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DENTAL STEM CELL DELIVERY THROUGH NEW INJECTABLE MATRICES FOR SPINAL CORD REGENERATION

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ABSTRACT

Traumatic spinal cord injury (SCI) is a global health problem involving complex pathophysiological cascade and afflicts both developing and developed countries. Transplantation of Mesenchymal stem cell population such as dental stem cells (DSC) have demonstrated preclinical potential for central nervous system (CNS) repair. The work presented in this thesis has evaluated the potential of dental stem cells from apical papillae (SCAP) in combination with different biomaterials for SCI repair.

ECM scaffolds were produced from different mammalian tissues including spinal cord, bone and dental hard tissue using different decellularisation processes. Scaffolds were then digested with pepsin to allow solubilisation and hydrogel formation. The ECM hydrogels were characterised and embedded with SCAP to investigate the effect of morphological and biochemical properties upon cell characteristics. All the hydrogels maintained high cell viability and an increase in the cell number with a satisfactory metabolic activity. However, only ECM hydrogels from decellularised spinal cord and bone tissue supported the expression of neural lineage and pro-angiogenic markers with stronger responses observed with spinal cord ECM hydrogels.

Biodegradable PLGA-Triblock (PLGA-TB) microparticles were fabricated to provide controlled release of glial cell derived neurotrophic factor (GDNF) and may facilitate SCAP attachment. An optimal PLGA-TB microparticle formulation was selected based on the size, surface morphology and release profile achieved. All commercial preparation of GDNF being stabilised in salt, a modified protocol was required to prepare microparticles. The formulation was modified with 10mM sodium acetate which led to a successful encapsulation and sustained release of bioactive GDNF. To support SCAP attachment and survival, PLGA-TB microparticles surfaces were coated with different ECM pre-gel solutions (spinal cord and bone tissue ECM) and laminin. Assessment of surface coating with ToF-SIMS showed protein adsorption on all the coated microparticles, with a higher adsorption on ECM pre-gel coated microparticles. All the surface modified PLGA-TB microparticles supported prolonged SCAP attachment and survival. Laminin and bone ECM pre-gel coated microparticles promoted a significant increase in SCAP number after 7 days.

Over all, the result in this thesis have shown that SCAP combined with decellularised mammalian tissue derived ECM hydrogels or GDNF loaded PLGA-TB microparticles may facilitate delivery of autologous stem cells to promote spinal cord repair.

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“गुरु गोबिंद दोऊ खड़े, का के लागूं पाय।

बलिहारी गुरु आपणे, गोबिंद दियो बताय॥”

“ Guru Gobind dou khade kake lagoon paay,
Balihari Guru aapne Gobind diyo batay ”

- Kabir Das’s couplet

Meaning,

“If both, Guru (teacher) and Gobind (God) were to appear at once, whose feet should I touch first?

It has to be the Guru’s feet first, because without him, how would I have recognized/known God?”

“A sincere dedication to all my teachers”

CONTENTS

ABBREVIATIONS

5-HT	5-hydroxytryptamine
AdMSC	Adipocyte-derived mesenchymal stem cells
AIS	ASIA impairment scale
Akt	Protein kinase B
ASIA	American Spinal Injury Association
ATP	Adenosine triphosphate
BBB	Beattie and Bresnahan
BDNF	Brain-derived neurotrophic factor
bECM	Decellularised bone
bECMh	Bone ECM hydrogels
bFGF	Basic fibroblast growth factor
BMSC	Bone marrow derived stromal cells
BSCB	Blood-Spinal Cord Barrier
c-Kit	Proto-oncogene c Kit
c-RET	Receptor tyrosine kinase encoding proto-oncogene
Ca^{2+}	Calcium ions
CD	Cluster Differentiation
chABC	Chondroitinase ABC
CINC	Cytokine-induced neutrophil chemoattractant
CNPase	2′, 3′-cyclic nucleotide 3′-phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
Coll IV	Collagen IV
COX	Cyclooxygenase
CPD	Critical point dryer
CSF	Cerebrospinal fluid

CSPG	Chondroitin sulphate proteoglycan
CST	Corticospinal tract
DAPI	4’,6-diamidino-2-phenylindole
DCM	Dichloromethane
DCX	Doublecortin
dECM	Decellularised dentine
dECMh	Dentine ECM hydrogels
DFSC	Dental follicle stem cells
DMMB	1, 9–Dimethyl methylene blue assay
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cells
DRG	Dorsal root ganglion
DSC	Dental stem cells
DSPG	Dermatan sulphate proteoglycan
ECM	Extracellular matrix
ECMh	ECM hydrogel-Hydrogels derived from ECM material
EDTA	Ethylenediaminetetraacetic acid
EEA	Excitatory amino acids
ES	Embryonic stem cells
ESB	European Society for Biomaterials
ESNPC	Embryonic stem cell-derived neural progenitor cells
FDA	U.S Food and Drug Administration
FIM	Functional independence measure
GAD	Glutamic acid decarboxylase
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary proteins
GFR-α1	GDNF- family receptor-α1
GK11	Gacyclidine

GM-CSF	Granulocyte–macrophage colony-stimulating factor
GMSC	Gingiva-derived MSC
GvHD	Graft vs. host disease
HAMC	Hyaluronic acid-methylcellulose
HBSS	Hank's balanced salt Solution
HCl	Hydrochloric acid
HDE	Humanitarian Device Exemption
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hNSC	Human neural stem cells
HSA	Human serum albumin
HSPG	Heparan sulphate proteoglycan
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IKVAV	Isoleucine-lysine-valine-alanine-valine
IL	Interleukin
iPSC	induced pluripotent stem cells
ISNSCI	International Standards for Spinal Cord Injury
K^+	Potassium ions
KSPG	Keratan sulphate proteoglycan
LCST	Lower critical solution temperature
LiCl	Lithium chloride
LIF	Leukemia inhibitory factor
M-CSF	Macrophage colony-stimulating factor
Mag	Myelin associated glycoprotein

MAPK	Mitogen-activated protein kinase
MBV	Matrix-bound nanovesicles
MCP-1	Monocyte chemoattractant protein 1
MDA	Malonyldialdehyde
MEP	Motor evoked potential
MHC	Major Histocompatibility Complex
MMPs	Matrix metalloproteases
MP	Microparticle
MPC	Mesenchymal progenitor cells
MPSS	Methylprednisolone sodium succinate
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MSC	Mesenchymal stem cells
NA	Normalised Absorbance
Na^+	Sodium ions
NaCl	Sodium chloride
NCAM	Neural cell adhesion molecules
NeuN	Neuronal nuclear antigen
NFM	Neurofilament-M
NG2	Neurone-glial antigen 2
NGF	Nerve growth factor
NK cells	Natural killer cells
NMDA	N-methyl-d-aspartate
NO	Nitric oxide
NSE	Neuron-specific enolase
NSPC	Neural stem cells and progenitor cells
NT	Neurotrophin
OEC	Olfactory ensheathing cells

$OH^{\bullet}$	Hydroxyl radical
Omgp	Oligodendrocyte myelin glycoprotein
OPC	Oligodendrocyte progenitor cells
p-DAB	4- p-dimethylaminobenzaldehyde
PAN-VC	Poly-acrylonitrile-co-vinylchloride
PBS	Phosphate buffered saline
PCL	Poly-ε- caprolactone
PCR	Polymerase chain reaction
$P_{DL}LGA$	poly (DL- lactic acid-co-glycolic acid)
PDLSC	Periodontal ligament stem cells
PECAM	Platelet endothelial adhesion molecule-
PEG	Poly-ethylene glycol
PES	Polyethersulfone
PGE	Prostaglandins
PGI	Prostacyclin
PHB	Poly- β-hydroxybutirate
pHEMA	Poly-2-hydroxyethyl methacrylate
PHPMA	Poly [N-(2-hydroxypropyl) methacrylamide]
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placental growth factor
PLA	Poly (lactide)
PLC	Phospholipase C
PLP	Proteolipid protein
PNiPAAm-PEG	Poly(N-isopropylacrylamide)-g-polyethylene glycol
PNS	Peripheral nervous system
PTMC-CL	Poly-trimethylene carbonate-co-ecaprolactone
PTS	Post-traumatic syringomyelia
PTSD	Post-traumatic stress disorder

PVA	Poly Vinyl Alcohol
RCT	Random clinical trial
RGD	Arginine-glycine-aspartic acid
ROS	Reactive oxygen species
RST	Raphespinal tracts
SC	Schwann cells
SCAP	Stem cells from the apical papilla
scECM	Decellulaised spinal cord
scECMh	Spinal cord ECM hydrogels
SCI	Spinal cord injury
SDF-1	Stromal-derived factor 1
SDS	Sodium dodecyl sulphate
sGAG	Sulphated glycosaminoglycan
SHED	Stem cells from human exfoliated deciduous teeth
Shh	Sonic hedgehog
SIS-ECM	Porcine small intestine mucosa
SKP	Skin-derived precursor cells
SLE	Systemic lupus erythematosus
Slug	Zinc finger protein SNAI2
Snail-1	Zinc finger protein SNAI1
Sox-9	SRY-box 9
SSEP	Somatosensory Evoked Potential
STS	Staurosporine
t_{95}	95% of gelation
$t_{1/2}$	50% of gelation
TCP	Tissue culture treated plastic
TE buffer	Tris-EDTA buffer
TGF-1	Transforming growth factor beta 1
TGPC	Tooth germ progenitor cells

t_{lag}	lag time
TM	Tirilazad mesylates
TNF-α	Tumour necrosis factor-alpha
ToF-SIMS	Time-of-Flight Secondary Ion Mass Spectroscopy
TRH	Thyrotropin releasing hormone
UCB-MSC	Umbilical cord blood derived mesenchymal stem cells
VEGF	Vascular endothelial growth factor
w/o/w	Water in oil in water
WD	Wallerian degeneration
YIGSR	Tyrosine-isoleucine-glycine-serine-arginine
Triblock copolymer, TB	PLGA- PEG- PLGA
SA- P_{DL}LGA-20% TB	Sodium acetate-P_{DL}LGA-20% w/w TB

