof of concept to target multiple endogenous regeneration and recovery mechanisms. These factors are neutrophic factor-3 (NT3) and basic fibroblast growth factor (bFGF).

NT-3 was reported to promote axonal regeneration in the corticospinal region, the largest tract harboring motor pathways (71,72). It was also reported to enhance sprouting and new synapse formation especially after injury in rats, (71,72). This is congruent to what was reported in the rat SCI model where significant functional recovery was noticed when NT-3 was injected (58). It was also reported that elevated levels of NT-3 following SCI stimulates the release of other growth factors such as NGF which in turn enhances cholinergic sprouting (73). In addition, the slow release of NT-3 stimulated the migration, and differentiation of neural stem cells into neurons in rat SCI model. These neurons formed neural relay networks and synapses that bridged the gap in the injured spinal cord and hence, significant functional recovery was observed (56).

bFGF was reported to promote proliferation and differentiation of ependymal cells into neurons and oligodendrocytes. In addition, it was reported to have neuroprotective, anti-apoptotic effects with enhanced repair of the blood spinal cord barrier following the injury (48,59,60,61,62,63,74,75,76). Moreover, bFGF was reported to increase cellular response to NGF which plays a major role in the spinal repair process (73). The use of NT3 and bFGF combination is expected to enhance the neural stem cell migration, recruitment, proliferation and differentiation into neurons at the lesion site with axonal regeneration. This combination

of GFs and its impact on SCI was not studied previously to the best of our knowledge. Hence, optimum results would be the synergistic effect that accelerates axonal regeneration and the formation of new synapses leading to significant functional recovery. Moreover, the use of a small cocktail rather than a larger mixture of GF should decrease the chances of any antagonistic effects. Finally, we aim to sustain the release of the loaded GFs for up to 14 days. The choice of the 14 days timepoint was based on several reports utilizing GFs for SCI in animal models. In these studies, the significant regeneration was noticed at the 14-day timepoint that seems to be a hallmark timepoint to assess the presence of significant repair (48,74,75,76).

Biomaterials and SCI

1.6.1 Natural and synthetic biomaterials

The use of a biomaterial-based delivery system has attracted a lot of attention as a potential treatment strategy for SCI. These materials can be used as a localized, sustained drug delivery system as well as a carrier for stem cells. They can be classified according to their origin into synthetic or natural materials. Natural materials have the advantages of abundance and feasibility as they are produced by biological systems (77). Examples of natural materials used for SCI involve collagen, fibronectin, hyaluronan and extracellular matrix derivatives such as Matrigel (77). Because of their natural existence in the body, these materials are biocompatible and show better cell proliferation and adherence compared to synthetic materials (77). On the other hand, synthetic polymers are easily synthesized with superior and controllable physicochemical and mechanical properties (43).

Interestingly, some studies utilized the advantages of both synthetic and natural materials for tissue regeneration. For instance, a poly (2-hydroxyethyl methacrylate-co-methyl methacrylate) hydrogel tube was filled with different matrices, loaded with FGF-1 or NT-3 growth factor and was tested in a rat SCI model (78). This tube strategy was designed to stimulate and guide axonal regeneration together with providing a suitable niche for cellular growth and proliferation. It was found that the synthetic scaffold filled with fibrin matrix and loaded with a GF promoted axonal regeneration and improved functional recovery after 7 weeks (78). This study together with the vast majority of the reports in the last 20 years utilized the use of both natural materials from mammalian sources or synthetic ones for the treatment of SCI. Few literature reports investigated the use of materials produced by non-mammalian resources for regenerative purposes in general and SCI treatment applications specifically (79). Table 2 shows some of the main natural materials with non mammalian origins that were used for different applications.

1.6.2 Cellulose and Biocellulose

Cellulose is the most abundant natural, non-mammalian derived material with about 1.5×10^{12} tons produced yearly. Besides being an essential component of plant cell walls, some bacterial species produce cellulose in the form of a biofilm (79). Figure 2 shows the structure and morphology of biocellulose ($C_6H_{10}O_5$). It is composed of a linear β-D-glucose linked by β (1→4) bond. Two glucose molecules form the cellobiose repeating unit of biocellulose (BC). The cellulose chains produced by bacteria collate together to form subfibrils of 1.5 nm in diameter. Those subfibrils assemble to form ribbons which then form the macrostructure of BC biofilms (80). Bacterial species that produce BC include Agrobacterium, Alcaligenes, Pseudomonas, Rhizobium, Sarcin, and Gluconacetobacter (79).

1.6.3 Characteristics of BC

An advantage of BC is its purity compared to other sources where it contains impurities such as lignin and hemicelluloses from plants (80). In addition, it was has been demonstrated to be biocompatible and has a porous fibrous structure, high water retention, high crystallinity and high modification capacities both chemically and physically (79). Moreover, it was demonstrated that BC sheets possessed superior mechanical properties with a Young's modulus higher than 15Gpa and a tensile strength of 91 to 256MPa depending on the drying method (81). The properties of the produced BC can be altered by changing culture conditions to customize it to specific tissue engineering applications (82,83).

1.6.4 Therapeutic applications of BC

BC was investigated for different applications such as tissue regeneration, and drug and GF delivery. For example, BC was used for perforated tympanic membrane in a human randomized clinical trial (84). BC was also used for bone regeneration after coating with bone morphogenic protien-2 (BMP-2) (85). In this study, coated BC scaffolds triggered the differentiation of c2c12 mouse cells *in vitro* with osteogenicity directly correlated with the concentration of the loaded BMP-2 (86). Finally, BC was tested for the topical delivery of some drugs such as caffeine and lidocaine with promising release and diffusion profiles (87,88). BC was reported multiple times for peripheral nerve regeneration. For instance, modified BC tubes for peripheral nerve grafts were demonstrated to be biocompatible with a minimal immunogenic response and promising axon guidance and neutrophic factor delivery in rats.

Table 1 Non mammalian natural materials used for SCI applications

Material	Origin	Advantages/Drawbacks	examples for loading
Chitosan	Crustaceans	-Biodegradable, biocompatible (89) -Provided sustained release for growth factors (56) -significant, long distance axonal regeneration and counter inflammation and neo vascularization (56,90)	Neurotrophin-3(NT3) (56,57,89)
Agarose	Red algae	-Axonal regeneration into the scaffold and through the lesion (91). Significant axonal growth (92)	Matrigel (91) BDNF (92)
Alginate	Brown algae	Can form injectable hydrogel (48) Axonal regeneration, nerve guidance (93). Enhanced neurite growth and axonal regrowth with decreasing cavity size (48)	Stromal cells expressing BDNF (93) EGF and bFGF (48) GDNF (94)
Xyloglucan	Plant cell wall	Supported differentiation of primary cortical neurons. Functionalization can control different parameters as neurite density and growth direction in vitro (95) Increased astrocyte infiltration (96)	Functionalized by immobilization of poly D-lysin (95)
Methyl cellulose	Chemically treated plan cellulose	Commercially available duraplasty for the brain Non-biodegradable unless combined with other materials (e.g. Hyaluronan (97)	stromal cell-derived factor 1α (29)

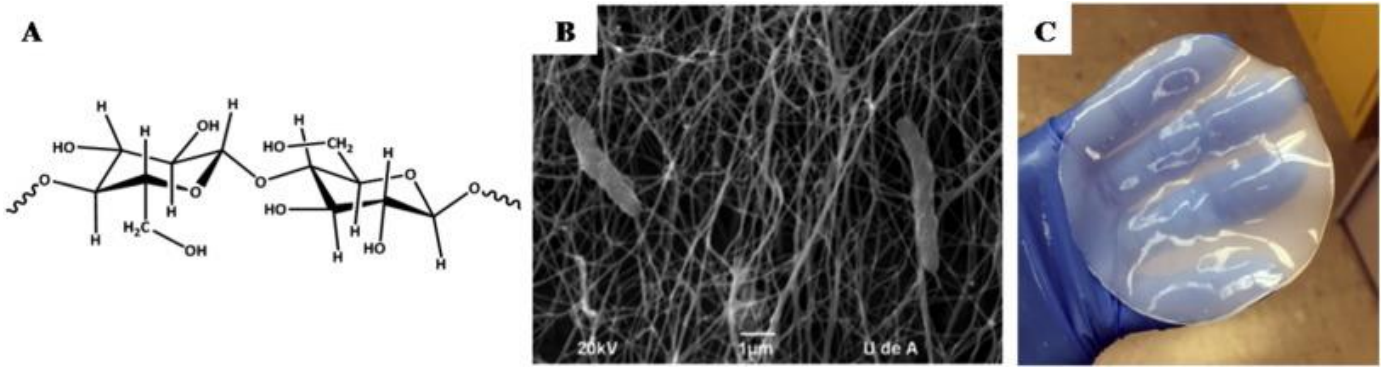


Figure 2 Biocellulose structure A) Chemical structure, B) Scanning electron microscope (SEM) showing the network structure of BC and C) BC membrane after purification (reprinted with permission from Elsevier).

However, functional recovery was non-significant compared to control groups (98). It was also reported that cellulose tubes promoted nerve regeneration in a complete transection model of rat femoral nerve (98). Nerve growth was observed inside the tube across the transection site with no immunogenic or inflammatory response. However, motor recovery was non-significant compared to the controls due to spontaneous recovery in peripheral nerves; a process that is minimal in central nervous system. In this work, it was argued that the tubes promoted the buildup and conduction of neutrophic factors at the injury site which enhanced the regeneration process (98). Similar findings were also reported using cellulose/soy protein tubes induced high levels of NGF (axonal regeneration), IL-10 and IL-6 (anti-inflammatory) with significant differences compared to the autographs over the period of 30 days (99). However, in this study no negative control was used to eliminate the effect of spontaneous recovery. In addition, functional recovery showed significant improvement (99). These studies demonstrate the benefits of BC tubes in peripheral nerve regeneration leaning on its biocompatibility, high mechanical strength and cost-effective production.

1.6.5 Limitations of BC

One of the main limitations of BC is its non-biodegradable nature where it requires surgical removal after serving its purpose. This might be challenging since the regenerated tissue tends to grow throughout the scaffold due to its porous structure. For instance, it was reported that connective tissue and blood vessels regenerated in and around the BC tubes used for peripheral nerve regeneration (98). In this case, the removal of the graft might reinjure the regenerated nerves. Interestingly, it was shown in several studies that BC can be rendered biodegradable by chemical modifications such as the biodegradable 2,3 - dialdehyde BC derivative (100). In

addition, BC like most hydrogels lacks the prolonged release profile beyond 7 days (101,102,103,104,105). This can be overcome using another sustained release delivery system that can be loaded onto the BC such as polymeric microspheres. This will include the advantages of both systems; sustained delivery of the microspheres and the carrier properties of BC. Despite its advantages and its limitations that can be easily overcome, BC has not been tested for the treatment of SCI. This makes BC a promising candidate scaffold material for investigation.

Microspheres as delivery systems

Microspheres (MS) are spherical multi-particulate, controlled drug delivery systems with a size ranging from 1-1000μm. They are matrix systems designed to troubleshoot the issues that can occur with conventional delivery methods which include the inability to produce sustained and site-specific delivery (106). They function by regulating and prolonging the release rate of the encapsulated therapeutic agents for several days, weeks and sometimes for months (107). Microspheres can be incorporated into various existing dosage forms targeting different routes of administration such as oral, topical and parenteral dosage forms.

1.1.1 Advantages of microsphere-based delivery systems

The use of MS offers multiple advantages. First, the design parameters can be adjusted to manipulate the release profile. They can provide constant sustained delivery over a long period of time, a burst localized release behaviour or a mixture of both. This can be used to boost local drug concentration above the minimum effective concentration followed by sustained maintenance dose for a longer period. This allows less doses to be administered and hence enhancing patient compliance. An example for this would be vaccine delivery, especially those

that need several booster doses like hepatitis B or toxoids such as diphtheria and tetanus (108,109). Second, drug encapsulation into the MS offer dual protection: It protects the active ingredient from being metabolized by the body and protects the body from undesirable side effects, especially if the drug is cytotoxic. This is useful for treatments with poor bioavailability to prevent their rapid destruction before they perform their action. In addition, MS offer site-specific delivery which minimizes toxic side effects. An example for this would be for therapeutic proteins with poor bioavailability such as insulin or GFs. The short half lives of these medications force the frequent administration; this can be overcome by encapsulation in MS systems. Finally, MS have a better local action compared to nanoparticles as they do not cross the interstitial fluid since their size is bigger than 100nm, preventing them from being transported by the lymph. MS size adjustment can be used for site specific targeting as well. An example for this would be MS size adjustment to target certain immune cells and uptake mechanisms (110). This method was proven to be more than 100-fold effective compared to conventional delivery methods for T cell activation used for cancer immunotherapy (110,111,112).

Despite all these advantages, MS have some disadvantages represented in the increased cost of medications due to the higher cost of production and more expensive excipients and equipment. In addition, the fabrication technique can lead to a significant loss of bioactivity of the loaded drugs. This can occur due to the organic solvents used, variable temperature conditions, prolonged fabrication time or a combination (113,114,115). Moreover, the technique used can also initiate MS degradation before administration, leading to an increased burst release that might exceed the minimum toxic dose (116). This is a major concern as it impacts the system in its biggest selling point which is the release profile. Interestingly, major

research was conducted to overcome these drawbacks mainly through the use of excipients during the fabrication to protect against the loss of bioactivity as well as degradation. In our research, we aim to study the effect of different fabrication parameters on the release profile, aiming to eliminate any degradation during MS synthesis.

1.1.2 MS fabrication

The basic structure of a MS is composed of a polymeric matrix where the drug is dissolved or uniformly dispersed (107). The ideal fabrication methods for MS should have three main criteria: Compatibility, reproducibility and scalability. A compatible method should be chosen to avoid decomposition or degradation of the drug used during fabrication. An example would be if the drug was known to be temperature sensitive or possess a certain sensitivity to organic solvents. Once compatibility is not an issue, the method chosen should ensure the reproducible production of MS with the same properties with minimum batch to batch variation. Finally, the chosen method should be scalable to be able to produce industrial size batches suitable for satisfying the demand. Several methods are being used that satisfy these requirements to produce polymer MS including: polymer extrusion, spray drying, coacervation, interfacial polycondensation and solvent evaporation (117,118,119,120). In this work, solvent evaporation method will be used while other methods were reported and reviewed elsewhere (107).