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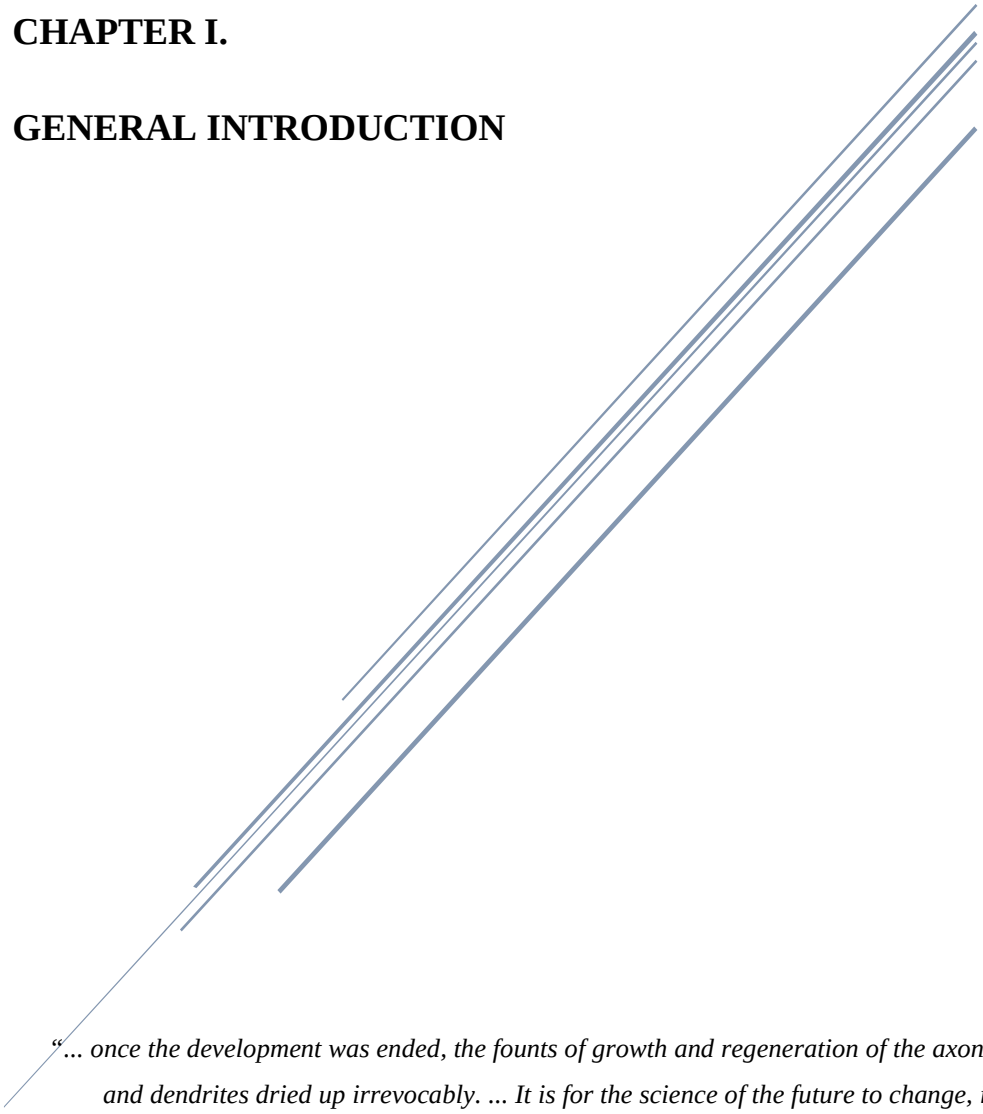
CHAPTER I.

GENERAL INTRODUCTION



"... once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. ... It is for the science of the future to change, if possible, this harsh decree".

-Ramón y Cajal, 1991

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1. SPINAL CORD INJURY

Spinal cord injury (SCI) is caused by trauma (e.g. car crash) or disease (e.g. polio, spina bifida) to the nerves at the end of the spinal canal or any part of the spinal cord. It often results in debilitating changes in nearly every aspect of the victim's life- physical health and well-being, productive employment, personal relationships and social activity [1]. The earliest reference to SCI dates back to 2500 BC [2]. Today, 4500 years later, the management of SCI remains broadly palliative instead of curative, involving prevention of secondary injury progression, managing spasticity, dysautonomia, deafferentation pain, complications of sensory loss and educating patients to manage their disabilities [3].

Worldwide, an approximate of 100,000 new SCI incidents are reported each year [4,5]. A significant number of spinal cord injuries are due to preventable causes, such as automobile crashes which have been a main cause these past four decades, falls and violence [6]. According to the demographic trends, the SCI incident rate is higher in adolescent/ young adults (15-29 years) and older age (60+ years) with a male-to-female ratio of 2:1 in adults [7,8]. Unfortunately, there are no therapies for SCI yet that provide a complete functional restoration. Thus, SCI often results in post-traumatic stress disorder (PTSD), depression/anxiety and lesser survival in most of the victims [9]. Herein, the different injury levels and pathophysiology of SCI alongside the various therapeutic and regenerative interventions utilised for the management of SCI was reviewed.

1.1. ANATOMY OF SPINAL CORD

The spinal Cord is a fundamental part of the central nervous system (CNS) and a major conduit through which motor and sensory information are transmitted between brain and body [10,11]. The spinal cord is sheathed in protective membranes of spinal meninges, i.e. the outer dura mater, middle arachnoid mater and innermost pia mater [12]. The whole spinal cord and its protective layers are together encased in the bony vertebral column [12]. The spinal cord can be divided into four parts based on the rostro caudal orientation: cervical, thoracic, lumbar and sacral; two of these parts are marked by an increase in size known as cervical and lumbar enlargement [12,13].

The H/butterfly-shaped central zone of cord named grey matter, comprises cell bodies and dendrites as well as myelinated axons of neurons [10,11]. Longitudinally oriented spinal tracts, the white matter surrounds the grey matter. It consists of myelinated axons responsible for the transmission of impulses [11,13]. The anterior nerve root originating from anterior horn of grey matter carries motor information [10,13]. These nerve roots converge and enter dorsal root ganglion (DRG) [10,13]. The posterior nerve root originating from the ventral horn carries sensory information [10,13]. The anterior and posterior nerve roots unite to form the spinal nerve immediately distal to the ganglion. There are usually 31 pairs of spinal nerves that subdivided into eight cervical (C1-C8), twelve thoracic (T1-T12), five lumbar (L1-L5), five sacral (S1-S5) and one coccygeal spinal segments in human [10,12,13]. The essential functions of each spinal nerve affected by an acute injury are displayed in Figure 1.

1.2. KEY CONCEPTS AND LEVELS OF SCI

A crucial factor in determining a successful prognosis after SCI is understanding the extent of natural recovery [15]. To achieve this requires a consistent classification of neurological impairment to facilitate accurate communication between clinicians and researchers for the development of goal-oriented treatment approaches [16]. A severity scale to determine the acuteness of SCI was first introduced in 1969: the Frankel scale [16]. Although the Frankel scale has been redefined multiple time, the key limitations including (1) exclusion of level of injury and (2) ambiguous criteria in determining what includes “useful” motor strength were addressed by the classification committee of American Spinal Injury Association (ASIA) in 1992 [17]. Since then, the term “ASIA impairment scale” (AIS) (Table 1) was used instead of the Frankel scale [18]. The ASIA classification system named International Standards for Spinal Cord Injury (ISNSCI) was revised in 2011 with an update in 2015. It remains the most recognised and standardised method for assessing the severity scale till date [15].

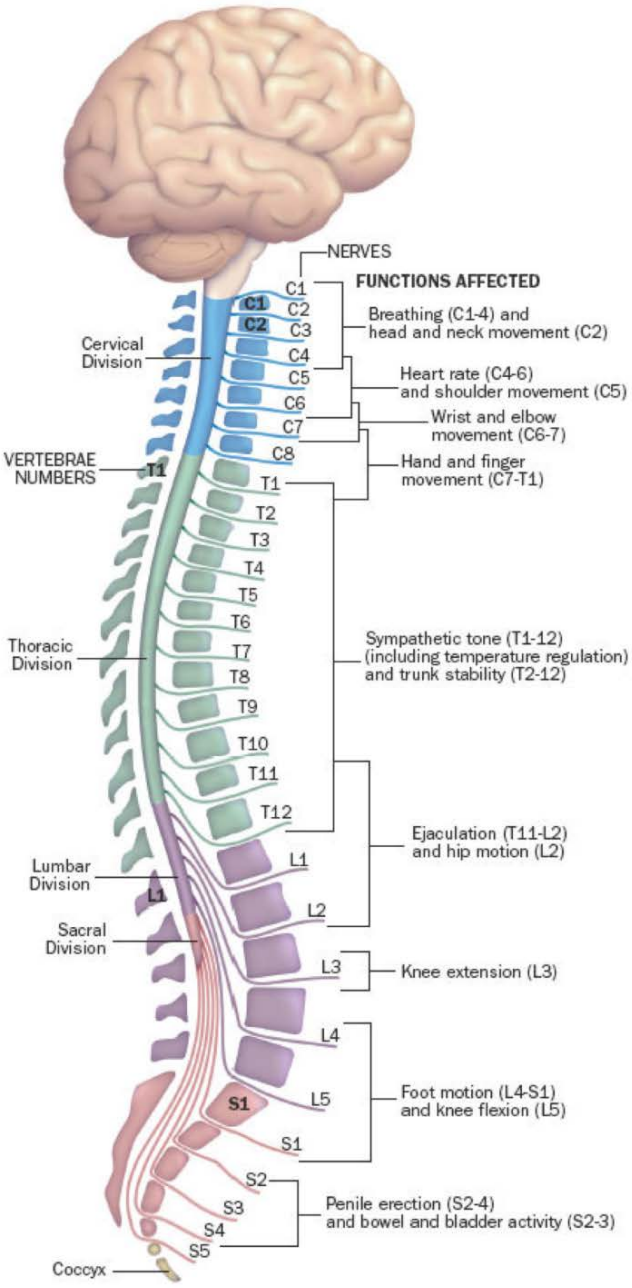


Figure 1: Illustration of spinal nerves and corresponding functions. Reproduced from [14]

Table 1: The American Spinal Injury Association impairment scale (AIS) for International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI).

	ASIA impairment scale (AIS)	Lesion
A	No motor or sensory function is present in the sacral segments (S4-S5)	Complete
B	Absence of motor function preserved below the neurological level and involves the sacral segments S4-S5. Presence of sensory function.	Sensory incomplete
C	Sensory function preserved at the most caudal sacral segments and presents of some motor function below the neurological level. Active movements and full range of motion (ROM) without gravity preserved in more than half of the muscles below neurological level	Motor incomplete
D	Motor function preserved below the neurological level with < half of the muscles possess active movements and full ROM against gravity	Motor incomplete
E	Normal motor and sensory function.	Normal

1.3. PATHOPHYSIOLOGY OF TRAUMATIC SCI

The SCI can be classified broadly into four types [3,19–21]: (i) solid cord injury, a rare type of SCI (ii) contusion injury, the most common injury characterised by haemorrhage, expanding necrosis and cavitation with no surface disruption of the spinal cord, (iii) cord laceration wherein a surface disturbance of spinal cord occurred via a sharp penetrating force and (iv) spinal cord maceration that happened due to a massive compression force and caused a severe distortion of spinal cord morphology. The pathophysiology of SCI can be described as triphasic, comprising a primary injury phase of initial mechanical trauma, followed by a delayed secondary phase with an onset of a biological events cascade leading to neurological damage [21]. Subsequently, the chronic injury/end phase which occurs from weeks to years after the primary injury [3,21,22]. The chronic injury causes both orthograde and retrograde neurological impairments [3,21,22]. The principal events in the pathophysiology occurring after SCI are illustrated in Figure 2 [23].

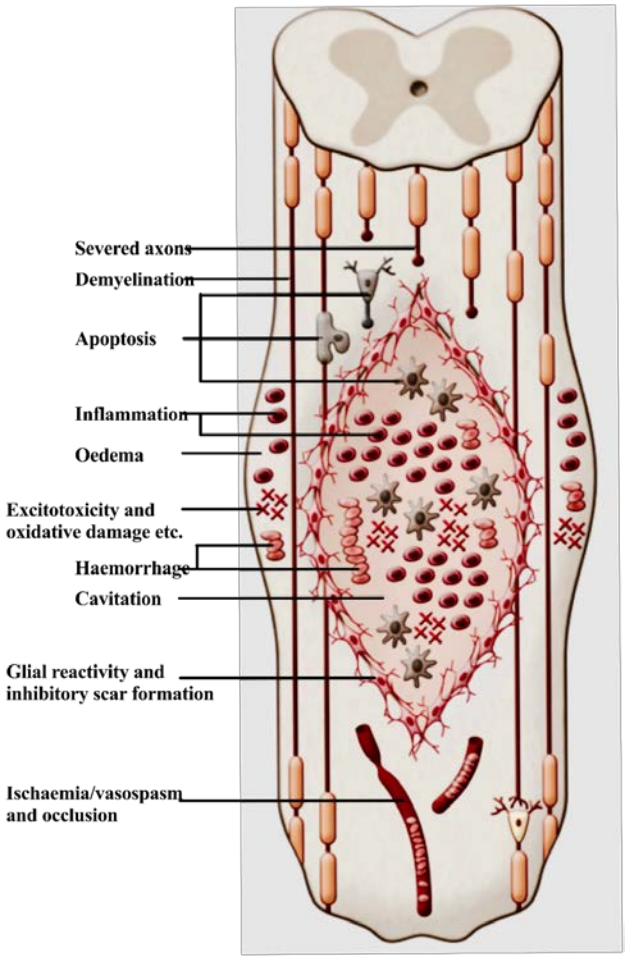


Figure 2: Pathophysiology of SCI. The layout depicts a multifactorial pathophysiological event occurring as the result of mechanical insult on the spinal cord. The primary and secondary events include oedema, haemorrhage, inflammation, apoptosis, necrosis, excitotoxicity, lipid peroxidation, ischaemia/vasospasm, electrolyte imbalance and blood vessel occlusion. Consequently, a fluid filled cyst forms in the centre of the cord surrounded by hypertrophic astrocytes. Source: reproduced from Mothe. A and Tator. C [23]

1.3.1. Primary injury

The physical mechanisms involved in the primary injury are shearing, laceration, acute stretching and sudden acceleration-deceleration injuries [24]. It is rare that primary injuries fully disrupt the continuity of spinal cord, except for cord maceration injury and the spared demyelinated axons are mostly seen in subpial rim [25]. The existence of these spared axons is therapeutically significant as they are likely to be re-myelinated by oligodendrocytes as well as Schwann cells [25,26]. Furthermore, a significant neurological recovery with up to 10% of preserved original axons was demonstrated in animal studies, increasing the interest in the optimisation of curative intervention for functional recovery with the existing connections [27–29]. The pathophysiological events occurring as the result of primary injury are characterised by vasodilation, congestion and petechial haemorrhage [19].

1.3.2. Secondary injury

After the initial tissue damage, a cascade of vascular, biochemical and cellular events occur that cause additional structural and functional damage, leading to a prolonged secondary phase of injury [21,30]. It is of the utmost important to understand the physicochemical processes involved in this phase as it could provide significant information for the development of promising therapies [3]. The secondary events are principally comprised of (a) Systemic Effect-Neurogenic shock, (b) vascular events, (c) biochemical and electrophysiological events and (d) cellular events. The mechanisms involved in post-traumatic injury of spinal cord are represented in Figure 3.

Neurogenic shock

Neurogenic shock occurs due to the paralysis of vasomotor input, thereby debilitating the balance of vasodilator and vasoconstrictor influence to the arterioles and venules, leading to an insufficient tissue perfusion [31]. It is characterised by prolonged bradycardia, hypotension and depressed cardiac output due to a combination of underlying abnormalities such as decreased sympathetic tone, unimpeded vagal tone and probably secondary changes in the heart [31,33].

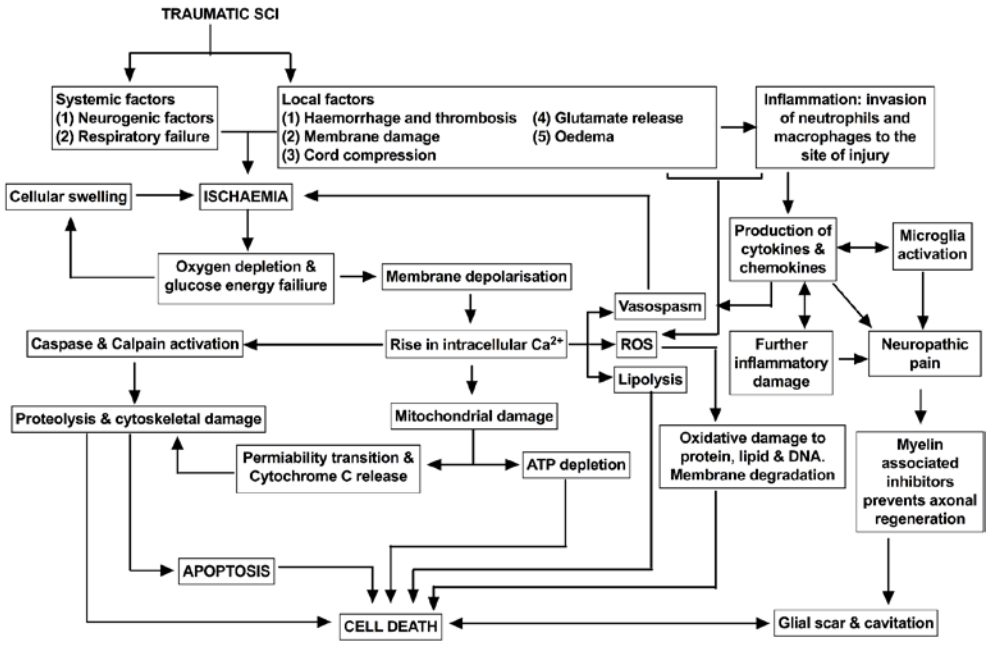


Figure 3: Major mechanisms involved in SCI. A simplified flowchart of different pathological event in secondary injury phase portrayed emphasising on ischaemia, apoptosis, cell death and glial scar formation [31,32].

Vascular events

The disruption of Blood-Spinal Cord Barrier (BSCB) after the initial injury facilitates the leakage of plasma fluid into the extracellular spaces instigating pressure-induced oedema formation [19]. An intracellular swelling, called cytotoxic oedema, might be seen in astrocytes from 3-hours to 3 days post-injury [34]. Although the exact reason behind the cytotoxic oedema is not known, it is likely due to the elevation of inflammatory factors such as potassium ions (K^+), lactate, reactive oxygen species (ROS), nitric oxide (NO), arachidonate and ammonia [19,35–37]. The cell swelling can also potentially influence the astroglial function and intensify the deleterious effects in part due to the release of excitatory amino acids (EEA) in the injured tissue [36,37]

Haemorrhage, which is directly proportional to the severity of the spinal trauma, happens predominantly in the grey matter and expands to parts of white matter [38,39]. Due to the softer consistency and high vascular nature, the grey matter is more susceptible to severe injury than white matter [33]. The initial stages after severe SCI are characterised by the production of many petechial haemorrhages due to diapedesis [33]. The bleeding often occurs from microcirculatory systems, particularly venules and capillaries, mostly sparing the anterior and posterior spinal arteries [33,39,40]. However, the rostral and caudal remote haemorrhage can happen at the base of the dorsal column as it is of venous origin [33]. Over 24 hours, the grey and white matter start to lose their integrity, worsening the haemorrhage. This induces a progressive necrotising process, leading to a major ischaemic zone [19,31,33]. Other important factors that stimulate post-traumatic ischaemia are vasospasm, intravascular thrombosis and deformities in the autoregulatory homoeostasis [31,41–43]. The endothelial swelling/damage due to mechanical trauma or the release of vasoactive amines can be the potential reason behind the vasospasm [28,42]. Similarly, the presence of thromboxane A2 facilitates the platelet aggregation leading to thrombosis of sulcal arterial network [31,41,43]. The autoregulatory mechanisms which maintain haemodynamic homeostasis within the spinal cord have been demonstrated to be affected by systemic hypotension, reduced sympathetic tone or bradycardia, consequently worsening the ischaemia [39,42].

Biochemical and electrophysiological events

Ironically, the early ischaemic stage as mentioned above is preceded by a transient increase in the blood supply to the lesion site called reactive hyperaemia [44]. Such reperfusion intensifies the damage at the injured site by generating free radicals. These are harmful as they are highly reactive to proteins, lipids and nucleic acids due to their unpaired electrons. The usual free radicals or ROS involved in the SCI pathology are superoxide (addition of one electron to the molecular oxygen), hydrogen peroxide (two electrons added to the molecular oxygen) and highly reactive hydroxyl radical (three electrons added to the molecular oxygen) [44,45]. Another highly reactive free radical is peroxynitrite, produced by the reaction of nitric oxide and superoxide [45]. These reactive species are often originated from multiple cellular pathways including nitric oxide synthases, xanthine oxidases, calcium-dependent activation of phospholipases, Haber-Weiss reaction (generation of hydroxyl radicals from

hydrogen peroxide and superoxide catalysed by free iron/protein-bound iron) and inflammatory cells [31].