Solvent evaporation method is the most commonly used method for MS production (figure 3). In this method, the organic polymer solution is emulsified with the drug using homogenization or sonication to form the primary emulsion. The formed emulsion is then added to an aqueous non solvent continuous phase containing a low concentration of a stabilizer and mixing

continues for a longer period of time. During mixing, the organic solvent is extracted and evaporates leading to microsphere hardening and the MS are then washed to remove solvent or stabilizer traces. Finally, the formed MS are dried. The drug can be added to the organic solvent as a solid or as a solution, forming a solid in oil (S/O) suspension or water in oil (W/O) emulsion, respectively. The final emulsion is either (S/O/W) or (W/O/W), depending on the state of the drug added. As demonstrated above, the solvent evaporation technique is one of the most extensively researched methods due to its simple setup and cost friendly equipment used as well as suitability with various polymers and drugs. However, the use of organic solvents, non-uniform particle size distribution and high cost of scale up are some of its main limitations.

1.1.3 MS drug delivery mechanisms

1.7.3.1 Introduction

Studies have been conducted utilizing MS for various drug delivery and tissue engineering applications. Most of these applications rely on the biocompatibility and the physicochemical properties of the polymer used. An ideal biopolymer should be biocompatible and biodegradable to prevent any immune reaction and avoid the need for its removal via surgery. Most importantly, it should be able to carry, deliver and release biomolecules at a specific rate suitable for the intended application. Several materials have been reported for MS fabrication intended for different biomedical applications. Most of the reported materials are biocompatible and biodegradable. However, the main factor that dominates the selection process is generally the release profile. The nature of the polymer used and its degradation

profile dictates the drug release behavior in a MS system, which can be classified into two main types: surface erosion and bulk degradation.

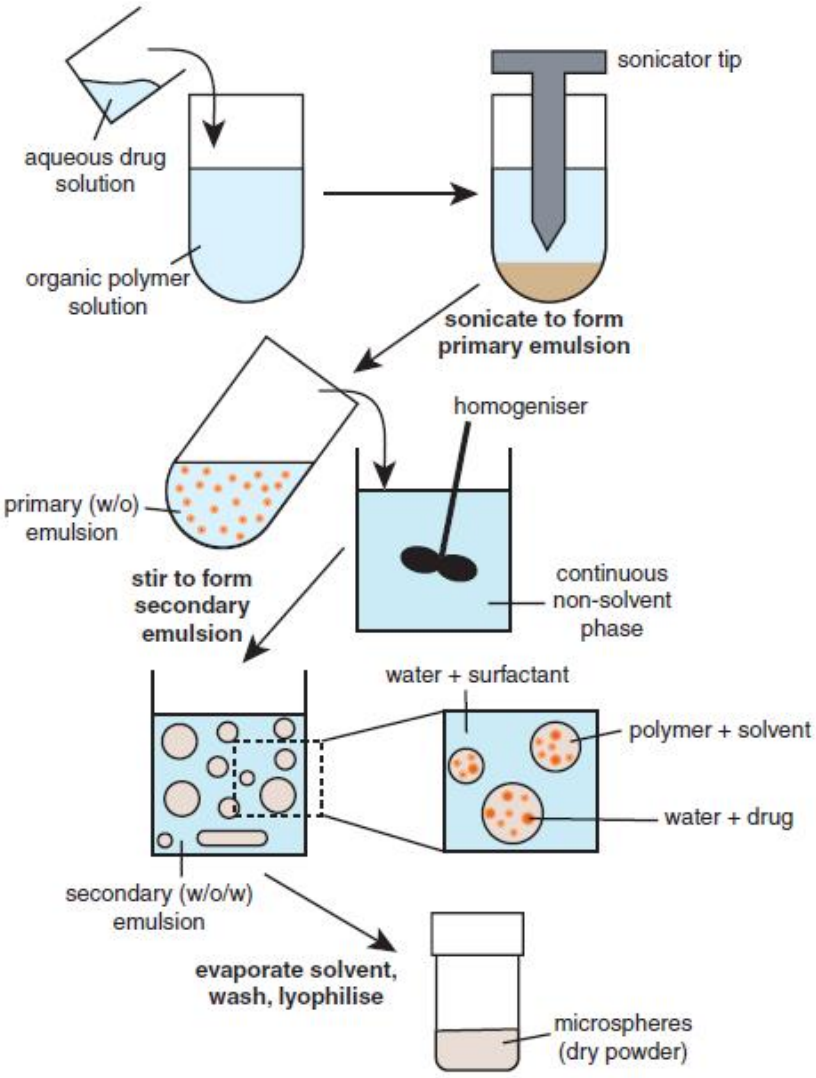


Figure 3 Schematic representation of MS fabrication using solvent evaporation technique. Figure reprinted from (107) with permission from Tylor & Francis Online.

1.7.3.2 Surface erosion

This mechanism occurs in highly hydrophobic polymers which resist diffusion of water deep into the MS. Consequently, only the surface layers undergo hydrolysis and the drug is released in a sequential layer by layer manner providing a constant release rate assuming uniform drug distribution. Examples of polymers that have a dominant surface erosion based release are poly(1,3-bis-p-carboxyphenoxypropane-co-sebacic acid) (p(CPP-SA)) and poly(1,6-bis-p-carboxyphenoxyhexane-co-sebacic acid) (p(CPH-SA)) (121).

1.7.3.3 Bulk degradation

This release profile occurs due to water diffusion through the MS leading to an overall degradation throughout the entire sphere. This creates channels and pores through which the loaded drugs as well as degradation products diffuse into the surrounding medium. These pores and channels grow deeper into the MS as polymer chain hydrolysis continues, until the whole structure collapses releasing all the loaded drug. From this we can conclude that the release profile is triphasic: The first stage is an initial burst, where drug molecules close to the surface of the MS are released within the first 6-8 hours leading to a sharp increase in the drug released. This is followed by a sustained release stage as the water channels and pores grow larger with the continuous polymer hydrolysis. Finally, hydrolysis reaches a certain stage where the whole structure collapses releasing all the remaining drug, leading to a final burst release behavior. This release mechanism usually occurs in polymers with a hydrophilic nature such as poly lactic co glycolic acid (PLGA) which is used in our work.

Both of the above-mentioned mechanisms happen simultaneously for all degradable polymers. However, one of them will dominate the degradation behavior and hence the release profile.

For example, surface erosion is dominant in poly anhydrides due to the presence of highly reactive functional groups, leading to the utilization of water molecules in the hydrolysis of surface molecules. In contrast, PLGA possesses less reactive functional groups, leading to a faster water permeation and a domination for a bulk degradation profile.

Both of the above mechanisms have well established mathematical prediction models that were extensively validated (121,122,123,124). In this work, we aim to modify the bulk degradation mechanism of PLGA to eliminate the initial burst and replace it with sustained delivery. This have been explored in previous reports and it will be discussed later in the discussion section (116,125).

1.1.4 MS utilization in biomedical applications

1.7.4.1 Introduction

MS based delivery systems have been researched for different biomedical applications for drug delivery, protein therapeutics and DNA encapsulation for the treatment of different conditions such as cancer, ischemia, bone fractures, Parkinson's, eye diseases, inflammatory bowel diseases, respiratory diseases and others (110,111,115,126,127,128,129,130,131,132). In addition, MS were utilized for treatment of different central nervous system related conditions such as stroke, epilepsy, migraines and more importantly SCI and regeneration (133,134,135,136).

Several polymers were reported for the fabrication of MS delivery systems. Some used natural materials due their biocompatibility such as gelatin, alginates, chitosan and cellulose derivatives (137,138,139). Other studies used synthetic polymers due to their physicochemical and mechanical properties together with their abundancy such as poly caprolactone, poly lactic

acid and poly (lactic-co-glycolic acid) (PLGA). Some of these systems have been approved and are commercially available to treat different conditions. Examples of commercially available MS formulations are Lupron Depot®, Nutropin Depot®, Eligard®, Enantone® andVivitrol® (140). Recent reports include 11 approved MS products in the US with over 1000 global publications and approximately 100 clinical trials (140).

1.7.4.2 PLGA as a polymer for microsphere systems

The PLGA based MS system is one of the polymers that was extensively studied for different applications. It was among the first MS delivery systems that was rendered biocompatible and biodegradable back in the 1960s when it was used for surgical sutures (141,142). PLGA degradation occurs mainly via the hydrolysis of ester bonds leading to its degradation to its original monomers: Lactic acid and glycolic acid. These are later metabolized by the body via the citric acid cycle (143). Their biocompatibility and biodegradability made them the first FDA approved polymer for biomedical and pharmaceutical applications (143).

PLGA degradation occurs by a bulk degradation mechanism explained in section 1.7.3.3. The rate can be adjusted by changing the ratio of its two acidic components. The increase in the hydrophilic glycolic acid content leads to more hydrolysis and hence a faster degradation rate. Consequently, several lactide:glycolide ratios were used ranging from 25:75 to 100:0 and including the 50:50, the one used in our study.

The release profile of PLGA microspheres was studied extensively and it was shown that it relies mostly on the degradation profile, generating a bi or triphasic sustained release profile (143,144,145). On the other hand, other studies revealed the possibility of a release profile modification through a modified fabrication of process, adding excipients or changing the

release conditions such as pH (146,147). The abundance and ease of fabrication, biocompatibility and flexible release profile is what made PLGA the most utilized candidate for drug release. However, there are certain challenges that accompany the use of PLGA such as low encapsulation efficiency and drug loading, degradation of the loaded molecules and difficulty in controlling the release rate (143,148,149). In this thesis, our work is focused on modifying and controlling the release rate to be suitable for stimulating endogenous stem cells in the spinal cord and regenerating the lost tissue in the case of SCI.

1.7.4.3 Biomedical applications of PLGA microspheres

PLGA has been successfully used in different drug delivery and tissue engineering applications. MS have been utilized in the sustained delivery of multiple drugs to treat different conditions in kidneys, liver, cardiovascular system as well as in the central nervous system that includes the brain and spinal cord. In addition, PLGA MS have been used commercially to deliver different medications. For example, Signifor Lar® for Cushing disease; Triptodur™ for prostate cancer; Zilretta® for osteoarthritis related knee pain; and exenatide, Bydureon Bcise® for diabetes and most recently Perseris ™ for the sustained release of risperidone for over a month for mental illness (140,150).

PLGA MS combined with GFs have been studied for the treatment of different CNS conditions. This was achieved through MS loaded with either drugs or growth factors, introduced solely or loaded on a composite membrane as shown in Table 3.

PLGA microspheres were used to deliver more than one GF. Yu et al. investigated the co-delivery of 3 factors: VEGF, bFGF and angiopoientin-1 and their effect on neuro-regeneration and angiogenesis following SCI in rats. This was done via incorporating the three GFs into

MS fabricated using a modified electrospraying technique (151). The GF release study demonstrated a biphasic profile with the initial burst occurring at one week and the sustained release occurring for 48 days. The bioactivity of the GFs released was confirmed via *in vitro* analysis for up to 2 months after the end of the release. In addition, the *in-vivo* study using a rat contusion model for SCI showed significant axonal regeneration, myelination, angiogenesis and preserved white mater compared to controls. Finally, open field testing demonstrated significantly higher motor and functional recovery for the treatment groups after 10 weeks compared to controls (151).

In another study, three sets of PLGA MS were loaded with one of three potential treatments for SCI: GDNF, chondroitinase ABC and Nogo-A antibody (152). This was to investigate the effect of a combination therapy of the delayed release of the three on a rat SCI model. Findings showed a continuous enhancement in motor recovery for 10 weeks following the treatment compared to un treated controls or those treated with each drug alone (152). However, this study had its limitations such as the absence of a release profile assessment. In addition, future work involving the effect of the treatment on axonal and neural regeneration on a macroscopic and microscopic levels is needed. Furthermore, additional work investigating the mechanisms of action of this combination therapy is essential to consider this system as a promising treatment for SCI. Finally, direct injection of MS into the injury site is one of the main limitations since the MS injected might escape from the injury site and could cause off target effects.

Table 2. Summary of studies utilizing PLGA microspheres for nerve and spinal cord regeneration

MS polymer used/fabrication method	Drug loaded	Carrier scaffold	Target condition	Release period	In-vitro	In vivo	References
-PLGA (50:50) -Modified electrospraying	VEGF/ Angiopoietin -1/ bFGF	None/ MS directly injected	Promoting angiogenesis and neural regeneration after SCI	Initial burst: 6-10 days Sustained release: 48 days	Bioactivity: 60 days following the release	-GFs release at injury site is significantly higher compare to control after 8 weeks -Axonal regeneration and myelination -Enhanced neuro regeneration and angiogenesis -Significant functional recovery after 12 weeks	(151)
PLGA (50:50) Solvent evaporation	NGF, BDNF, GDNF, bFGF, NT-3	Collagen gel	Nerve regeneration	Initial burst: Day 0 up to day 8 Sustained release: 35 days	Increased neurite growth in dorsal root ganglia and spinal cord explants, exceeding controls after 1 week for BDNF and GDNF	Tested on rat sciatic nerve transection model -significantly higher sensory and motor neuron regeneration with NT-3, NGF, GDNF and BDNF MS compared to free factors -Axon myelination with NGF	(153)

MS polymer used/fabrication method	Drug loaded	Carrier scaffold	Target condition	Release period	In-vitro	In vivo	References
Block copolymer: PLGA (50:50) conjugated with Poly(ε-carbobenzoxy-L-lysine) and poly (ethylene glycol) -Double emulsion technique or spontaneous emulsification	BDNF	None	Neurodegenerative disorders	-Double emulsion PLGA MS: Immediate release. -Spontaneous emulsification: 50% burst in first 2h followed by sustained release for 65 days -Block copolymer MS had 5% initial burst, low release for 2 weeks and sustained release until 65 days	-PC12 cells expressing TrkB -significant neurite number and length after 7 days	N/A	(154)
PLGA (50:50) MS with GF loaded heparin/poly(l-lysine) nanoparticles -Poly ionic complex method with double emulsion for MS	bFGF	None	Nerve regeneration	-Initial burst: 20% in the first 2 days Sustained release: 30 days	-PC12 cells -Significant induction of cell proliferation, neuronal differentiation and neurite outgrowth		(155)
PLGA (50:50) Spontaneous emulsion	BSA	The MS form the membrane use a fusion method. MS are the	Tissue engineering	Initial burst: first 2 days, 40% Sustained release: up to 20 days	Scaffold 3D structure allowed cell attachment into the scaffold after 4 hours	N/A	(156)