An increased oxidative stress occurs due to the imbalance between production of ROS molecules and the cellular antioxidant capacity, leading to a progressive oxidation of membrane lipids (lipid peroxidation), protein and deoxyribonucleic acid (DNA) [31,44–46]. Consequently, a geometric increase in the generation of free radicals ensues through lipid peroxidation (Figure 4) [44,45].

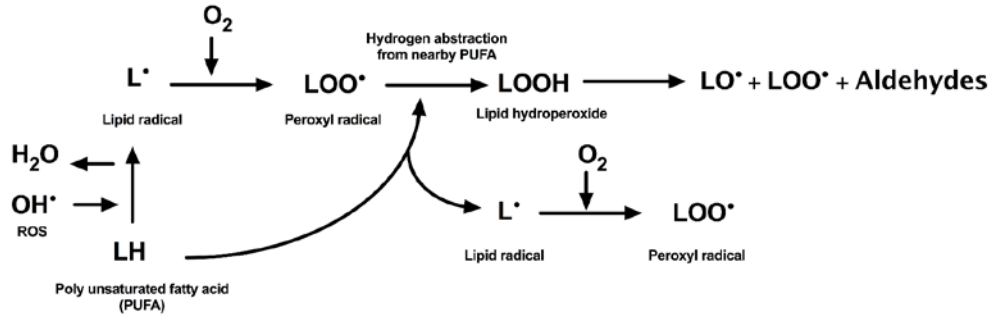


Figure 4: lipid peroxidation- a chain reaction of free radical production and cell membrane disruption. The reactive oxygen species $OH^{\bullet}$ (hydroxyl radical) generate a lipid radical $L^{\bullet}$ and a molecule of water from the lipid membrane. $L^{\bullet}$ undergo oxidation to produce peroxyl radical $LOO^{\bullet}$. Subsequently, $LOO^{\bullet}$ reacts with the nearby PUFA and abstract a hydrogen molecule leaving yet another lipid radical resulting in a gradual membrane disruption. The lipid hydroperoxide breakdown to reactive aldehydes [44,48].

Malonyldialdehyde (MDA) formed by lipid peroxidation exerts toxic stress in the cells and forms carcinogenic covalent protein adducts and DNA adducts [47]. The oxidative stress consequently accelerates metabolic collapse by inactivating mitochondrial respiratory chain enzymes, including glyceraldehyde-3-phosphate dehydrogenase, sodium ions (Na^{+})-K^{+} ATPase and sodium membrane channels [46]. A rapid increase in extracellular EEA occurs in response to the ischaemia and membrane depolarisation [46,49]. Thus accumulated EEA leads to a condition whereby the death of neurons happens due to prolonged excitatory synaptic transmission called excitotoxicity [46,49].

Glutamate, a prototypic EAA that serves vitally important metabolic and neurotransmitter functions in the CNS is released markedly after injury [49–51]. The excitotoxic actions of

glutamate is mediated through its receptor subtypes such as (i) ionotropic N-methyl-d-aspartate (NMDA) and ± alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptors and (ii) metabotropic glutamate receptors [49–51]. Interestingly, it was reported that unlike cortical neurons, spinal neurons are more vulnerable to AMPA/Kainate than to NMDA and in general, cells become sensitive to glutamate under hypoxic conditions [52,53].

Upon glutamate activation, the metabotropic glutamate receptor (G-protein coupled receptors) induces an accumulation of extracellular calcium ions (Ca^{2+}) and Na^{+} in the cytosol [54]. Also, the receptor activation leads to an excessive production of intracellular Ca^{2+} and to a release to the cytosolic space [54]. The elevated Ca^{2+} concentration in turn lethally alters the cellular metabolism by activating lytic enzymes such as calpains, phospholipase A2, lipoxygenase, free radical generation and deregulating mitochondrial oxidative phosphorylation, leading to apoptotic cell death [55–57]. Similar to the calcium dysregulation, a high intracellular influx of Na^{+} destroys the axonal and glial segments of the white matter [57]. An increase in the glutamate concentration and adenosine triphosphate (ATP) depletion can inhibit the efflux of Na^{+} back into the extracellular space [44]. As a consequent, a massive depolarisation happens that leads to the toxic accumulation of Na^{+} and water within the axon [44]. The depletion of intracellular magnesium, which is responsible for the neuronal protection via blocking NMDA receptors and metabolic processes such as glycolysis, protein synthesis and oxidative phosphorylation can also hasten the pathophysiological process summarised above [31].

Cellular events

In addition to the oxidative stress and electrolytic shifts, many cellular processes play pivotal roles in the progression of spinal cord injury.

(i) Neutrophil invasion:

Neutrophils are listed as an unfavourable factor in CNS inflammation and accumulate in the lesioned tissue [58]. They are usually found in the blood stream and are the first inflammatory cells to reach the injury site. The infusion of neutrophils to the section of damage requires three steps (i) rolling on the endothelial membrane through carbohydrate based ligands E-

selectin and P-selectin [59], (ii) tethering to the endothelial wall mediated by intracellular adhesion molecule-1 (ICAM-1) and platelet endothelial adhesion molecule-1 (PECAM-1) [32,59] and (iii) lastly, a chemotactic gradients induced by chemoattractants such as interleukin (IL)-8), interferon-gamma (IFN-α) and C5a [58,59]. The adhesion molecules are usually upregulated by the proinflammatory cytokines IL-1 and tumour necrosis factor-alpha (TNF-α). Additionally, neutrophils secrete many cytokines, matrix metalloproteases (MMPs) such as neutrophil elastase and ROS (superoxide dismutase, myeloperoxidase) that digest the extracellular matrix, leading to an enhanced vascular permeability and hence haemorrhage [60].

(ii) Microglial activation

Microglia cells are resident macrophages of the CNS and consist of approximately 12% of glial cellular offset and are distributed in a non-overlapping manner throughout CNS [61]. They are derived from progenitors in the embryonic yolk sac and annexed to CNS during embryonic development [62]. Microglia are highly sensitive and rapidly respond to disturbances in the CNS microenvironment. Hence, they are referred as sensors of pathology [63]. In normal physiological conditions, microglia cells function to maintain the CNS tissue integrity by scanning and scavenging minor tissue alterations through their stochastic movements [64,65]. Microglial function in acute and chronic neural dysfunctions appears to be paradoxical, as they can be both detrimental and beneficial [66–68]. In response to the systemic and biochemical cascade following severe mechanical injuries, ramified microglia undergo morphological changes and become proliferating, non-phagocytic cells called reactive microglia [61,63,69,70]. Post-traumatic activation of microglia is evident after 24 hours and mainly sited in the grey matter. The presence of reactive microglia reaches its peak in the first seven days following SCI then plateaus after 2-4 weeks post-injury [32,71].

The activated microglia together with invading peripheral blood macrophages (generated by the differentiation of neutrophils) enhance the concomitant expression of specific cell surface molecules, for example Major Histocompatibility Complex (MHC) antigen class-II, secretes cytokines such as IL-1, IL-6 and TNF-α [32,68]. Consequently, an expression of cyclooxygenase(COX) 2 and lipoxygenase induced by the cytokines mentioned above

initiates the breakdown of arachidonic acid into prostanoids (Prostaglandins (PGE), prostacyclin (PGI) and thromboxane) and leukotrienes (eicosanoids) [72–74].

The deleterious effect of these chemoattractants significantly contributes to the acceleration of ischaemia, oedema and inflammation. PGI 2 act as vasodilator, thromboxane A2 as vasoconstrictor, platelet aggregation factor and prostaglandin as well as leukotrienes are responsible for membrane hydrolysis, enhancing the BSCB permeability [31]. Furthermore, overexcited microglia produce neurotoxic cues such as glutamate and superoxides which in turn expedite neuronal cell death and inflammation [61,69]. In contrast to this it has been shown that activated microglia in CNS participate in the removal of myelin debris, secrete various neurotrophins, including glial cell-derived neurotrophic factor (GDNF), and transforming growth factor beta 1 (TGF-1) that significantly enhance neuronal survival, neurite growth and regeneration [63,68,69,75].

(iii) Lymphocyte infiltration

Lymphocytes are produced in bone marrow and matured in the thymus (T-lymphocytes) and bone marrow (B-lymphocytes). It has previously been reported that a marked reduction in the B-lymphocyte number in the peripheral lymph nodes and spleen after SCI corresponded to an increase in the proximity of the lesion within an hour [76]. Although B-lymphocytes are responsible for the production of antibody against the injury and memory in adaptive immunity, due to the paucity of data, it is premature to comment on the effect of B-lymphocytes in the SCI pathophysiology [77].

On the other hand, T-lymphocytes are scattered through the uninjured spinal cord. Their number progressively increases in SCI epicentre, in parallel with the microglial activation and peripheral macrophage infusion over 7 days post injury [32,77]. The T-lymphocytes, both cluster differentiation (CD)-4+ helper cells and CD-8+ cytotoxic cells play a crucial role in the progression of damage and repair mechanisms. The T-cell function is usually determined by the microenvironment to which they are attracted. It was reviewed by Schwartz that the autoimmune T-cells, i.e. T-cells which display immunity to CNS antigen myelin basic protein (MBP), affected the injured tissue in a protective fashion [68]. In another study, a single injection of autoimmune T-cells aimed against MBP significantly diminished the loss of fibres, thereby hastening the recovery in rats suffering from partial SCI (contusion

injury) [32,68]. Furthermore, combined data from numerous studies insinuate that a slow longitudinal degeneration within the spinal cord due to axoplasmic disruption might be both advantageous or disadvantageous to the overall functional result [68,78–80].

(iv) Astrocyte activation/glial scar formation

Astrocytes are the primary glial cells in the CNS providing the metabolic support of neurones [81,82]. Microenvironmental changes linked with the inflammatory responses and cellular damages trigger astrocytes to undergo a radical shift in the morphology and molecular perturbations to form reactive astrocytes (or reactive astrogliosis) [83]. In most types of injury, these radical changes involve hypertrophic process including swelling of astrocytes and up-regulation of the expression of intermediate filaments such as glial fibrillary proteins (GFAP), vimentin and nestin [83–87]. At the border of the damaged site, the hypertrophied astrocytes, fibro meningeal cells and glial cells are restructured to form a thin mesh-like layer of tightly entangled filaments [83]. These filaments further bound together by tight and gap junction and form a scar like structure, called glial scar [83,85]. The glial scar act as a physical barrier to the axonal regeneration in long descending or ascending tracts [83,85,86]. The idea of an obstruction to CNS regeneration due to the glial scar formation was recognised for the first time by Ramón y Cajal in 1928 and later proposed by Reier *et al.* in 1983 [19]. Astrocytes produce four classes of proteoglycans: chondroitin sulphate proteoglycan (CSPG), heparan sulphate proteoglycan (HSPG), dermatan sulphate proteoglycan (DSPG) and keratan sulphate proteoglycan (KSPG) [88]. Among these proteoglycans, the CSPG family (aggrecan, brevican, neurocan, neurone-glial antigen 2 (NG2), phosphacan and versican) plays a decisive role in the progress of the glial scar, being extremely inhibitory to the axonal growth as shown in *in vitro* studies [85].

Nevertheless, recent developments in the perception of glial scar formation and astrocyte activation have suggested that the astrocyte scar functions as a neuroprotective barrier to the inflammatory cells and harmful agents to preserve healthy tissues surrounding the lesion [81,85,89]. It has been demonstrated that upon SCI, further to their pernicious effects, the reactive astrocytes contribute to the neuroprotection by 1) protection from ammonium toxicity, 2) production of glutathione against nitric oxide toxicity, 3) BSCB repair, 4) amyloid β peptide degradation, 5) glucose transport, and 6) stabilisation of extracellular fluid

and ion balance [81]. Furthermore, astrocytes secrete the extracellular matrix proteins laminin and fibronectin as well as a number of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) that influence the metabolic activity and physiological functions of central neurons [81,90].

(v) Apoptosis

Apoptosis is a prominent hallmark in SCI which occurs aftermath of various systemic, biochemical and cellular events. Two different apoptotic pathways have described in mammalian cells related to SCI (i) mitochondrial dysfunction pathway initiated by caspase-9, and (ii) death receptor pathway initiated by caspsae-8 [32,91]. Apoptosis was observed both in the grey and white matter during the first 1-8 hours [57,92]. Interestingly, 24 hours-72 hours post-injury, the cell death in the grey matter reduces considerably with a corresponding increase in the white matter [57,92]. A high number of apoptotic cells are found near to the lesion epicentre and spatially linked with degenerating neurones [93,94]. Since an extensive numbers of oligodendrocytes dies due to apoptosis, retaining these cells and facilitating remyelination should have a large impact on functional improvement after SCI [95–97].

1.3.3. Chronic injury/ end phase

The later phase of SCI which begins around six months and may be prolonged for years is defined as the chronic or end phase. This phase is characterised by Wallerian degeneration and formation of cysts/syrinx [19,27,98]. The principal aim of therapeutic strategies employed in the chronic phase is to improve spinal cord plasticity and regeneration as well as to promote the function of spared axons [27].

Wallerian degeneration

Subsequent to an injury on the proximal part, degenerative events occur in the distal axon of a neuron called Wallerian degeneration (WD) [99]. WD is characterised by axonal degeneration, an increase of the BSCB permeability, myelin sheath breakdown, high influx of macrophages to the lesioned site, and finally, an expulsion of myelin debris distal to the

site of axonal injury [100]. Several cell types (oligodendrocytes, neurones, non-myelinating microglia and monocyte lineage cells) are involved in the intricate histological changes in WD [100]. It is noteworthy that WD in the mammalian CNS is dramatically slow (months to years) compared to what is observed in the peripheral nervous system (1- 2 weeks) [101]. The delay in WD in CNS is essentially due to the continued presence of myelin-associated inhibitors such as Nogo-A, myelin associated glycoprotein (Mag) and oligodendrocyte myelin glycoprotein (Omgp), slowing the gradual removal of myelin debris, hence the failure of axon regeneration [102,103].

Fibrous/ Mesenchymal scar

Another type of scar observed during SCI is the fibrous or mesenchymal scar [104]. A variety of cellular and molecular factors, both inhibiting as well as growth promoting are associated with the fibrous scar. Among them, the most abundant molecules involved are collagen, laminin, entactin/nidogen, fibronectin, hyaluronic acid, tenascins, CSPG and sema 3A [104–106]. Although collagen IV (Coll IV) is deemed to be the predominant collagen type in the scar, Maxwell *et al.* proposed that depending on the location of the lesion, deposition of collagen I, III or V was detected [106]. The majority of the cells contributing to the scar (invaded meningeal fibroblasts, endothelial cells and astrocytes) are capable of expressing collagen IV [107–109]. In the lesion site, the secreted protocollagen chain of Coll IV is self-assembled spontaneously to form a dense Coll IV matrix [110]. The Coll IV matrix then constituted the backbone of basal lamina and progress into an impenetrable mesh-work [106,110].

Cysts and cavity formation

The cysts, girdled by a thin astroglial wall, filled with cerebrospinal fluid, residual macrophages and a little connective tissue, as well as blood vessels, symbolise the healing stage of the necrotic process [19]. Although these cysts are presumed to be clinically non-problematic, they fail to be a favourable substrate for regeneration [19]. However, another form of cavity, the syrinx, which has a superficial resemblance to the cysts but with more compact gliotic walls are usually problematic [19,111]. A syrinx is composed of pressurised cavities that expand when the pressure increases with subsequent compression of the nearby

parenchyma and exacerbates the neurological deficit [19,111]. The specific mechanism behind the development of syrinx is unknown, and the remedies available for this lesion include surgical drainage of the accumulated fluid, shunting and division of adhesions [111–113].

2. CURRENT AND CONVENTIONAL MANAGEMENT OF SCI

Medical advancement and extensive knowledge regarding the complexity of SCI has improved patient care. Currently, SCI management focuses on preventing secondary complications and empowering the victims to return to an active and productive life. A summary of various degenerative phases in the pathophysiology of SCI and possible treatments that best suited for each phases are outlined in figure 5. Commonly adopted practices for SCI management after the incident include immobilising patients at the site of injury and spine boards to reduce further tissue damage [114]. Another indispensable approach to stabilising cervical spinal fracture dislocation and reconstruction of anatomical alignment by applying tensile force is the traction technique (Figure 6). Neurological improvement, as well as a decrease in the injuries observed in approximately 80% of conscious patients, have endorsed the safety and effectiveness of the closed reduction by traction [115–117]. A review of literature was exposed two documented cases of transient neurological deterioration associated with closed reduction [115,118]. But, the clinical trials failed to establish the relationship between reduction induced herniated disc and succeeding neurological deterioration in awake patients [119].

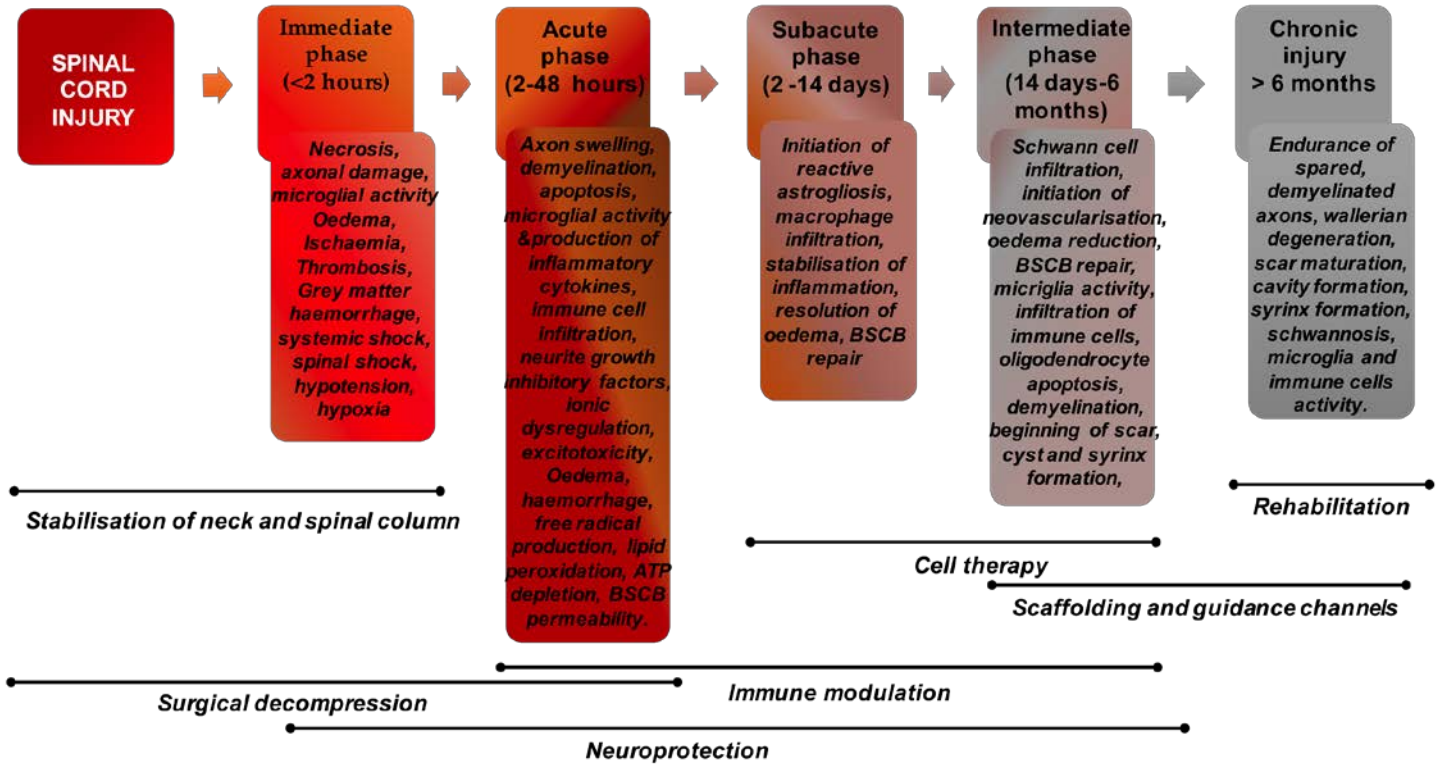


Figure 5: Overview of the principal degenerative events and timeline summarising possible treatments best suited for each phase. The elemental changes in the spinal cord microenvironment describe these phases include inflammation, haemorrhage, oxidative stress, apoptosis and BSCB permeability. Hence, certain therapeutics aim to target these events are found to be beneficial in SCI repair [19,27,250].