MS polymer used/fabrication method	Drug loaded	Carrier scaffold	Target condition	Release period	In-vitro	In vivo	References
		building block					
-PLGA -Double emulsion method	C3 transferase fused with trans-activating transcription factor, to form a membrane permeating form of C3 transferase	None	Central nervous system injuries	Initial burst: first 4 hours, 25% Sustained release: up to 30 days	-Bioactivity: up to 21 days tested on neuroblastoma cell lines	None	(157)
PLGA (85:15)	NGF with BSA	Poly (2-hydroxyethyl methacrylate-co-methyl methacrylate) channels	Nerve repair	-Initial burst: 5.5% -sustained release: 70 days -total cumulative release percent: 6.5%	-Sustained release was achieved with either NGF loaded in microspheres and incorporated into the channel as well as NGF directly loaded on the channels -total protein release is lower in case of MS.	None	(158)
PLGA	Tacrolimus (FK506): FDA	Fibrin gel	Peripheral nerve repair	Initial burst: lower than 5%	-Bioactivity: Enhanced neurite extension for 28 days	N/A	(159)

MS polymer used/fabrication method	Drug loaded	Carrier scaffold	Target condition	Release period	In-vitro	In vivo	References
	approved immunosuppressant			Sustained release: 30 days			
PLGA	Neurolegin-1	None/ direct injection	Endogenous neural stem cell stimulation following SCI	Initial burst: minimal Release pattern: Porous and non-porous MS were tested by slat leaching using sodium carbonate as a porogen Release quantity was significant with more porous MS Sustained release for 38 days for porous MS	Loaded MS enhanced neural stem cell survival and proliferation compared to control Bioactivity was retained -Significant increase in differentiation to oligodendrocytes with decrease in astrocytes -	-Rat compression SCI model -In vivo release: Significant boost in neurolegin-1 using the MS system after 28 days. -Release pattern was sustained for 28 days -Significant reduction of scar formation and regeneration inhibitory molecules after 28 days -Significant reduction in inflammation after 14 days -significant differentiation of stem cells to oligodendrocytes with axonal protection	(160)

MS polymer used/fabrication method	Drug loaded	Carrier scaffold	Target condition	Release period	In-vitro	In vivo	References
PLGA	NGF, BDNF, GDNF, FGF-2, or NT-3	Laminin, Collagen or fibronectin in a silicone tube for in vivo studies	Peripheral nerve regeneration	Initial burst: Minimal Sustained release: 35 days	-Significant increase in neurite length and number of axons using MS loaded with NGF, BDNF and GDNF -Significant increase in the number of neurons	-Significant increase in number of myelinated axons with NGF MS in rats -Significant increase in motor and sensory neurons 20 days after injury - significant increase in sensory neurons 75 days post injury -significant long-term functional recovery with BDNF loaded MS embedded in fibronectin matrix -laminin with NGF MS promoted sensory functional recovery	(161,162)
PLGA	GDNF	Fibrin matrix	Delayed repair in peripheral nerve injury	Initial burst: Less than 20% at day 2 compared to 25% in control Sustained release: 16 days	The fibrin-MS system is biocompatible with no neurotoxicity	-transection of peroneal nerve -significant enhancement of motor and sensory nerve regeneration 60 days after injury.	(163)

MS polymer used/fabrication method	Drug loaded	Carrier scaffold	Target condition	Release period	In-vitro	In vivo	References
PLGA	VEGF and BDNF	Tubular hyaluronic acid hydrogel with anti Nogo antibodies bound to the surface	Regulation of microenvironment at the injury site following SCI	Release profile: sustained release over 2 weeks -initial burst: about 200pg in the first 24 hours out of total 500 pg for VEGF and 900pg for BDNF	Bioactivity: GFs remained active after 2 weeks	-rat hemisection SCI model -significant levels of both factors compared to control at injury site - significant functional recovery at 2 to 10 weeks post injury -enhanced angiogenesis 8 weeks after implantation -reduced inflammation 4 weeks after treatment -reduced scar formation -significant nerve regeneration and axon myelination 8 weeks post treatment	(164)

1.7.4.4 Composite delivery systems: Combinatorial approach for SCI treatment

Research utilizing PLGA MS evolved with time. This was achieved through the use of composite membranes where the MS are incorporated in another matrix or polymeric scaffold. The use of a carrier scaffold had several added advantages as well such as: adding another layer of release rate control that can allow for sequential release, increasing the amount and number of drugs that can be delivered, regulating the release environment to have maximum bioactivity and the addition of nerve guidance capabilities while maintaining a sustained release of different drugs. These, in addition to the advantages of the MS, made this approach one of the most investigated when it comes to SCI.

PLGA microspheres were also used for drug delivery applications to treat SCI. For example, minocycline is a neuroprotective antibiotic that works via anti-inflammatory, antioxidant and antiapoptotic effects and was used to treat different neurological conditions (165,166,167). In addition, paclitaxel was reported to induce axonal regeneration and neuroprotection through various mechanisms at low doses (168). In a study by Nazemi et al., the effect of combination therapy of both drugs on SCI was investigated (169). This was via a composite delivery system, where paclitaxel was encapsulated into PLGA MS which were then loaded into an alginate hydrogel containing minocycline. The release profile assessment showed a biphasic release for both drugs with an initial burst followed by a sustained release period for up to 60 days (169). This composite system was injected into a hemisection rat SCI model. In vivo results showed reduced inflammatory cell response together with reduced fibrotic scar formation and axonal regrowth and neuron regeneration. Finally, this combination therapy showed significant functional recovery over a course of 21 days compared to the controls. Although this study shows significant

improvement on the microscopic scale, the functional recovery was comparable to the overall scores of untreated controls (169). On the other hand, the delivery system was proven to be effective for the delivery of any dose small molecule drugs and therapeutic agents, with a release profile that is tunable and superior to systemic administration.

Composite systems were also used in combination therapy involving stem cells with a differentiation inducing growth factor. For example, PLGA microspheres loaded with dibutyryl cyclic-AMP was embedded on chitosan channels pre-loaded with neural stem cells to be studied in a rat SCI model (170). Dibutyryl cyclic-AMP is an analogue to cyclic AMP that can permeate the cell membrane and induce neuronal differentiation of neural stem cells (171). In this report, this molecule was encapsulated in PLGA MS using a solvent evaporation technique with the addition of 10% NaCl in the continuous phase to regulate osmotic pressure. The i*n vitro* release profile was sustained for over 11 days using the MS alone and 5 days when MS were incorporated in the channel. This was due to a decreased microsphere retention during MS incorporation which was not quantified in the study (170). In addition, the prolonged release of dibutyryl cyclic-AMP induced neural stem cell differentiation *in vitro*, characterized by the increased beta-3 tubulin positive population that was accompanied by a decrease in nestin positive cells after 7 days. When this system was tested *in vivo* in a rat transection model, it did not enhance neural stem cell survival compared to controls. However, the composite system induced stem cell differentiation to oligodendrocytes, astrocytes and to neurons at a lesser extent. Open field testing showed an improved score compared to the untreated control but was non-significant. This study provides a proof of concept that relies on both the introduction and the stimulation of neural stem cells via a

composite scaffold to regenerate injured spinal cord tissue in SCI. It was proposed that the outcomes may be improved via varying the dose and the release profile of the differentiation agent.

A similar approach was followed to deliver drugs that counteracted the effect of inhibitory molecules at the injury site and hence minimize the effects of secondary injury. In this report, PLGA MS loaded with myelin-associated inhibitor (NEPI-140) and lipid microtubes loaded with chondroitin sulfate proteoglycan (chondroitinase ABC) were incorporated in a fibrin matrix and used in a rat hemisection model. PLGA MS allowed the sustained release of the loaded drug for 5 days with a minimal initial burst compared to controls. The released drug maintained its bioactivity represented as enhanced neurite extension in chick dorsal root ganglia compared to controls. This combination therapy provided several synergetic effects represented as an increased neuron population, reduced axonal growth inhibitors and decreased scar formation in a rat SCI model with no immune reaction (172). Despite the significant outcomes reported, some limitations were present. Examples for this include the absence of MS retention assessment, low encapsulation efficiency and short release period. The MS formulation procedure used 10% NaCl (w/v) in both the organic and the aqueous phases which was reported to impact both the encapsulation efficiency and the release profile (116).

2. Gap analysis, Hypothesis and Objectives

As shown above, the combination of exogenous growth factor treatment PLGA MS and biomaterial carrier scaffolds have demonstrated promising outcomes for neural regeneration for SCI. However, certain limitations were found in the previous work summarized by the high initial burst, difficulty to control encapsulation efficiency and release rate, and stability of the loaded compounds during fabrication and storage.

Utilization of BC as a carrier material offers multiple advantages including cost effective and scalable production, high purity compared to plant sources of cellulose, and hydrophilicity with high capability to absorb water and biocompatibility. In addition, our lab has created a validated procedure to synthesize BC membranes for GF delivery as a potential duraplasty material for stroke treatment (103). BC non-biodegradability represents both an advantage where the mechanical properties are unaffected through time, and a disadvantage due to the unknown long-term body reaction when implanted *in vivo*. However, the main limitations were the release profile, where there was a high initial burst and a total release period of 7 days (103).

In this thesis, we modified this procedure to form tubes to be used for SCI. Afterwards, we aimed at optimizing a manufacturing protocol for GF loading using PLGA MS. This procedure has the following specific requirements: A) high encapsulation efficiency, b) minimal initial burst and c) provision of a sustained release pattern for 14 days or longer. To achieve this, we aim to investigate different factors affecting the protein encapsulation and release profile of the synthesized MS.

2.1 Hypothesis

In this work, we hypothesize that our BC composite delivery system incorporating drug loaded PLGA microspheres will provide a minimal initial burst with a sustained drug release profile

suitable for stimulating endogenous neural stem cell driven repair of injured axons following spinal cord injury. This will be achieved by optimizing the fabrication procedure for both drug loaded PLGA MS and BC tubes. Afterwards, characterization of this composite delivery system will be done. Finally, effect of different fabrication variables on both encapsulation efficiency and release profile will be investigated using a model protein.

2.2 Objectives

Objective 1: Fabrication of the BC tubes from membranes with characterization of swelling ratio, morphological characterization and release profile assessment using model protein.

Objective 2: Optimization of the fabrication procedure of PLGA microspheres. This involves testing the effect of different fabrication variables on encapsulation efficiency, morphology, and release profile using model protein.

Objective 3: Fabrication of the composite BC tubes with full morphological and mechanical characterization with a sterilization procedure suitable for future *in vitro* testing.

3. Materials and Methods:

3.1 Materials

3.1.1 Bio-cellulose Tubes

Gluconacetobacter hansenii was obtained from American Type Culture Collection (ATCC©, Manassasm VA). DWK Life Sciences Kimble^{TM} KIMAX^{TM} Buchner Kimflow^{TM} fritted disc filters with coarse porosity (60 mL borosilicate glass) were obtained from Fisher Scientific Company L.L.C. (Ottawa, ON) for the filtering process. Phosphate buffered saline (-) used for *in vitro* release study was obtained from VWR (Mississauga, ON) containing 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer. 0.02 % (w/v) analytical grade sodium azide (NaN_3, mw: 65.01 $gmol^{-1}$) was added to the PBS, obtained from BDH Chemicals (Mississauga, ON).

3.1.2 Microspheres

PLGA 50:50 (inherent viscosity: 0.76-0.94 dL/g) was purchased from Durect Corporation (Cupertino, CA). BSA (66.3 kDA) was purchased from MP Biomaterials, Inc (Solon, OH). PVA (mw: 44.053 $g*mol^{-1}$) was purchased from Sigma-Aldrich (Burlington, MA). Analytical grade chloroform (mw: 119.38 $gmol^{-1}$) was purchased from OmniSolv® (Etobicoke, ON). Sodium chloride (NaCl) was purchased from VWR (Mississauga, ON).

3.1.3 Sterilization

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS) and penicillin-streptomycin (pen/strep) were purchased from Thermo Fisher Scientific (Ottawa, ON). All materials were used as received unless mentioned otherwise.

3.2 Methods

3.2.1 BC tubes

3.2.1.1 Fabrication of BC tubes

BC sheets were prepared as reported previously (103). In brief, an inoculum concentration of 1.3×10^5 CFU*mL^{-1} of *Gluconacetobacter hansenii* was seeded in culture media containing 20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L Na_2HPO_4 and 1.5 g/L citric acid for 7 days at 26°C. Afterwards, purification of BC sheets was performed by rinsing with distilled water followed by overnight soaking in 0.1M NaOH at 50^{o}C to remove any remaining bacteria or endotoxins. The membranes were then washed with distilled water until the pH was neutral. Afterwards, 10-12 membranes with a final weight of 320 g were blended with water until a homogenous pulp was formed.

To fabricate BC tubes, several trials were conducted as shown in Appendix 2. The successful fabrication of the tubes was performed as follows: 70 g of pulp were vacuum filtered for 60 mins to produce BC composite membranes. The membranes were then cut to rectangular shapes (1.2cm× 1cm) and wrapped around a mold to form a tube similar to what was previously reported (173). This was followed by freeze drying for 48 hours and storage at 4^{o}C.

3.2.1.2 BC tube characterization

The swelling ratio was assessed by measuring the weight of dry BC membranes and the membranes were then submerged in distilled water. At predetermined times points, the membranes were tap-dried using a towel and weighed. The below equation was used to calculate the swelling ratio of the membranes.

$$SR\ (\%) = \frac{(Wwet - Wdry)}{Wdry} * 100\ \%$$

Where SR is swelling ratio, W-wet is the weight of the wet membrane at a specific timepoint, and Wdry is the initial dry weight of the membrane.

Scanning electron microscope (SEM) imaging was performed (Phenom Pro, Phoenix, AZ) on the BC membrane to assess the structure.

3.2.1.3 BC tubes release profile assessment

The release profile of BC tubes was assessed using different loading concentrations of BSA solutions in PBS. This was in an attempt to use the BC tubes as a delivery system alone without MS. Freeze dried, blank BC were soaked into 0.1 ml of the BSA solution for 5 minutes followed by placing them in a dry 24 well plate for air drying for 2 hours. Tubes were then placed in 2 ml PBS and were incubated at 37^{o}C. Samples of 470 µl were withdrawn at serial timepoints (1h, 2h, 4h, 6h, 8h, 12h, 24h, 48h, 96h and at 7, 10, 14 days) and fresh PBS was added to replace the withdrawn volume. BSA concentration was determined using a micro BCA kit (ThermoFisher Scientific, Ottawa, ON, Canada) following the manufacturer's instructions. Three loading concentrations of BSA solution were used: 3 mg/ml, 5 mg/ml, 10 mg/ml. Cumulative BSA release was calculated via the equation

$$R = (cn * vo) + \sum_{i=1}^{n-1} (ci * vi)$$

Where R is the cumulative amount of BSA released at each time point, cn is the measured concentration from the BCA assay, vo is the total volume of the release medium, ci is the

previously measured concentration, and vi is the sample volume withdrawn at each time point *i*.

3.2.2 Microspheres

3.2.2.1 Microsphere fabrication

MS preparation was performed using a double emulsion, solvent evaporation technique (W1/O/W2) (174). In brief, PLGA in chloroform (1g in 4.77ml) was stirred for 1 hour until a clear organic phase solution was obtained (O). Afterwards, BSA (50mg/ml solution in distilled water (W1) was added with stirring for 30 minutes to ensure uniform dispersion of the drug with theoretical drug loading of 4.8%. This was followed by emulsification using a high-speed homogenizer (Omni international, Georgia, USA) at 7000 rpm for 45 seconds to form the primary emulsion (W1/O). This emulsion was immediately injected dropwise into cold PVA 0.5% w/v (W2) with mixing using a propeller mixer at 400rpm. As described below, some formulations involved the addition of a variable concentration of an osmotic agent in the external aqueous phase, while others involved addition of PVA to W1 prior to homogenization. The formed W1/O/W2 emulsion was mixed for 3 hours to allow the evaporation of organic solvent and the subsequent microsphere hardening. Finally, the solution was centrifuged. MS were washed 3 times using cold distilled water followed by lyophilization for 48 hours. The final dried MS were stored at 4^{o}C for further testing.

Different variations of the MS were fabricated for comparative purposes to test the effect of changing different formulation parameters on the final MS. The alternative procedures involved:

A) Variation of BSA form added to W1: BSA Powder (50 mg) as well as BSA solution in distilled water (50 mg/ml) was used. In the case where BSA powder was used, the primary emulsion became a solid in organic phase (S/O) and the final emulsion was S/O/W2.
B) Addition of different volumes of 1% PVA prior to the first homogenization step to form W1 as previously reported (175).
C) Addition of variable concentrations of osmotic agents in W2 prior to solvent evaporation. Here, NaCl was added with concentrations: 0%, 2%, 5%, 8% and 10% (w/v).

Yield percentage was calculated by measuring the weight of the final freeze-dried microspheres followed by using the below equation:

$$Y\% = (Wa / Wt) * 100$$

Where Wa is the actual weight of MS produced and Wt is the total theoretical weight of MS and BSA used.

3.2.2.1 Morphological Characterization

Scanning electron microscope (SEM) imaging was performed (Phenom Pro, Phoenix, AZ) on MS produced to assess the particle size as well as differences in surface morphology. Size analysis and distribution was assessed using ImageJ software (NIH, USA) where the diameter of MS within five SEM images representative of different fields in all MS formulations was determined.

3.2.2.2 Encapsulation Efficiency

Encapsulation efficiency (EE) was measured to identify the total amount of BSA successfully encapsulated in the microspheres relevant to the initial loading amount. This was achieved through MS hydrolysis (176). In brief, 20 mg of MS were placed in 10 ml of 0.1M NaOH solution and was incubated at 37ºC for 48 hours. When the solution was clear, samples were centrifuged and the total BSA concentration was measured using the BCA kit according to manufacturer instructions. The EE was calculated according to the below equation:

$$EE\ (\%) = \frac{actual\ BSA\ content}{theoretical\ BSA\ content} * 100\ \% \quad (1)$$

3.2.2.3 Microsphere release profile assessment

0.1 g of MS were weighed and placed in 15 ml falcon tubes in PBS solution containing 0.01% sodium azide preservative. At specific time intervals, 0.5 ml samples were withdrawn and replaced with same volume of fresh PBS. Samples were collected at 1hr, 4hr, 8hr, 12hr, 24hr, 48hr, 96hr and 7, 10, 14, 21, 24, and 30 days, and the BSA concentration was measured using micro BCA following the manufacturer instructions. Finally, cumulative release was calculated using the following equation:

$$R = (cn * vo) + \sum_{i=1}^{n-1} (ci * vi)$$

Where R is the cumulative amount of BSA released at each time point, cn is the measured concentration from the BCA assay, vo is the total volume of the release medium, ci is the previously measured concentration, and vi is the sample volume withdrawn at each time point *i*.

3.2.3 Composite membranes

3.2.3.1 Composite membrane fabrication and characterization:

Composite membranes were fabricated as shown in Figure 2 using the same procedure mentioned above for the blank membranes with few modifications. Briefly, 70 g of pulp were mixed with 0.1 g of MS with stirring for 5 mins to ensure uniform distribution of MS. Afterwards, the loaded pulp was vacuum filtered and the produced membrane was cut, re-shaped into a tube and lyophilized as in blank membranes. The final composite tube was loaded with MS and was used for further testing.

3.2.3.2 Composite membrane characterization

Microsphere retention was calculated to determine any loss of microspheres during the fabrication process. Retention (R) was calculated using the below equation:

$$R\ (\%) = \frac{(Wc\ -\ Wb)}{Wms} * 100\ \%$$

Where Wc is the weight of the composite membrane, Wb is the weight of the blank membrane with no microspheres manufactured at the same conditions and Wms is the initial weight of microspheres added.

SEM imaging to confirm the successful incorporation of MS into the BC tube was performed using the method described above in section 3.2.2.1.

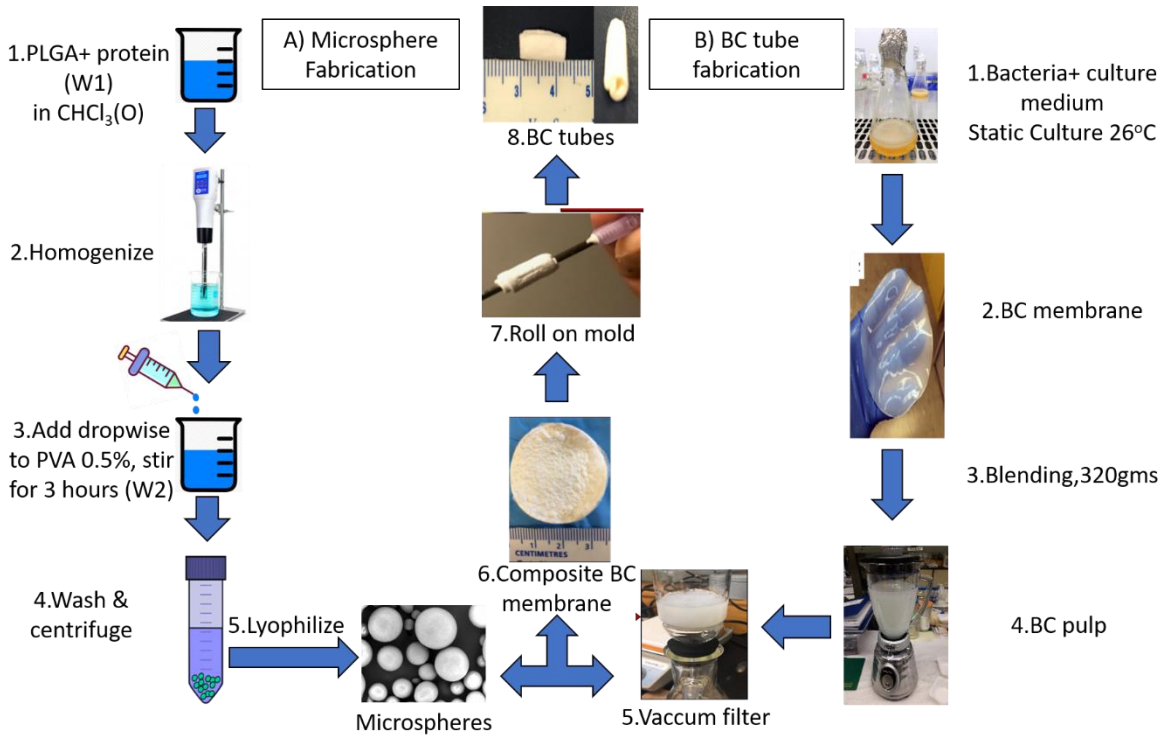


Figure 4 Schematic representation of different fabrication protocols. A) fabrication protocol for PLGA microspheres and B) Fabrication protocol for blended BC. Both products are mixed resulting in the final composite BC tube loaded with PLGA microspheres.

Mechanical testing was performed using an Instron mechanical tester (Instron, Norwood, MA, USA) to determine the compressive modulus of the composite BC tubes. In summary, BC tubes were hydrated for 24 hours with distilled water in preparation for the testing. Hydrated tubes are then placed between the two platens and were compressed at a rate of 0.1mm/second until 100% compressed where the lumen is completely collapsed. Stress strain data were recorded, and compressive modulus was calculated via the slope of the linear portion of the curve for each sample at n=10. PLGA loaded BC tubes were tested and the results were compared to the human and rat spinal cord moduli as reported previously (177).

3.2.3.3 Composite tube sterilization

To test if the composite tube can be sterilized by 70% ethanol, both blank tubes and composite tubes loaded with 0.1 g of PLGA MS were submerged in 70% ethanol for 10 minutes followed by extensive washing with PBS, after which the tubes were placed into complete cell culture media (DMEM with 1% antibiotics). This was performed in sterile culture plates which were placed in 37^{o}C and were checked for media turbidity due to contamination daily for 7 days (N=3).

3.2.3 Analysis

The results are presented as mean ± standard error of the mean. All the data were treated statistically using a one-way analysis of variance (ANOVA) with Tukey's post hoc analysis except for the release profile section where data was analyzed using a split-plot ANOVA. A statistically significant difference was considered at $p < 0.05$. Statistical analysis was performed using IBM SPSS® (SPSS software, Chicago, IL, USA).

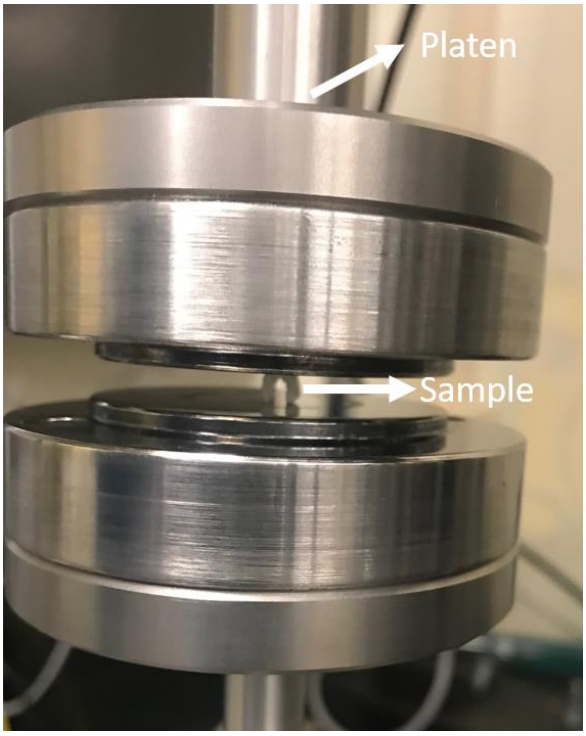


Figure 5 Compressive modulus assessment machine setup for composite biocellulose tubes. Hydrated samples were placed between platens and were compressed at a predetermined rate until the lumen was completely collapsed.

4. Results and Discussion

4.1 BC tubes characterization

BC tubes were successfully fabricated using the method reported previously (178). Morphological characterization using SEM showed the nanofibrous structure of BC tubes with nanosized pore sizes as shown in Figure 6. In addition, the random orientation of the fibers was seen as a result of the blending process.

The assessment of swelling of the BC tubes revealed valuable information reflecting the hydrogel nature of BC. As shown in Figure 6, BC tubes had a ratio of 340.6% ± 13.9 in the first 10 minutes which is a statistically significant weight gain ($p<0.05$). The swelling ratio kept increasing after 5 hours (722.9%± 45.8) reaching 967.5% ± 50.4 at the end of the 72 hours. This shows the hydrogel behavior of our material demonstrated in its ability to absorb a significantly large amount of water. This behavior causes swelling of the cellulose fibers, increasing the mesh size and therefore creating larger pores leading to faster drug diffusion out of the material (179). This potentially has a determinant effect on the drug release profile of the BC tubes if the tubes are directly loaded with drugs or proteins.

To further asses the potential of directly loading proteins on the BC tubes, the BC tubes were loaded with different amounts of BSA as a model protein and their release performance was assessed over 2 weeks. The release profile shown in Figure 7 demonstrates the cumulative BSA release over time.