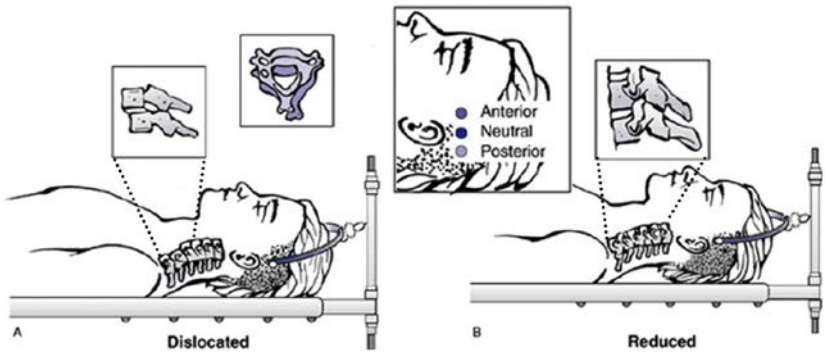


Figure 6: Traction technique for stabilising cervical dislocation and alignment. A) showing the position of tong at a distance of 1cm above pinna; B) Posterior placement of pin induce flexion. Source: Reproduced from Wang *et al.* [116].

Surgical decompression potentially relieves many symptoms caused by pressure and compression of the spinal cord [120]. The timing of early decompression remains controversial as it is defined differently in animals and human [120]. Fehling *et al.* suggested that to perform an early decompression (6-8 h) in animals enhanced neurological recovery [121]. Notwithstanding, sub-acute surgical decompressions in human patients (24-72 h) have failed to produce satisfactory results due to irreversible tissue damage experienced [30,122].

Administration of the steroid anti-inflammatory drug methylprednisolone for neuroprotection has been investigated as a therapy for SCI [123]. However, methylprednisolone when administered as either a 'high dose' (1000 mg bolus) or 'low dose' (100 mg bolus) for ten days in patients with acute injury, did not demonstrate motor or sensory outcome [124]. Also, severe complications such as corticosteroid myopathy, pneumonia, and sepsis appeared with the intravenous administration of methylprednisolone at a very high dose after SCI [125,126]. People with SCI may also benefit from a patient-centred approach called physiotherapy rehabilitation aiming to minimise psychological distress for patients and their families [127]. It involves maintenance of blood pressure, respiration, circulation, bowel care, bladder drainage, nutrition as well as body temperature [127]. Rehabilitation procedures initiated soon after the medical stabilisation of SCI aims to empower the patient return to his normal productive life [128]. Physiotherapy predominantly focuses on improving motor tasks such as walking, transferring and using upper limbs and

pushing the wheelchair [128]. Nonetheless, lack of high quality, randomised and conclusive clinical trials with SCI patients significantly influence the reliability of the treatment [129].

3. THERAPEUTIC INTERVENTIONS AFTER SCI

Remarkable progress in molecular biology has facilitated a better understanding of the pathophysiological processes and mechanisms behind the control of axonal growth and sprouting. This knowledge, in turn, has helped to develop new therapies focused on altering secondary injury mechanisms to stimulate spinal cord repair and regeneration. Drug therapies emphasis primarily on alleviating key injury mechanisms in the cascade of the degenerative events and thereby halt secondary complications [130]. The efforts to find an appropriate drug therapy that mitigates the secondary injury cascade have generated several promising molecules.

With growing evidence from the preclinical experiments, some molecules have been trialed in clinical research (Table 2). Also, other pharmacological agents that have demonstrated modest but promising outcomes in pre-clinical studies and clinical trials to determine their neurological efficacy are underway [131]. The neuroprotective pharmaceutical agents that are currently under human clinical trials are detailed in Table 3. Nevertheless, on account of its complexity, spinal cord injury is highly unlikely to be cured by a single therapy. Also, given the diversity of the factors and events involved, a multifaceted approach to ensure adequate replacement of the missing tissue and a boost in the natural healing of tissue may be more suitable.

Table 2: Summary of clinical trials of neuroprotective pharmacological agents in acute SCI

Type	Pharmaceutic agents	Proposed mode of action	Treatment approach	Conclusions
Glucocorticosteroid	Methylpredni-solone sodium succinate (MPSS) [132–135]	Suppression of inflammatory cell functions such as chemotaxis, phagocytosis, production of inflammatory mediators and lysosomal enzyme release	NASCIS-1, Phase I/II IV administration of a mega dose (100 or 1000mg) and a lower dose (25 or 250mg) of MPSS within 8 hours	No significant difference between the groups treated with the high dose and the low on neurological deficit improvement.
			NASCIS-II, Phase II 30mg/kg body weight bolus for initial hour followed by an infusion of 5.4mg/kg for 23 hours	Improvement in the neurological recovery was observed in the patients treated within 8 hours post injury
			NASCIS-III, Phase II Initial bolus of MPSS (30mg/kg) before randomisation into subgroups: (i) MPSS infusion of 5.4mg/kg per hour for 24 hours, (ii) MPSS infusion 5.4mg/kg per hour for 48 hours	An enhancement of the motor functions for 48hours MPSS delivery initiated within 3-8 hours post-injury. The incipience of severe sepsis and pneumonia observed with MPSS 48-hour delivery

21-Aminosteroids	Tirilazad mesylates (TM) [135,136]	Prevention of radical-induced, iron-catalysed lipid peroxidation	NASCIS-III, Phase II MPSS 30mg/kg + TM 2.5mg/kg body weight bolus in every 6 hour for 24 hours	No significant improvement in neurological functions compared to MPSS
Gangliosides	Maryland GM1 [137]	Inhibition of EAA associated neurotoxicity	IV administration of GM-1 100mg/ day within 72 hours for 18-32 days.	Improved neurological recovery with GM-1 treatment compared to placebo
	Sygen GM-1 [138]		NASCIS-II MPSS dose+ low dose GM-1 NASCIS-II MPSS dose+ high dose GM-1	Improvement in bladder/bowel function, sacral sensation and anal contraction. High mortality rate.
Opiate blockers	Naloxone [139]	Proposed to antagonise the rise of opiates by attacking opiate receptor sites	NASCIS-II, Phase II Bolus of 5.4 mg/kg initially followed by an infusion of 4mg/kg for 23 hours	Improved neurological recovery was observed in patients with incomplete SCI, treated within 8 hours post injury

Glutamate receptor antagonists	Gacyclidine (GK11) [24,140]	A non-competent NMDA receptor agonist preventing glutamate-induced neuronal death.	Gacyclidine (0.005, 0.01 or 0.2 mg/kg) i.v administration within 2 hours post-injury. A second dose administrated within next 4 hours.	Improved neurological recovery was observed in incomplete SCI patients treated with 0.2mg/kg GK-11. No beneficial long-term neurological scores compared to placebo.
Ca^{2+} channel blockers	Nimodipine [24,141]	Prevention of vasospasm by acting on vascular smooth muscles.	Nimodipine 0.015mg/kg/h for 2 hours following 0.03mg/kg/h for 7 days MPSS 30mg/kg bolus following 5.4mg/kg over 23 hours. Nimodipine+MPSS treatments	No beneficial neurological improvement in patients treated with nimodipine, MPSS or combination compared to placebo.
Na^{+} channel blockers	Riluzole [142]	Na^{+}- channel blockade and regulation of presynaptic Ca^{2+} channel dependent glutamate release	50mg of Riluzole twice/day within 12 hours post injury, for 14 days	Improvement in the in the impairment grades was observed in the patients with cervical SCI. No adverse effects or death was reported.

K^+ channel blocker	4-aminopyridie (Frampridine-SR) [143]	Restoration of action potential conduction in damaged nerve fibres.	Frampridine-SR 25mg twice daily.	No adverse reactions were observed and the drug was well-tolerated. No significant difference was observed between frampridine-SR treated patients compared to placebo.
Thyrotropin releasing hormone (TRH) [144]		Reverse the effects of endogenous opioids, platelet activating factors, peptidoleukotriens and EAA	0.2 mg/kg intravenous bolus followed by 0.2 mg/kg/h infusion over 6 h of TRH given within 12 hours post-injury	Improved neurological recovery observed in patients with incomplete SCI.

Table 3: Current SCI clinical trials of therapeutic agents to improve neurological and related functional outcomes. Source: ASIA clinical trial-Spinal Cord Outcomes Partnership Endeavour [145]

ID	Approach	Treatment	Phase	Primary outcome
NCT01597518	Riluzole 2×100mg oral or feeding tube within 24hours, followed by 2×50mg for 13 days after injury Vs placebo acute SCI. 351 subjects	Acute SCI ≤ 12hours Follow up= 6months	Phase II/III RCT Double blind	Efficacy/Safety Change in ISNCSCI total motor score from baseline to 6months of follow up
NCT02524379	72-hour IV infusion of glyburide (RP-1127) started within 6 hours of SCI. 10 subjects	Acute SCI SCI≤6hrs Follow up= 1yr	Phase not specified. Open Label	Adverse Events, Preliminary Efficacy (not specified.), Single group early phase safety study of IV glyburide in acute SCI

NCT01828203	Minocycline IV twice daily against placebo for seven days. All patients receive decompressive spinal surgery and blood pressure management. 248 subjects	Acute SCI ≤ 12hours Follow up= 12 months	Phase III RCT	Efficacy/Safety ISNCSCI Motor Score recovery from baseline to examination between 3m and 1yr post injury; ISNCSCI Sensory Scores, AIS; SCIM; QoL: SF-36 then continued twice daily x7days
NCT02096913	Dolormin extra (ibuprofen) 800mg 3 times per day for 4 weeks and 12 weeks for 6 subjects each. total 12 subjects	Acute SCI 4d≤SCI≤21d Follow up= 6m	Phase I Open label	Safety/Efficacy, Severe Gastroduodenal Bleed, Modified Ashworth Scale Neuropathic Pain Scale, ISNCSCI
NCT02669849	VX-210 (3mg or 9mg dose) in fibrin sealant application to the dura at the time of spinal decompression/stabilization surgery within 72hr of SCI. 150 subjects	Acute SCI SCI≥72hr Follow up= 6 months	Phase II/III Double blind RCT	ISNCSCI, UEMS/Motor Level, SCIM III, GRASSP, AIS, Pharmacokinetics

NCT01753882	Escitalopram (Lexapro) 10mg PO Daily x 4 weeks' vs Placebo in patients enrolled in gait training regimen (3x per week for 6 weeks—2 weeks prior to initiation of study med, then 4 weeks combined gait training and study med) 88 subjects	Subacute/Chronic 1month≤SCI≤9 month Follow up= 10-12wk	Phase I Double blind	Safety/Efficacy Modified Ashworth, Berg Balance, Peak treadmill Velocity, 6 Minute Walk, LEMS. Studying the combined effects of gait training and escitalopram on motor function and increase in locomotor capability
NCT01621113	Oral dalfampridine (Sustained release 4-aminopyridine) vs. placebo for 10 weeks in chronic motor incomplete SCI receiving locomotor therapy. 46 subjects	Chronic SCI SCI>12m Follow up 22wks	Phase II Double-Blind RCT	Change in 6-minute walk test at 10 weeks and 22 weeks follow up. Test of whether dalfampridine improves walking outcomes in chronic motor incomplete SCI

Abbreviations:

SCIM/SCIM II/SCIM III: The Spinal Cord Independence Measure is a measure of a person's ability to perform certain activities independently. **SF-36**: The Short Form-36 is a patient-reported survey of health status, commonly used as a measure of Health-Related Quality of Life. **GRASSP**: Graded Redefined Assessment of Strength, Sensibility, and Prehension is a clinical measurement of upper limb function for use in person with tetraplegia (quadriplegia). **TMS/UEMS/LEMS**: Total Motor Score/Upper Extremity Motor Score/Lower Extremity Motor Score are components of the ISNCSCI that include the ASIA Motor Index Score and the sub-components of the UEMS and the LEMS which are commonly analyzed and reported separated.