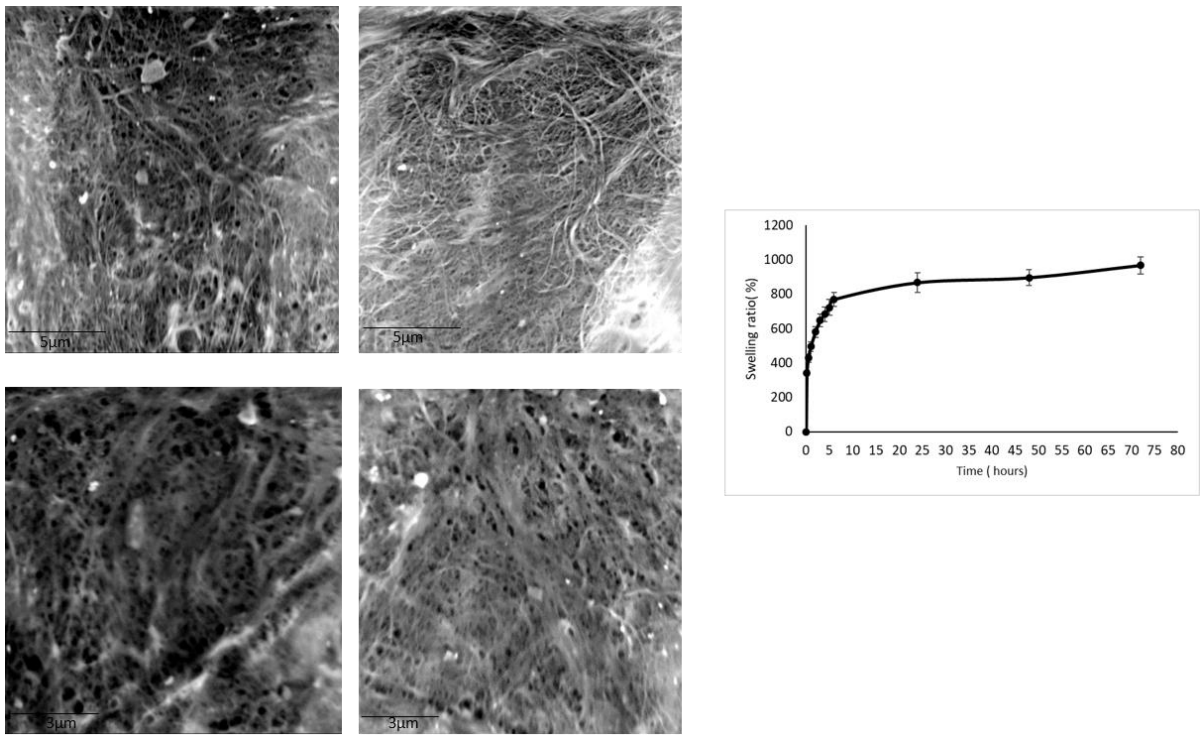


Figure 6 Characterization of BC tubes. Left: SEM images using different magnifications. Scale bars are 5µm (upper) and 3µm (lower) Right: Swelling ratio percentage of BC tubes after different incubation times in PBS. Data represented as mean ± standard error of mean (n=12).

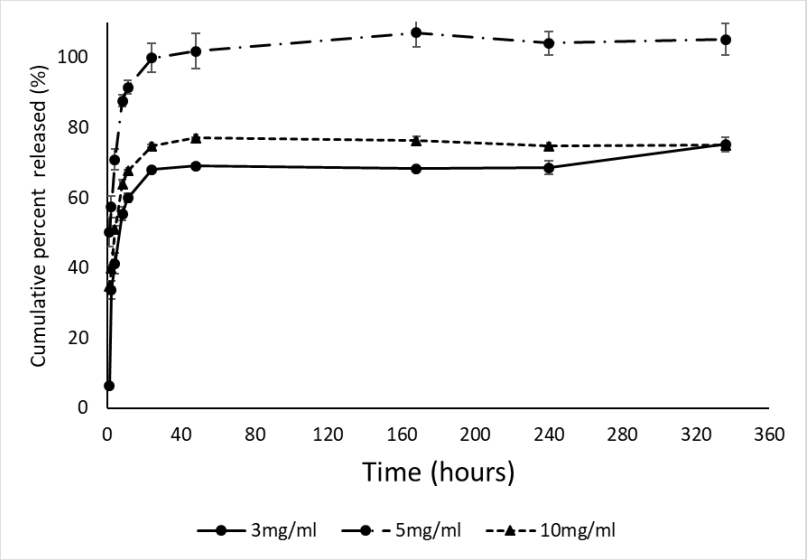


Figure 7 Cumulative release of different loading concentrations of BSA from BC tubes. Data represented as mean ± standard error of the mean (n=3).

A fast release rate was detected in all groups where 69.7% ± 0.3%, 99.8% ±4% and 74.7%±0.414% of the loaded amount was released after 24 hours from the 3 mg/ml, 5 mg/ml and 10 mg/ml groups, respectively. It is noted that the release was minimal after 48 hours.

The results appear to be a consequence of the hydrogel behavior of BC where the high swelling ratio corresponds to an enlarged mesh size leading to higher rate of diffusion and hence, faster drug release. This release behavior will not be suitable for our intended applications as the main aim of using the biomaterial is to produce localized sustained release of growth factors while maintaining their bioactivity. This should reduce the hostility of the microenvironment at the injury site while providing the needed stimulation of endogenous stem cells. Consequently, modification of the material should be carried out to prolong the release. This can be done by producing composite materials where the release is controlled by another polymer.

4.2 Optimization of the fabrication of PLGA MS

4.2.1 Encapsulation efficiency varied with changing the fabrication procedures

The initial fabrication method for MS was the double emulsion/solvent evaporation method used by our lab and previously reported (175). However, one of the major challenges was the low EE of the produced MS. This showed a need to modify the fabrication method to investigate the main factors affecting EE. To achieve this, several parameters were changed and the effect of these variables on both EE and MS morphology was monitored.

First, PLCL was used in this stage to confirm whether the type of polymer used could impact the EE. Table S1 shows different formulations and their compositions and Figure S1 shows the EE for these formulations. As expected, the type of polymer had no impact on the EE.

This was demonstrated by the low EE where the highest EE was around 6%. This was very low compared to other reports that varied in value reaching up to 90% in some cases (116,125,176,180). On the other hand, it was congruent with other papers that reported low EE with variable surfactant types and concentrations (181). This low EE was attributed to the diffusion of the loaded drug to the continuous phase which was possible due to several factors such as: drug vs. polymer ratio, volume of the inner phase, nature of the loaded protein and the amount of organic solvent used (175,182,183,184).

We then investigated the effect of drug vs. polymer ratio. Our initial preparation was 0.2:1, which is a high ratio due to the high amount of the drug used. This led to leakage of BSA from the organic phase to the continuous aqueous phase during microsphere formation before complete sphere hardening, yielding low EE in the final product. Hence, lowering down the initial amount of BSA was expected to regulate the osmotic pressure difference, elevating EE (116,184).

Consequently, we changed the amount of BSA added from 0.2 g to 50 mg. In addition, we used both solid and liquid forms of BSA, with changing the volume of BSA solution used in the inner phase as shown in Table 3. As shown in Figure 8, the EE was significantly increased, reaching 82.4%. However, it was observed that when the total volume of BSA solution was smaller, it led to increasing the viscosity of the organic phase after the first homogenization step. This was due to an increase in polymer concentration due to evaporation of chloroform leading to a loss of the spherical morphology of the MS as shown in Figure 8. To resolve this, a higher volume of BSA solution was added but was accompanied by a significant drop in EE while the MS structure was preserved. Moreover, NaCl addition caused a significant increase

in EE when used with solid BSA compared to the same preparation without NaCl. On the contrary, a decrease in EE occurred when NaCl was used with liquid BSA but was statistically insignificant. From these findings we can conclude that a smaller BSA solution (i.e. inner phase) volume produced MS with higher EE with some morphological irregularities.

The impact of changing the procedure was prominent on the MS morphology as was shown in SEM micrographs in Figure 8. When solid BSA was used, particles showed a smooth surface. This was not the case when liquid BSA was used. In the absence of NaCl, MS had a rough porous surface. These pores were reduced upon addition of NaCl as shown in Figure 9 forming a smoother surface compared to batches without the salt addition. It was also observed that some of the particles had a spindle or thread shape in addition to the spherical shape. This can be attributed to solvent evaporation during the homogenization process as well as the decreased volume of BSA liquid used, leading to an increase in viscosity and the injection of polymer in the form of a continuous stream rather than droplets. In addition, the low surfactant concentration in the continuous phase (i.e. PVA) may not be sufficient to separate that stream of polymer into spheres. These two possibilities were tackled in the last 2 formulations with more volume for BSA liquid and higher PVA concentration.

Table 3 Different formulas and their composition after the second modification

Formula/composition	F1	F2	F3	F4	F5	F6
PLGA	1g in 3.77 ml Chloroform					
BSA	Powder/ 50mg		Liquid/50mg/0.5ml in water		Liquid/50mg in 1ml water	
PVA continuous phase	0.1%/300ml				0.5%/300ml	
PVA inner phase	1%/20ml	1%/20ml	-	-	-	-
NaCl	-	5%	-	5%	-	5%

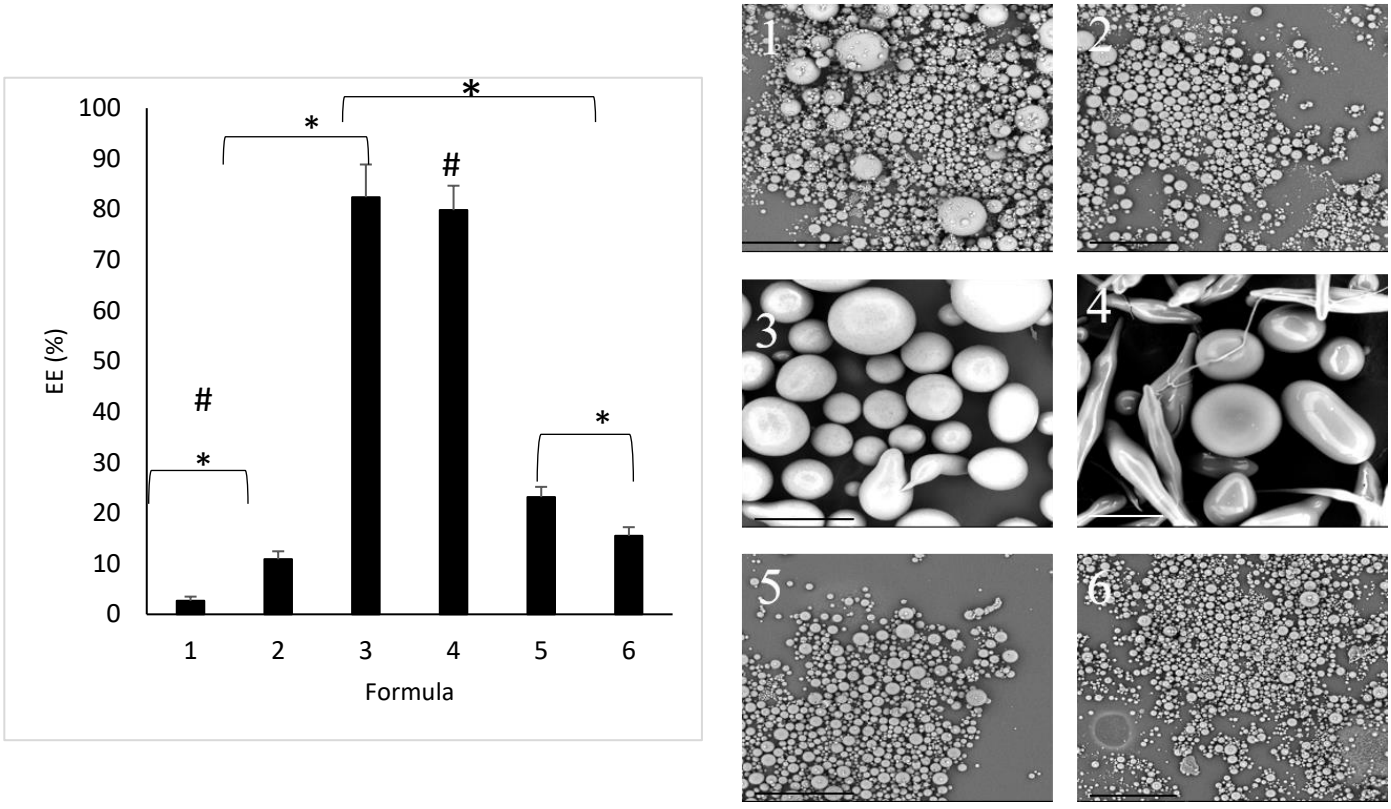


Figure 8 Encapsulation efficiency and SEM micrographs of different formulas from table 3 (Left). Data represented as mean ±STD (n=3) and scale bars are 100µm. * and # represents statistical significance ($P<0.05$).

Three main variables were changed to preserve the spherical morphology of MS without affecting EE: Increasing the solvent amount, PVA concentration in the continuous phase and using BSA in liquid form. When the amount of solvent was increased from 3.77 ml to 4.77 ml, it compensated for the loss of solvent during fabrication. This, in addition to the use of liquid BSA, lead to adjusting the viscosity of the solution prior to its addition to the continuous phase, forming spherical droplets instead of a continuous stream. Finally, elevating the PVA concentration in the continuous phase from 0.1% w/v to 0.5% (w/v) aided in preserving the spherical morphology. This came in agreement of what was reported previously (116).

The effect of the addition of osmotic agents such as NaCl on the morphology can be observed in the SEM micrographs (Figure 9). MS produced using the optimized method had a rough surface full of micropores as stated above. When NaCl was used, those pores were eliminated and the MS had smooth surface. This can be attributed to the nature of the solvent evaporation technique, that relies on the emulsification of the BSA aqueous solution into an organic phase followed by dispersion in the continuous aqueous phase (116). The presence of PVA leads to the formation of spheres where the polymer shell surrounds the protein. This stage is transient since the polymer shell is still soft due to the incomplete evaporation of the organic solvent. During this stage, water penetrates the MS leading to the diffusion of BSA to the continuous phase, reducing the EE and forming a rough porous surface. Addition of an osmotic agent such as NaCl lead to the saturation of the continuous phase, reducing the osmotic pressure difference and hence minimizing BSA diffusion and forming MS with smooth surface. This

is congruent with previous reports where presence of osmotic agent produced denser MS with a smooth, less porous surface MS (116).

No NaCl

NaCl

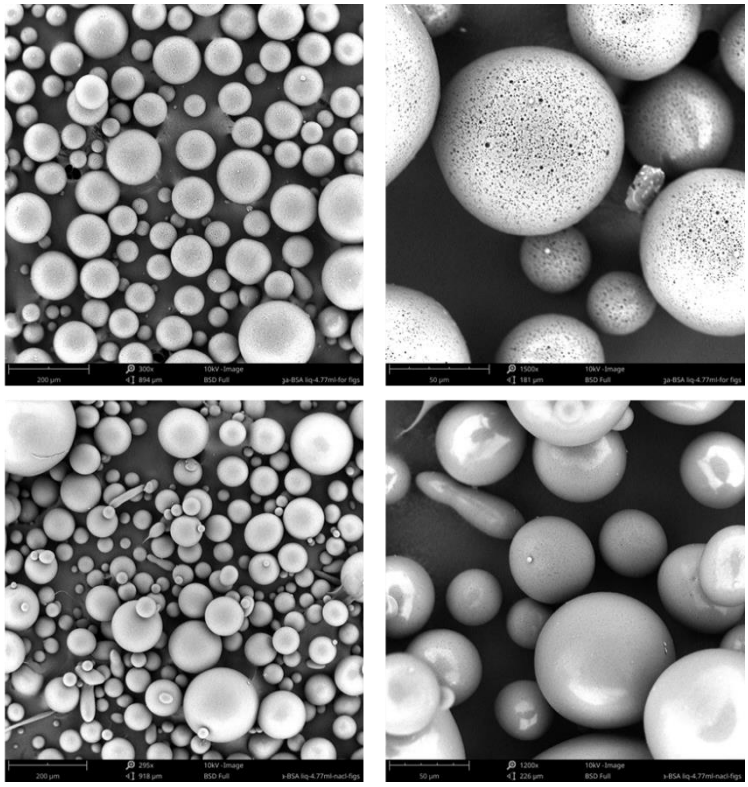


Figure 9 SEM micrographs of PLGA microspheres prepared using liquid BSA with inner phase volume 0.5ml in the absence and presence of NaCl. Scale bars are 200µm and 50µm, respectively.