4. RESEARCH STRATEGIES TO STIMULATE SPINAL CORD REPAIR AFTER SCI

Regenerative medicine is a discipline that focuses on developing methods to regrow, repair or replace damaged or diseased cells, organs or tissues [146]. The moderately diminished regenerative capacity of the human system, the complexity of endogenous regeneration and a shortage of organ donors have generated a high interest in regenerative medicine [147]. The complex series of reactive changes occurring after spinal trauma and subsequent tissue scaring impede the innate efforts of myelination and repair of axons. Hence regenerative strategies for SCI aim to facilitate neuroregeneration either by directly replacing the lost/damaged cells or via secreting factors to provide trophic support for neural cells and/or to alter the microenvironment making it more favourable for regeneration [148].

4.1. CELL THERAPY

The use of cell therapy for treating SCI dated back in late 1970's, where Richardson *et al.* demonstrated that an autologous sialic nerve graft containing Schwann cells stimulated the regeneration of transected CNS axons in rats [149]. Although the study was inadequate to determine whether the myelinated axons innervated the nerve graft, re-entered to the spinal cord to form synapses, it showed the capability of CNS neurones to regenerate in the presence of a beneficial neuroglial environment [149]. Later, Bregman and Reier supported the idea by demonstrating the potential of an embryonic spinal cord tissue graft in preventing massive retrograde cell death of naive axotomised neurones in new-born rats [150]. The authors proposed that, in the presence of a favourable terrain provided by the transplanted tissue, the preserved neurones might contribute to the gradual elongation of axons and re-establish a stable connection [150]. Since then, the cell therapy has been considered a promising strategy for SCI as it can potentially ameliorate some secondary complications through neuroprotection and regeneration of damaged tissue [148,151].

The different applications of cell therapy that have been investigated for the treatment of SCI based on their cell sources, include

i. Cell transplantation to stimulate bridging of damaged axons by serving as growth and neurotropic factors secreting scaffold for sprouting nerve fibres [152].

ii. Cell induction to produce oligodendrocyte precursors responsible for remyelination of damaged axons [153].

iii. Immunomodulation through removal or inactivation of self-destructive immune cells and growth inhibitory factors [152].

iv. Expediting axonal regrowth by promoting neo revascularization [5].

Different types and sources of stem cells and non-stem cells have been investigated and/or are being investigated in both clinical and pre-clinical studies for SCI. These cell sources are Schwann cells, (SC), olfactory ensheathing cells (OEC), activated macrophages, embryonic stem cells (ES), Neural stem cells and progenitor cells (NSPC), mesenchymal stem cells (MSCs), skin-derived precursor cells (SKP) and induced pluripotent stem cells (iPSC) [23]. The advantages and disadvantages of each cell type have been considered in SCI clinical trials are extensively described elsewhere [23,148,154], and their clinical potential is compared in Table 4.

Table 4: Comparison of clinical potentials of different stem cell and non-stem cell sources for SCI therapy [23,155–159]

	SC	**OEC**	**Activated macrophage**	**ES**	**NSPC**	**SKP**	**iPSC**	**MSC**
Ease of isolation	No	No	Yes	No	No	Yes	No	Yes
Autologous donors	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Differentiation potential	-	-	-	Pluripotent	Neural	non-neural, peripheral neurones and schwann cells	Pluripotent	Multipotent (non-neural and potentially neural)
Ethical concern	Yes	Yes	Yes	Yes	Yes	No	Yes	No
Tumorigenicity	Yes	No	No	Yes	No	Unknown	Yes	No
Pathotropism	Unknown	Unknown	Yes	Unknown	Yes	Unknown	Unknown	Yes
Efficacy in pre-clinical studies	Yes, in some studies	Yes, in many studies	Yes, in many studies	Yes, in some studies	Yes, in many studies	Yes, in very few studies	Yes, for preclones	Yes, in many studies
Safety in human clinical trials	Yes	Uncertain	Yes	Yes	Yes	Untested	Uncertain	Yes

The present thesis work has focused specifically on the most comprehensively studied progenitor cells currently in clinical trials for SCI: MSC.

4.1.1. Mesenchymal Stem Cells

Mesenchymal stem cells, also identified as marrow stromal cells (MSC) or mesenchymal progenitor cells (MPC) are multipotent, self-renewing cells that have potential to differentiate into several discrete mesenchymal lineages, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells) [160]. MSCs have been investigated extensively for their efficacy and safety in both pre-clinical settings and small clinical trials of diseases, including acute myocardial infarction, haematopoietic malignancies, graft vs. host disease (GvHD) and systemic lupus erythematosus (SLE) [161–163]. Furthermore, preclinical evidence suggests that MSC can migrate or dock preferentially to the lesion site when implanted into animal models of injury and exert their therapeutic benefits largely through immunomodulation and trophic activities [164,165]. The immunoactivity of these cells has been demonstrated to be arbitrated by both secreted soluble factors and by cell-cell interaction, including dendritic cells and B and T cells (Table 5) [162,166]. The paracrine effects are due to MSC-secreted biomolecules that inhibit apoptosis and scar formation, stimulate angiogenesis as well as mitosis [162,164,166].

Importantly, many MSC-based products have been approved for clinical applications and reached the market, including Cartistem® by Medipost™ (allogeneic umbilical-cord MSC for degenerative arthritis), Cupistem® by Anterogen™ (autologous adipose tissue MSC for Chron's disease) in Korea and Prochymal® by Osiris™ (allogeneic bone marrow MSC for acute paediatric GvHD) in Canada as well as in Newzealand. Bone marrow and umbilical cord blood are perceived to be the richest source for MSC in humans. However, MSCs can be isolated from other sources, including adipose tissue, dental tissue, umbilical cord, placenta, amniotic fluid and skeletal muscles [168–175]. Although MSCs derived from various tissues exhibit similar morphology and immunophenotypical properties, their proliferation potential, colony formation frequency and capability to differentiate along specific lineages are different [176].

Table 5: Principal soluble factors secreted by MSC *in vitro*, and their potential role as immunomodulation and trophic support [163,165,167]**.**

Immunomodulatory effects	Inhibition of T-cell (CD4+ and CD8+) proliferation	Human leukocyte antigen-G5 (HLA-G5), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2(PGE 2), TGF-β1, iNOS.
	Natural killer (NK)cell inhibition	IDO, PGE 2, TGF-β1
	Inhibition of Dendritic cell maturation	PGE 2
	Stimulation of regulatory T cell proliferation	Leukemia inhibitory factor (LIF)
Trophic support	Anti-apoptotic factor	Vascular endothelial growth factor (VEGF), stanniocalcin 1, insulin-like growth factor 1 (IGF-1), granulocyte–macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF, aka FGF2), HGF, TGF-β
	Anti-fibrosis/scarring	HGF, bFGF
	Angiogenesis	VEGF, IGF-1, bFGF, IL6, placental growth factor (PlGF), monocyte chemoattractant protein 1(MCP-1, aka CCL2)
	Mitotic stimulator	Macrophage colony-stimulating factor (M-CSF), stromal-derived factor 1 (SDF-1, aka CXCL12), Angiopoietin-1, LIF

In SCI, MSC have thought to be advantageous due to following reasons: (i) relatively easy isolation and cryopreservation, (ii) maintenance of regenerative potential and viability following cryopreservation, (iii) rapid cell replication with high-quality progenitor cells retaining excellent multilineage differentiation potential, (iv) autologous transplantation, (v) minimal immunoreactivity and graft vs. host reaction in allogeneic transplantation and (vi) minimal risk of tumorigenesis [160,177]. Furthermore, the beneficial aspects of MSC specific to SCI repair are: (i) reduced neural inhibitory molecules, (ii) promoted axonal regeneration and (iii) guided axonal growth, and remyelination [27,177]. The common sources of MSC evaluated for the potential SCI repair are discussed below.

Bone marrow derived MSC

Bone marrow derived stromal cells (BMSC) are the most widely studied non-neural type cells for transplantation in experimental SCI. These cells are isolated from the mononuclear fraction of bone marrow and expanded *in vitro* to refine the cell population that is adherent to plastic. They express distinct cell surface markers (CD 105, CD 73, CD90) but no hematopoietic markers (CD34, CD14, CD11b, CD79a, CD19 and human leukocyte antigen - antigen D related (HLA-DR)) [165,178]. The neural transdifferentiation and transplantation of BMSC to support SCI regeneration has been extensively reviewed elsewhere [148,152,179,180]. In brief, numerous studies have demonstrated locomotor function improvement, at least in part, in experimental rodent SCI models following BMSC transplantation [181–186].

The immunosuppressive nature of BMSC is attributed to their ability to influence macrophage activation and astrocyte reactivity. Following BMSC transplantation in SCI rats, Nakajima *et al.* have described a significant rise in IL4 and IL3 concentrations with a corresponding decline in TNF-alpha and IL6 [187]. Consequently, a shift in the macrophage polarity, from M1 to M2 phenotype, resulted in less scar tissue formation and increased axon and myelin sparing at the lesion site [187]. Ohta *et al*. suggested that infusion of BMSC into the cerebrospinal fluid (CSF) can help the recovery of locomotor function with reduced cavity formation in contusive SCI rats [188]. The authors showed that BMSC might have contributed to the tissue repair via trophic support rather than functioning as a structural component [188]. Furthermore, the BMSC has shown to amplify the neuroprotection by

effectively interacting with stressed neurons, leading to a halt in the staurosporine (STS) or amyloid beta peptide-induced cellular apoptosis [189]. The BMSC was also reported to stimulate the endogenous survival pathways both in neurons and oligodendrocytes [189]. Despite these promising data, BMC therapy has some limitations like the morbidity at the site of harvest, isolation of low cell number [190], a difference of efficacy depending on the donor that limit the efficiency of BMSC as a transplantation source for SCI repair [191]. Nevertheless, ongoing clinical trials are investigating the safety and efficacy of BMSC transplantation in SCI individuals.