4.2.2 Modified fabrication enhanced the EE with minimal effect on the yield

We assessed the EE for formulas shown in Figure 10. As expected, the use of liquid BSA without the primary emulsification step significantly enhanced the EE from 4.9% ±1.7 to 69.9%± 2.1 without NaCl and from 10.4% ± 1.4 to 68%±2.5 with NaCl in continuous phase. Interestingly, the addition of NaCl significantly increased EE when BSA solid was used. In addition, there was no significant change in the yield for all formulations. On the other hand, increasing the solvent amount did not affect the EE significantly when compared to previous experiments. This is because solvent effect is non-significant within a certain range (180).

The two main factors influencing the encapsulation efficiency of MS are the drug/polymer ratio and the volume of the inner aqueous phase used (185,186). When the 1% PVA is used in the internal organic phase prior to homogenization, BSA tends to move from the organic phase towards this aqueous phase, leading to a decreased EE. When this was eliminated, BSA stayed in the organic phase, thus resulting in enhanced EE. Other effects were noticed upon the elimination of the 1% PVA including: An increase in the particle size and a decrease in yield. These effects can be attributed to the higher amount of encapsulated BSA and increased viscosity due to volume reduction.

Finally, we assessed the effect of inner phase volume when using powder BSA. This was done to identify the main cause of low EE. Batches using solid BSA were fabricated with variable volumes of internal phase as shown in Table 5 and Figure 11. It was found that the presence of a high internal phase volume of 20 ml produced a low EE of 9.6% ± 0.5. When the volume was reduced to 0.5 ml, the EE was significantly increased to 70.5% ± 2.5. Interestingly, when the internal phase was eliminated, EE was further increased to 75% ±4.5 but lead to an increased viscosity, difficulty

in handling and reduced yield. These results confirmed the above explanation regarding osmotic pressure and BSA diffusion to the aqueous phase. In addition, it showed that a low internal phase volume of 0.5 ml did not affect the EE significantly. This is the same amount of liquid that is used to make the BSA solution in the other formulations and was the volume of choice for further experiments. Moreover, this was in congruence of previous findings showing the impact of the inner phase volume on EE (185,186).

4.2.3 Effect of salt concentration on microsphere morphology and particle size

We further analyzed the effect of variable salt concentration on both MS morphology, particle size and later on the release profile. As shown in Figure 7, MS preserved their spherical shape in all concentrations. However, particle size distribution was impacted. Particle size was non uniform for concentrations below 5%, where particles showed a broader size distribution. Interestingly, 5% NaCl use showed a uniform distribution which appears to be a cut off value for size uniformity as higher NaCl concentrations produced similar results.

4.2.4 Addition of osmotic agent significantly reduced the initial burst

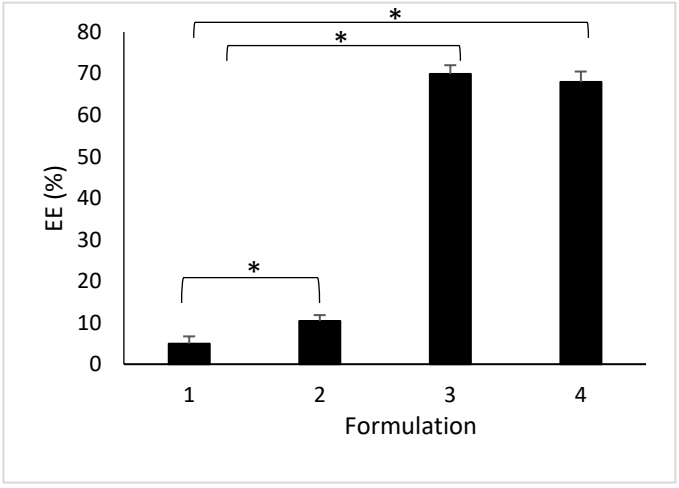
Cumulative release was assessed in the presence and absence of NaCl over a period of 30 days. As shown in Figure 13, the cumulative release in the case of the absence of the osmotic agent followed a biphasic profile. This can be seen with a significant burst release in the first 12 hours followed by a sustained release for the remaining time points reaching a plateau stage. Interestingly, the presence of NaCl significantly reduced that burst release, with a profile following a sustained release behavior continuing for 30 days.

To further explore the effect of NaCl on the release profile, we used different concentrations of NaCl in the continuous phase during fabrication of MS and assessed the morphology and the

release profile over 30 days. Interestingly, the release profile showed a significantly low burst release in all NaCl concentrations (Figure 13) compared to the formula without NaCl ($p<0.05$). This suggested that the presence of NaCl significantly reduce the initial burst regardless of concentration. Further analysis demonstrated a higher release rate for the 2% NaCl with a significant increase at day 2 ($p<0.05$) that did not occur with the other concentrations. In addition, the 5% NaCl showed the slowest release rate of all formulations, with significantly lower BSA amounts compared to the 2% NaCl from day 7 to day 27 ($P<0.05$). Interestingly, the difference was non-significant between formulations with 5% and above.

Table 4 Composition of different MS formulations tested for final optimization

Formula	F1	F2	F3	F4
PLGA	1 g			
$CHCl_3$	4.77 ml			
BSA	50mg solid		50mg/0.5ml	
PVA (inner)	1%w/v/20ml	1%w/v/20ml	n/a	n/a
PVA outer	0.5%			
NaCl	0%	5% w/v	0% w/v	5%w/v



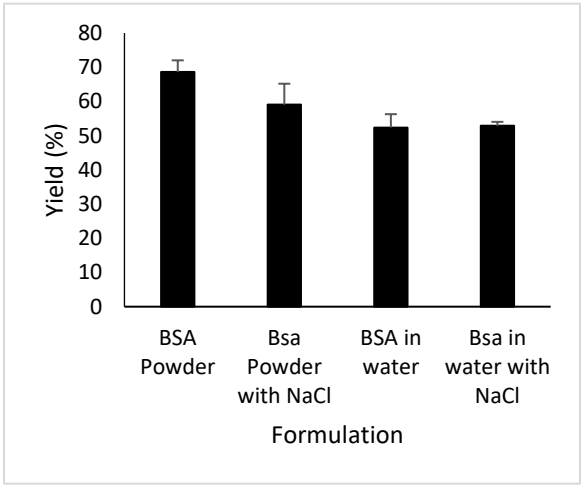


Figure 10 Encapsulation efficiency and yield of different MS formulations. Data represented as mean ±SEM (n=3). * represents statistical significance ($P<0.05$).

Table 5 Composition of MS batches for proving the effect of inner phase volume

Composition	A	B	C
PLGA	1g		
BSA	50mg		
PVA 1% inner	20ml	n/a	0.5ml
PVA 0.5% (outer)	300ml		
NaCl	n/a		

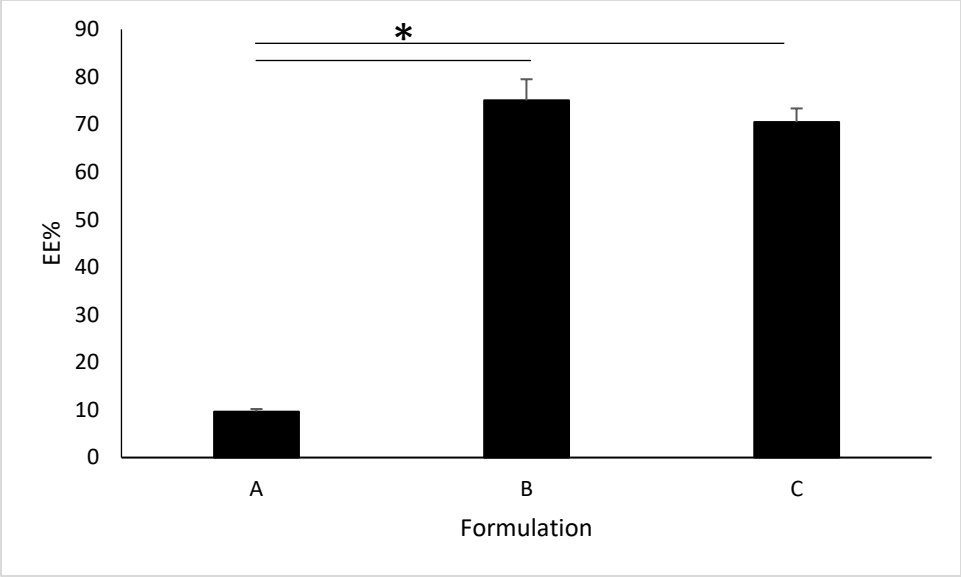


Figure 11 Encapsulation efficiency of 3 microsphere batches with different inner phase volumes (n=3). Data represented as mean ±STD. * represents statistical significance ($P<0.05$) where group A is significantly lower than both B and C while groups B and C are not significantly different.

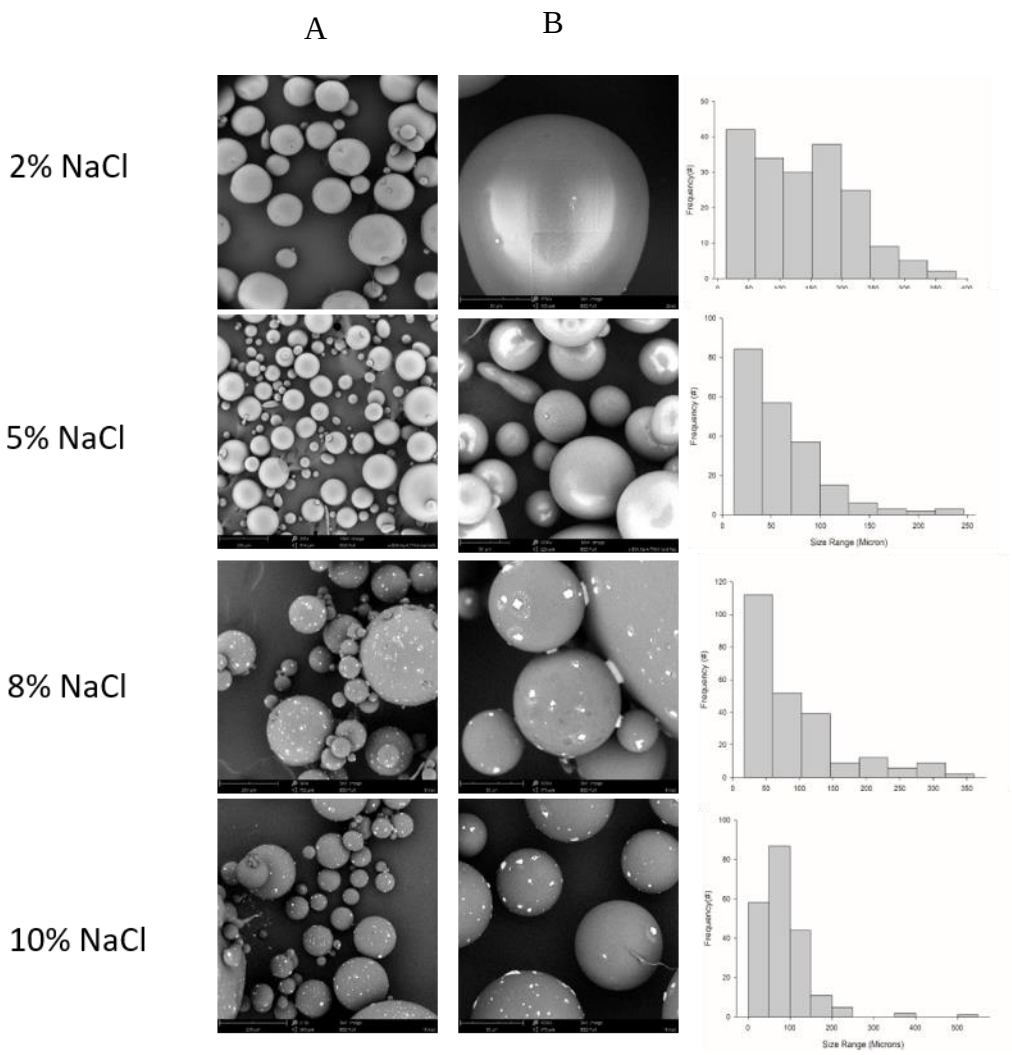


Figure 12 SEM micrograph and size distribution for microspheres formulated using different NaCl concentration in the continuous phase. Scale bars are 200µm (A) and 50µm (B).

Finally, it was found that comparisons of the cumulative amount released with each formulation had one significant spike that varied between each NaCl concentration. This spike was demonstrated at different timepoints, where it showed at day 2 for the 2% formulation, day 21 for the 5% formulation and at day 30 for both the 8% and the 10% formulation ($P<0.05$).

From the above findings we can conclude that the presence of NaCl minimized the initial burst release by the mechanism explained above. In addition, there seems to be a cut off value for the NaCl concentration where any further increase does note have an effect on the release rate. The cut off value for our study was 5% NaCl where increasing the concentration does not have an observable effect on the BSA release.

PLGA microsphere release systems are characterized by a triphasic release profile: A) an initial burst release followed by B) sustained slow release phase and finally C) another burst stage following complete sphere degradation. The first stage is diffusion controlled where a large amount of the loaded drug is released in a period of 6 to 8 hours. This can be explained by the release of drug molecules adsorbed on or present near the inner surface of the MS. In addition, the porous surface that was seen in the SEM images facilitated the penetration of the release medium into the MS, enhancing the diffusion-controlled release (116). This changes in the second stage where the release is degradation controlled and depends on the slow degradation of PLGA layers.

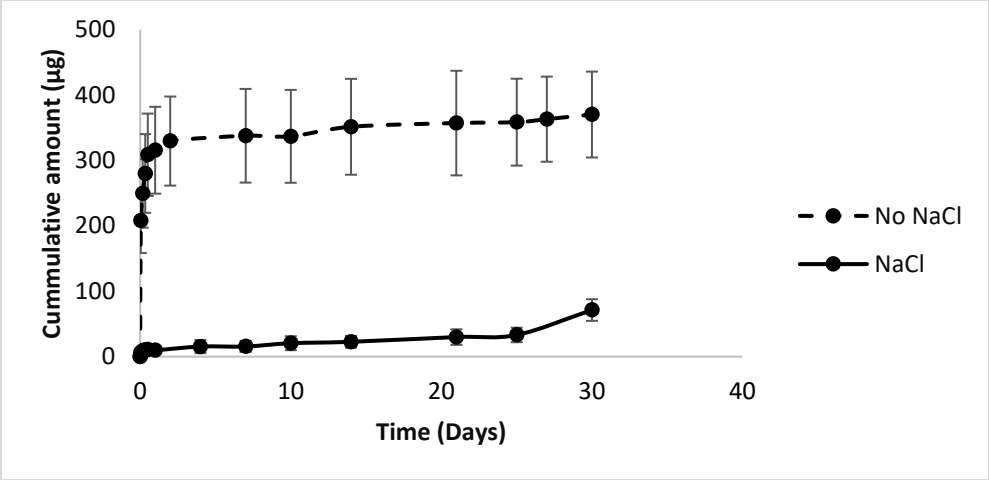
The scope of this study is two-fold: a specific focus on the first 2 stages (A and B) that occur within the first 30 days together with minimizing the diffusion dependent Stage A and maximizing the degradation dependent extended release. This was the reason to proceed in sample analysis for 30 days only with a special focus on sampling during the first 12 hours.

Our findings were in agreement with previous studies that identified 5% NaCl as the ideal concentration for further experiments (116,125,185). However, our release profile is different from these reports owing to the minor differences in the preparation procedure such as the method of homogenization. In addition, other studies found that 10%NaCl was the most suitable (187). On the other hand, some studies have mentioned other osmotic agents such as glucose leading to similar effects (187). These findings demonstrate that the smallest difference in fabrication procedure can result in variable effects, which explains the differences in our results compared to some previous literature.

A successful growth factor delivery system requires stabilizers to preserve the bioactivity of the loaded growth factors during and after formulation. Several stabilizers were reported in literature, some of which were generally beneficial to all proteins while others were specific to the growth factor loaded. For example, a mixture of BSA/ Mg(OH)2, heparin, EDTA and sucrose was used when loading bFGF in PLGA microspheres (188). This mixture was aiming to neutralize the acidic niche, chelate trace metal ions and preserve the structure (188). In another study, polyethylene glycol, BSA and a gelatin derivative were used to stabilize IGF in PLGA spheres (189). In addition, other therapeutic agents may require incorporation in the system, such as anti-inflammatory agents to target the inflammation at the injury site.

Although these additives enhanced GF encapsulation and stability, the increased salt concentration in the internal phase solution lead to non-uniform distribution of GFs, increased MS size, porosity and a significant initial burst release (185,190). The introduction of an osmotic agent in the outer phase as in our work neutralized this difference in osmotic pressure and minimized the initial burst release. This is important when loading the MS with growth factors as there would not be a

significant effect on osmotic pressure from adding stabilizers in the internal phase. This will guarantee maximum encapsulation, preserved morphology and a sustained release rate.



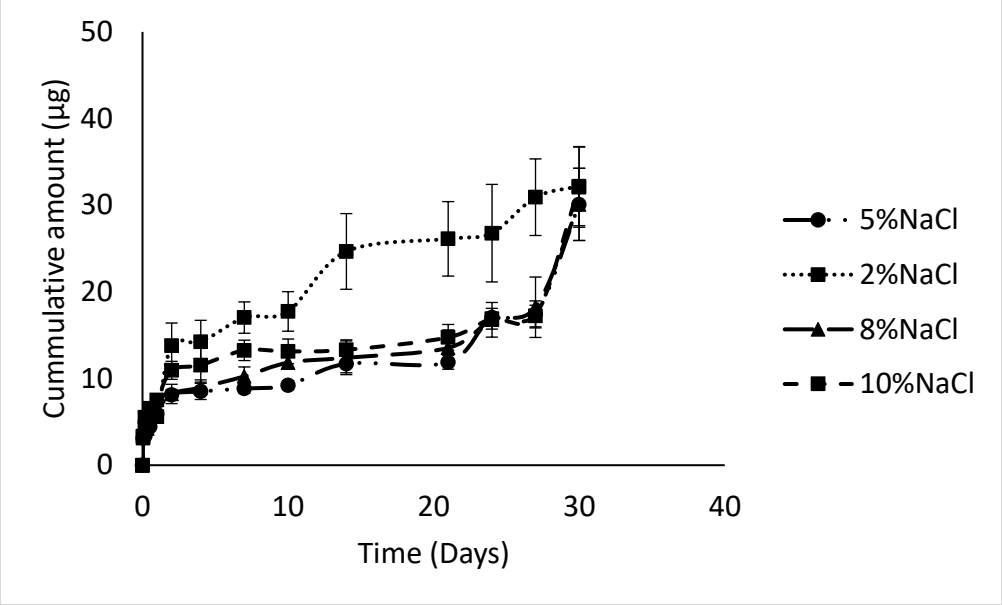


Figure 13 Effect of osmotic agent addition on the release behavior of PLGA microspheres. MS were fabricated in absence and presence of different concentrations NaCl and BSA release was assessed at predetermined time intervals. Data represented as mean ±SEM (n=3).

4.3 Composite Tube were successfully fabricated and characterized

Figure 14 shows the successful and homogenous incorporation of MS into the BC tubes. This is advantageous in terms of release as the use of MS aims to produce a degradation-based release profile where the MS that are exposed to water first will degrade before the ones inside the tube, aiding in sustained release. In addition, there was no structural or size change in both MS or membrane internal structure after incorporation. Assessment of microsphere retention showed 96.66 ± 12 % (n=3) of MS initially loaded to the pulp was preserved after tube formation and drying.

4.4 Mechanical properties of BC tubes are suitable for SCI applications

Compressive modulus was used to evaluate mechanical properties of BC tubes loaded with PLGA and PLCL MS that were used for comparative purposes as shown in Figure 15. Our material has the advantage of being non-biodegradable, which means it will possess stable mechanical properties over time. As shown in Figure 15, composite BC tubes possessed a similar compressive modulus of 0.11 ±0.01 MPa and 0.13 ±0.03 MPa for BC tubes loaded with PLCL and PLGA MS, respectively. From the above data we can conclude that the MS type and amount have a nonsignificant effect on the compressive modulus of the composite tubes.

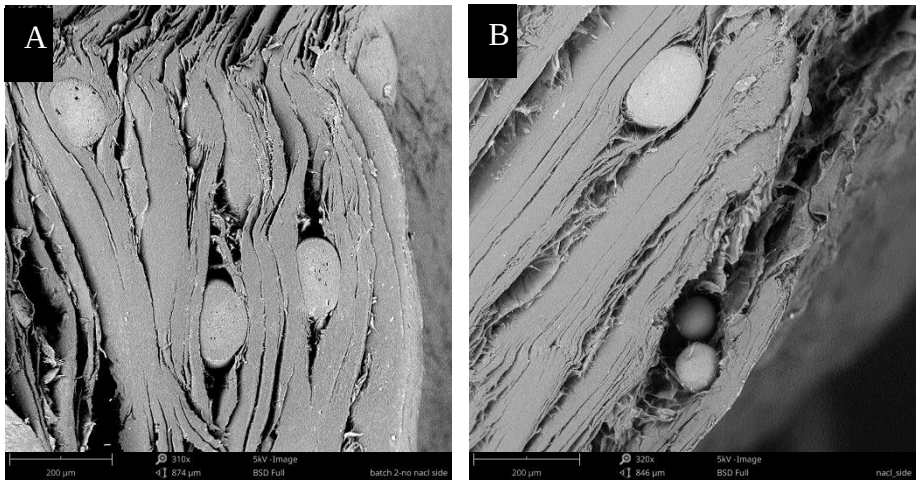


Figure 14 SEM for composite membranes loaded with MS prepared without sodium chloride (A) and with sodium chloride (B). Scale bar is 200µm.

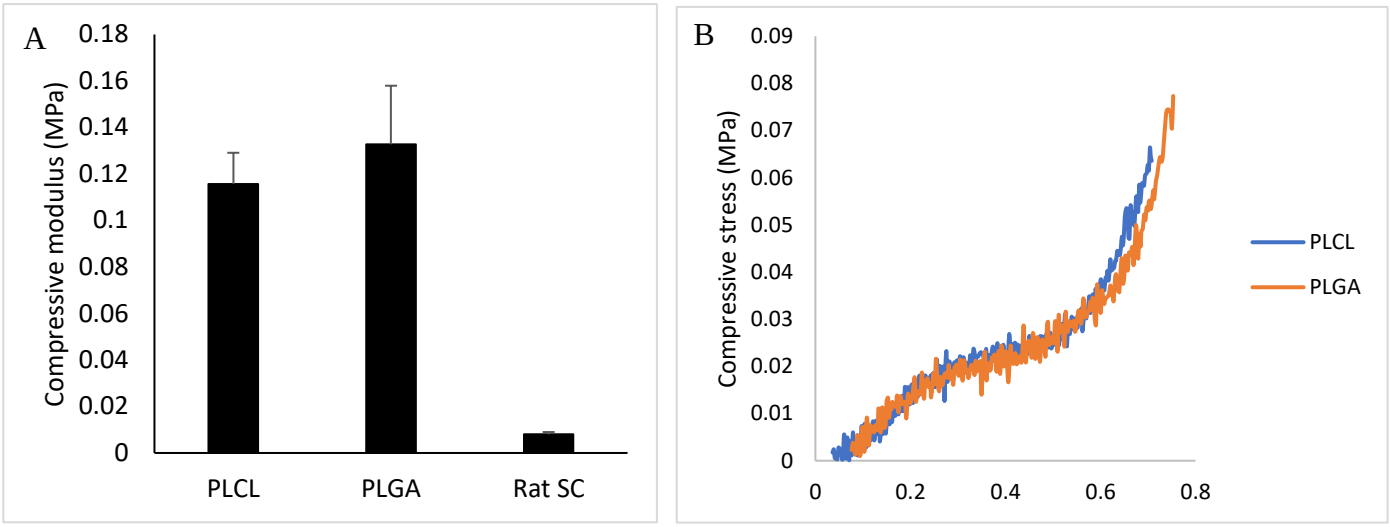


Figure 15 Mechanical properties of microsphere loaded BC tubes. A) Compressive modulus of PLCL and PLGA loaded BC tubes compared to rat spinal cord (177). Data represented as mean± SEM (n=10). B) Representative stress strain curve for BC tubes loaded with PLGA and PLCL microspheres.

Compressive modulus is critical for tubes aimed to be used in the treatment of SCI. This is because material mechanical properties were reported to have a prominent effect on cell adhesion, spreading and differentiation, especially with respect to stem cells (191). In addition, uninjured central nervous system tissue belongs to the class of the softest tissue among organisms and the use of soft materials would be preferable as it mimics the natural tissue (192). However, the mechanical properties of injured tissue vary and so is its reaction to different biomaterials. In the case of SCI where tissue is partially or completely transected, soft material may collapse and destroy any nerves growing inside and prevent regrowth (193,194). On the other hand, a highly stiff material can causes a foreign body reaction that induces microglial activation followed by elevated levels of inflammatory mediators and neural cell death (195). In order to determine whether a material mechanical property is suitable, it should be tested *in vitro* and *in vivo* due to the conflicting data in literature regarding the ideal values (196). Interestingly, our material showed a higher modulus compared to rat spinal cord which is sufficient to protect any regrowth of tissue or nerves within the tubes (197). Moreover, these results were consistent with other materials that were tested and utilized for the same application with promising results (198,199).

The model used to measure the compressive modulus in this study is the same model used for other biomaterial tubes intended for SCI (177,200,201). In these studies, the products were produced by molding the material into a full circular tube shape. In contrast, our tubes were a C-shape that is opened from one side. This provides an advantage of easily fitting onto the cut spinal cord stumps when tested in vivo. This difference in shape could be causing some challenges in the mechanical property characterizations that could not be carried out using

conventional methods. This model was used as a proof of suitability for our tubes for SCI as it is a simple model that can be compared with other studies available in literature.

4.5 Composite tube sterilization

The feasibility of sterilizing the composite tubes in preparation for future *in-vitro* work was explored. This was done through a proof of concept experiment by incubating the BC tubes in complete cell culture media with and without sterilizing them with 70% ethanol. As shown in Figure 16, both ethanol sterilized and non sterilized did not show any signs of contamination after 7 days of incubation.

Sterilization should be done while maintaining structural and mechanical properties of the fabricated insert. There are several sterilization techniques used to sterilize biomaterial scaffolds. Some of these methods such as ethylene oxide, gamma irradiation, and autoclaving are extensively used. However, these methods have been reported to cause changes in mechanical properties and increased degradation (202,203). Other methods such as super critical CO_2 are promising methods due to the low temperature and high penetration. However, it is still under testing and validation for different materials. Details of different sterilization methods and their pros and cons have been reviewed elsewhere (204).

One of the simple, low cost and effective methods for sterilizing biomaterials is ethanol sterilization. It offers room temperature sterilization which is critical for some materials and loaded drugs. For our material, BC is non-degradable but the MS material is degradable and irradiation might affect the release rate (204). In addition, super critical CO_2 may be suitable but it requires special equipment and safety training (204). Interestingly, ethanol sterilization was chosen for its simplicity as well as effectiveness and absence of any significant effects on

the molecular weight and structure of PLGA (181,204). Our results show that ethanol sterilization does not produce a significant effect on contamination prevention. This is most probably due to the antibiotic component of the cell culture media which supresses the growth of any bacteria or contaminants. From this we can conclude that our inserts can be used for in vitro testing. However, the only way to render our tubes suitable for in vivo work is to perform the whole manufacturing process in a GMP facility.

Ethanol sterilized

Control

Media Only

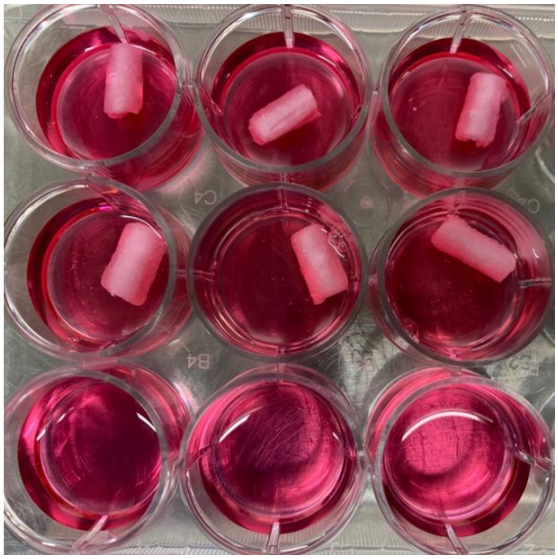


Figure 16 Effect of ethanol sterilization of composite tubes on eliminating contamination. BC tubes were immersed in 70% ethanol for 10 minutes followed by three washes with PBS and incubation in complete media for 7 days at 37ºC. Control group was immersed in PBS only and all media were checked visually for contamination or turbidity.

4.6 Biocompatibility of composite membranes

One of the concerns about using BC tubes as carriers for MS for SCI was whether they were biocompatible where axons, neurons and stem cells are able to grow into and around the tube. This was investigated by our group in a study recently published (103,190) . In this work, Stumpf et al. investigated the *in vitro* bioactivity of growth factors released from BC membranes using rat neural stem/progenitor cells (NSPCs). It was demonstrated that NSPCs formed neurospheres in presence of BC membranes both blank and loaded with GFs. In addition, the number of neurospheres increased from week 1 to week 2 in presence of blank BC. Moreover, neurospheres showed a significant increase in their number and diameter when GF loaded BC membranes were used, demonstrating enhanced proliferation. NSPCs also showed the capability to differentiate into oligodendrocytes, astrocytes and neurons when the GF loaded BC membrane was used. Finally, the *in vivo* biocompatibility of BC membranes was assessed in rat brains and showed no sign of any unfavorable reactions after 4 weeks of implantation. From these results we can conclude that the presence of the BC did not hinder the proliferation and differentiation of NSPCs demonstrating their biocompatibility and their ability to deliver bioactive GFs (103,190).

Our BC tubes were expected to have similar properties since they were fabricated with a similar process. The main difference was that we were using PLGA MS as carriers for the GFs rather than loading them directly on BC. This was aimed at increasing the overall release period while minimizing the initial burst release. Interestingly, PLGA was reported to be biocompatible and hence became FDA approved (205,206,207,208). Therefore, our delivery system is likely biocompatible and is ready for further *in vitro* and *in vivo* investigations.

5. Limitations

Despite all the advantages, our delivery system has some limitations. First, there is a limit to the amount of MS that can be loaded on the BC tubes. If the amount of loaded MS exceeds the capacity of the membrane, the MS retention will be significantly reduced. In our trials we used up to 0.1g of MS loaded on the membrane with minimal effect on MS retention. However, if the amount of MS per tube was to be much higher, the retention could be impacted. Secondly, the long term body reaction is unknown. Although our material is biocompatible, the long-term reaction of the animal or human body is uncertain as the material is still a non biodegradable foreign material. This can be further investigated during *in vivo* studies by imaging the tubes after an extended period following implantation. Finally, the total percentage of protein release compared to the initial loaded amount is very low. This is due to the continuous degradation of the PLGA microspheres overtime leading to two main effects: First, an increase in the acidic degradation products in the sphere release niche which increases protein binding to the core of the polymer, increasing its retention and preventing its release (209). This was further assessed in a previous study which showed an increase in the number of acidic functional groups in PLGA determined after degradation (210). This increase in acidity resulted in a significant increase in protein binding to the polymer through interaction between basic amino acids of protein and the acidic carboxyl groups of the polymer (211,212). In addition, the acidic microenvironment was reported to reduce the stability of the loaded protein (213,214). Second, long term degradation changes the microspheres into a sticky lump leading to the blockage of microporous structure, preventing the release of protein molecules in the core of the MS (209). This can be overcome through the addition of different excipients such as sodium carbonate to neutralize the acidity in the medium and

stabilize the protein (214,215,216). Finally, the release profile needs to be retested after the addition of the therapeutic agents. Although our data are promising, it was done using a model protein. This system is intended to be loaded with GFs, each of which have different properties such as molecular weight and charge. In addition, the therapeutic doses for GFs are significantly less than the doses tested using the model protein. The high loading dose of BSA was justified by the detection limit of the used assay. Consequently, there are expected to be differences in the release profile that needs to be investigated using low therapeutic doses and suitable detection assays prior to *in vitro* and *in vivo* testing of the delivery system.

6. Conclusion and future directions

The focus of this study was to fabricate a composite, BC based delivery system to be used for the localized sustained release of GFs in case of SCI. This was achieved through the successful optimization and fabrication of multiple components. First, the BC tubes were fabricated and characterized. This was followed by testing the release behaviour of a model protein loaded directly on the tubes. The release behaviour was found to be an immediate release profile that was not sustained and so was not suitable for our intended application. Second, optimization and successful fabrication and characterization of PLGA microspheres was performed. In addition, a detailed investigation of the effect of different fabrication parameters on the encapsulation efficiency was conducted. We found that a lower inner phase volume and drug: polymer ratio lead to a significant increase in the amount of protein encapsulated. In addition, the presence of 5% NaCl regulated the osmotic pressure, leading to a reduced porosity of the MS surface rendering it smooth. Release profile assessment revealed a cut off concentration of salt above which the initial burst is significantly reduced while the sustained release continued for 30 days which is suitable for our intended SCI applications. Finally, the MS were successfully loaded onto the BC tubes and the composite system was characterized.

Future studies involve loading the composite system with GF combination followed by *in vitro* testing with neural stem cells. This is to test the effect of the released GFs on proliferation and differentiation of these cells. Ultimately, additional studies would include *in vivo* testing on a rat transection SCI model and examining the regenerative capabilities of our release system and its potential for further studies.

Appendix 1

Table S1 Different formulations to overcome low EE

Formula	Polymer	BSA	NaCl	PVA conc. In the internal phase	PVA conc in the cont. phase
1	PLCL	-	5% w/v	1%	0.1% w/v
2		Liquid/ 0.2g/ml	5% w/v		
3			-		
4		Powder/0.2g	5% w/v		0.1% w/v
5		Powder/0.2g	-		1% w/v
6	PLGA	Powder/0.2g	5% w/v		0.1% w/v

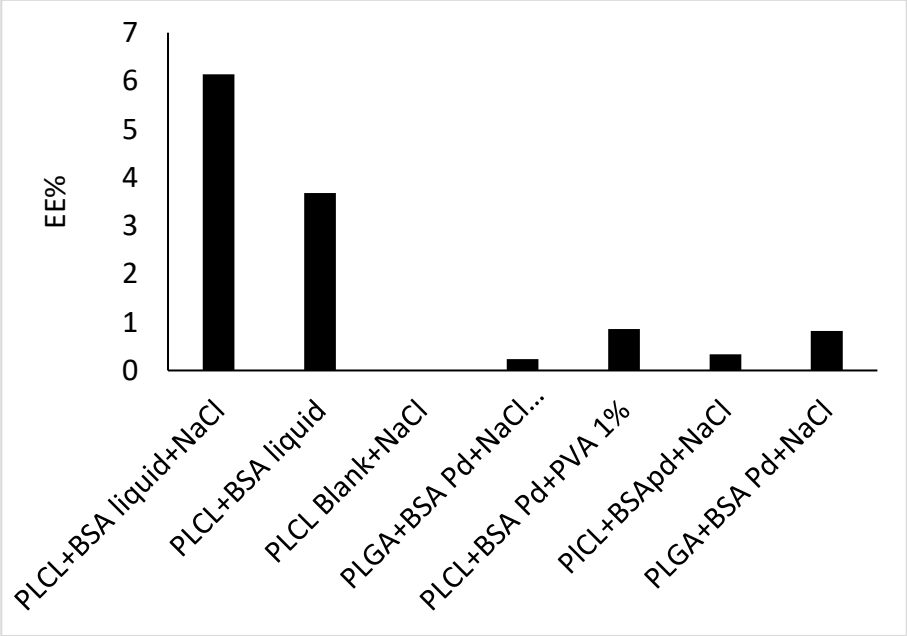


Figure S1 Encapsulation efficiency of different formulations after the first modification.

Appendix 2

Different methods and trials used to produce the BC tubes

2.1 Methods

Method 1: dissolving and molding BC

The vacuum filtered membranes weree cut down into small pieces using scissors and added at a concentration of 3.5wt% to a precooled solution (-10^{o}C) of NaOH (7wt%) and urea (12wt%). The mixture was stirred until BC was completely dissolved and the solution was then degassed and injected into a mold which was frozen at -80^{o}C for 24 hours. The frozen molds were freeze dried for 24 hours. The BC tubes were removed from the molds and placed in a NaOH neutralizing solution (5% acetic acid). Afterwards, BC tubes were washed with distilled water for 4 hours, frozen at -80oC, freeze dried again and autoclaved (25min, 120^{o}C) for further use.

This method was reported in several occasions to successfully dissolve cotton linter derived cellulose within minutes (217,218). Low temperatures allowed cellulose fibers to relax and expand, facilitating more penetration by water and solvent molecules. In addition, NaOH hydrates binds to cellulose, whereas urea hydrates act as hydrogen bond donor/ acceptor that catalyzes the reaction. This reduces hydrophobic interactions between cellulose fibers, forcing them stay far from each other in solution, leading to their dispersion and hence, a clear dissolved solution. When this solvent system was used on our BC membranes using the mentioned concentrations, cellulose was partially dissolved with a slight increase in solution viscosity. This is clear in Figure S2 where chunks of BC were still visible in solution. Consequently, several concentrations of NaOH (5%, 7%, 8%, 10%, 12%) were attempted while keeping urea concentration constant (Table S2).

However, complete dissolution was not achieved. To confirm whether there was a partial dissolution, 5ml NaOH/ urea / cellulose solution was frozen at -20^{o}C followed by freeze drying. This resulted in a porous chunk of dry BC (Figure S2) that collapsed upon rehydration due to its high porosity. This confirms the partial dissolution of cellulose in the solvent system. However, the product was of low cellulose content which is fragile and unsuitable for our application. High and low concentrations of BC (1%, 3.5%) were used with this solvent system producing similar results. It was reported that this solvent system is efficient up to a certain degree of cellulose polymerization above which dissolution is not possible, which is apparently the case in our membranes. This can be attributed to the degree of polymerization of our BC which appears to be high. This is confirmed by previous reports stating that the aqueous alkali systems are limited to cellulose of relatively low DP (typically below 300) (218).

Method 2: Stacking and reshaping

Vacuum dried membranes were cut into disc shaped pieces and then loaded onto a stainless-steel needle or glass capillary tube (4mm diameter, approximate diameter of rat spinal cord). The layered discs were treated with NaOH (20wt%) for 24 hours until they become compact in size due to water loss. The formed tubes were then washed, taken off the needles and placed in distilled water.

Method two was originally attempted on bacterial cellulose that was mercerized and mechanically shaped (219). When our material was cut and placed on a needle followed by alkaline treatment (Figure S2b), the pieces shrank in size without fusion. In addition, they became harder in structure similar to what was reported previously (219). It was expected that those layers would fuse to form

one tube which was not the case. Interestingly, the mechanical properties were modified just by alkaline treatment which may be useful in further modifications of mechanical properties.

Table S2 Different compositions of the alkali-based system and the relevant solubility of BC

NaOH Conc. (%)	Urea Conc. (%)	Cellulose (wt%)	Temperature	Comment
5	12	1 or 3.5	-10/ -5/ room	Insoluble
7	12		temp.	Partial
8.5	12			Insoluble
10	12			Insoluble
12	12			insoluble

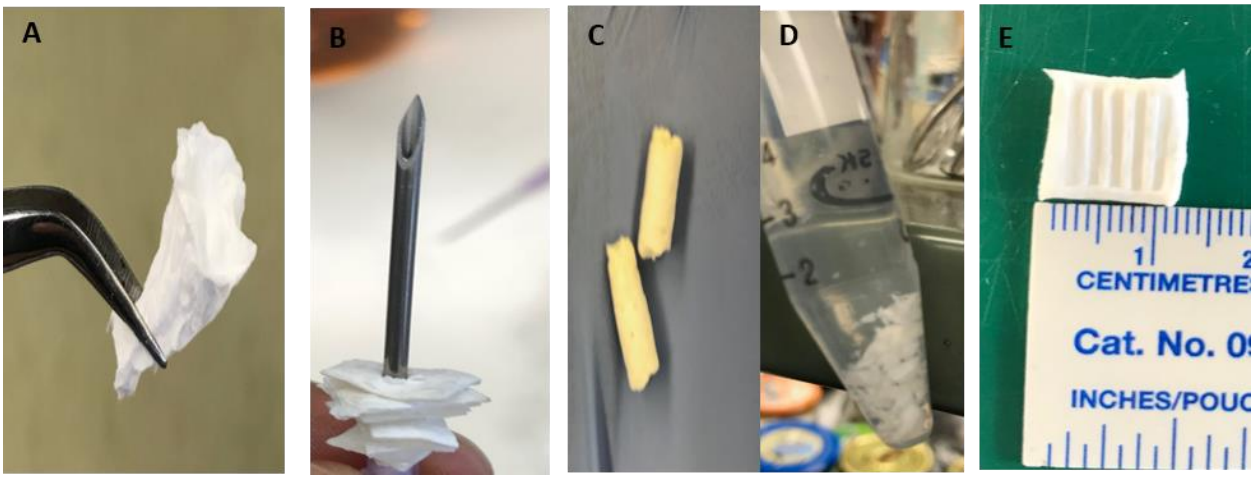


Figure S2 Final product after attempting to synthesize BC tubes using different methods. A) Freeze drying a portion of NaOH (7%) and Urea (12%), B) Stacking and reshaping and C) molding drying after freeze drying and D) after hydration

Other unsuccessful attempts and techniques were used to produce the tubes. For example, the vacuum dried membranes possessed a paste like structure which was molded into a tube and inserted into a mold before freeze drying (Fig 1a). However, any modification to the structure of the wet membrane lead to irregularity of the final product after freeze drying. One attempt to make this work was through alkaline treatment but the tube structure was already compromised.

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