Adipose tissue derived MSC

The stem-progenitor cells from the stromal fraction of adipose tissue that are capable to differentiate into adipogenic, chondrogenic and osteogenic lineages refer as adipocyte-derived mesenchymal stem cells (AdMSC) [160,192]. The AdMSC can acquire effortlessly from the fat tissue through minimally invasive liposuction procedure [193]. Furthermore, upon specific induction, AdMSC can differentiate into neural lineages *in vitro* [194]. Kang *et al*. reported that intravenous administration of OPC derived from rat AdMSC improved the motor function in rat SCI model [195]. The injected AdMSC were survived, migrated into the injured region and partially differentiated into neurons and oligodendrocytes [195]. Although the intravenous delivery of AdMSC resulted in an unspecific migration, a significant accumulation of cells was observed in the spinal cord attributed to the role of SCI-induced chemoattractants to guide the AdMSC migration [195]. Similarly, studies by Ohta *et al.* showed that systemic administration of AdMSC stimulated the motor function recovery in SCI rat model [196]. The gradual migration of AdMSC to the lesion site and a short-term survival of the cells indicated the uncertainty of the cell to differentiate into beneficial neural cells *in vivo* [196]. However, the study reported elevated levels of cytokine-induced neutrophil chemoattractant (CINC)-1 as well as GDNF leading to the angiogenesis [196]. Interestingly, a comparison of human BMSC and human AdMSC transplanted into a rat model of SCI demonstrated similar surface protein expression and migration into the lesion site without differentiating into specific neural cells [197]. However, AdMSC exhibited increased proliferation with higher expression of VEGF, HGF and BDNF compared to BMSC. This resulted in greater axonal preservation, enhanced angiogenesis, and reduction

in cavity formation [197]. Overall, the study identified human AdMSC as a more suitable transplantable cell source for SCI than BMSC [197].

The potential to proliferate and the likelihood of unbridled differentiation of stem cells has raised the concern about inherent toxicity and tumorigenicity after transplantation. Ra et al. reported that systemic transplantation of a high number of human AdMSC (2.5×10^8 cells/kg body weight) into immunodeficient mice did not cause any toxicity nor tumour development up to 13-26 weeks post transplantation [198]. The study also reported no serious adverse reaction in human SCI patients after a one-time autologous hAdMSCs (4×10^8 cells) transplantation during the three months follow-up period [198].

Umbilical cord blood derived MSC

Umbilical cord blood derived mesenchymal stem cells (UCB-MSC) are self-renewing multipotent cell populations with a potential to differentiate into chondrocytes and osteocyte lineages, but, not adipocyte lineage [176,199]. Although morphology and immune phenotype of BMSC, AdMSC and UCB-MSC are similar, UCB-MSC exhibited poor success rate during isolation and a high ratio of cells experiencing senescence in the early passages, compared to the other two cell types [176]. However, the cells can be cultured longer and possess a high proliferation capacity [176]. Similar to BMSC and AdMSC, UCB-MSC are also capable of neuronal differentiation when cultured under specific growth conditions [199]. Saporta *et al.* demonstrated that after an infusion up to 5 days post-injury into the SCI rats UCB-MSC migrated to the lesion site and benefitted in reversing the behavioural effects and promoted healing of neurological deficits caused by SCI [200].

Systemic infusion of human UCB-MSC into a compression model of SCI in rats receiving no immunosuppression demonstrated an elevation of the neuroprotective cytokine IL-10, endogenous VEGF and GDNF concentrations, accompanied by a decline in the serum levels of TNF-α [201]. Thus, infused human UCB-MSC promoted an environment conducive for revascularisation and ameliorate SCI-induced hind limb dysfunction in animals [201]. Besides, studies have reported that transdifferentiated UCB-MSC downregulated Fas as well as caspase-3 and stimulated the messenger RNA (mRNA) expression for NT3, BDNF, MBP and proteolipid protein (PLP) [202,203]. The stimulation of these bioactive factors in turn aided in the remyelination of injured axons and recovery of hind limb function in SCI rats

[203,202]. The major concerns in UCB-MSC transplantation include cell purity, mode of delivery, safety issues due to the human leukocyte antigen (HLA) mismatch mediated immunogenicity, unchecked efficacy and *in vivo* differentiation [204].

Dental tissue derived MSC

Dental tissue derived MSC also known as dental stem cells (DSC), are a unique population of postnatal stem cells, capable of both self-renewal, and tri-lineage differentiation including, but not limited to the osteocyte, chondrocyte, adipocyte and neurocyte phenotypes. [170,205]. Depending on the prime location of their niche, DSC have been classified into six types. These are: i) dental pulp stem cells (DPSCs), ii) periodontal ligament stem cells (PDLSCs), iii) stem cells from human exfoliated deciduous teeth (SHED), iv) dental follicle stem cells (DFSCs), v) stem cells from the apical papilla (SCAP) and vi) tooth germ progenitor cells (TGPCs). Recently, gingiva-derived MSC (GMSCs) have also been identified as dental stem cells [205]. The origin and differentiation potential of different types of DSC are summarised in Table 6.

DSC are considered to be one of the neural crest descended cells. They are a temporary group of cells originated from the ectoderm at the rims of the neural tube and, following an epithelial-mesenchymal transition phase as well as widespread migration, interspersed in various parts of the vertebrate body [206,207]. These cells express both MSC and neuroectodermal stem cell markers, making them a cell source of interest for applications in neuroregeneration [207]. Unlike skeletal tissues, dental tissues do not experience continuous remodelling and are specialised tissues. Furthermore, DSC can efficiently be acquired through non-invasive conventional clinical procedures such as extraction of third molar or premolar teeth [207]. Additional latent advantages of DSC over other MSC sources (BMSC and AdMSC) include: (i) higher proliferative capacity (80-100 fold more than BMSC) enabling quick *ex vivo* expansion into sufficient cell numbers, (ii) capacity to produce single cell derived CFU that survive for extended periods without experiencing senescence [208,207,209–211].

Table 6: Origin and characteristics of dental stem cells [205,208,212]**.**

Stem cell type	**Location**	**Proliferation rate**	**Differentiation potential**	
			In vitro	***In vivo***
DPSC	Dental pulp tissue	Moderate	Odontoblast, Adipocytes, neural cells, osteoblasts, chondrocytes, cardiomyocytes, active neurons, melanocytes, hepatocyte-like cells	Odontoblast Adipocytes, endotheliocytes, neurocytes, myocyte,
SCAP	Apical papilla of developing root	High	Odontoblast, osteoblast, neurocyte, adipocyte,	Odontoblast/osteocyte like cells
SHED	Remnant pulp of exfoliated deciduous teeth	High	Odontoblast,osteoblast, chondrocyte, myocyte, neurocyte, adipocyte, endothelial cells, hepatocyte.	Odontoblast, osteocyte, endothelial cells, neurocyte,
DFPC	Dental follicle of developing tooth	High	Odontoblast, osteoblast, adipocyte, neural cell, chondrocytes	odontoblast, osteoblast
PDLSC	Peridontal ligament	High	Odontoblast,osteoblast,chondrocytes, neurocyte cementoblast,	Odontoblast, osteoblast
TGPC	Dental mesenchyme of the third molar	High	adipocytes, osteoblasts/odontoblasts, chondrocytes, neurons, hepatocyte, endothelial cells	Osteocyte, hepatocyte

DSC have shown to promote angiogenesis and neurogenesis through the production of distinct growth factors, cytokines and immunomodulatory activities [213,214]. Among DSC, cell populations such as SCAP, DPSC and SHED display significantly higher angiogenic and neurogenic potential than the other members of DSC family [207]. DSC have reported expressing neural stem cells marker nestin abundantly while being specific to the other significant neural crest stem cell markers including musashi-1, p75, snail-1, -2, slug and Sox-9 [207,215,216]. Interestingly, most of the DPSC and SHED express diverse neural lineage markers aside from what has mentioned above. This includes beta III tubulin (early neuronal cell), doublecortin (DCX, neuronal progenitor cells), NeuN (mature neurones), S-100 (Schwann cells), GFAP (neural stem cells and astrocytes), A2B5, and CNPase (oligodendrocyte progenitor cells, OPC) [207,217].

Several researchers have investigated the ability of DSC to secrete and to be impacted by neurotrophic factors. Nosrat *et al.* showed that DPSC could express mRNA transcripts for NGF, BDNF and GDNF *in vitro* [218]. Further, in co-culture system in the absence of exogenous neurotrophic factors, DPSC promoted the survival of trigeminal neurones and facilitated a robust neurite ramification specifically orientated towards them [218]. Transplantation of DPSC to the anterior chamber of the rat's eye demonstrated an excellent graft innervation and a heightened catecholaminergic nerve fibre mass [218]. When transplanted into hemisected SCI rat model, DPSC remarkably improved motoneuron sparing [218]. Additionally, neurotrophins such as GDNF, NGF and BDNF secreted by DPSC from both rat and human origin promoted the dopaminergic phenotype of motoneurons as well as improved their survival upon contact with the neurotoxin, 6-hydroxydopamine *in vitro* [219]. Taking into account these results, the neurotrophic factors produced by DSC might confer them a dual role in the SCI repair via directly influencing the endogenous cell types to differentiate into best-suited cell populations or accelerate the secretion of other neurotrophic factors from resident endogenous cells to promote tissue regeneration [212].

Sakai *et al*. described that transplantation of SHED and DPSC in a completely transected rat spinal cord has facilitated a significant recovery of the hindlimb locomotor function [217]. They showed a multifaceted regenerative action of DSC [217]. These include (i) inhibition of SCI-induced apoptosis in neural cells leading to an enhanced neurofilament and myelin

sheath preservation, (ii) suppression of anti-growth inhibitors (CSPG and MAG) through paracrine signalling, and (iii) differentiation into mature oligodendrocytes under hostile microenvironment of SCI [217]. Furthermore, the transplantation of DSC, notably SHED, promoted regeneration of descending axons, including corticospinal tract (CST) and 5-hydroxytryptamine (5-HT), beyond the lesion epicentre and showed good survival with no teratoma formation at eight weeks post-transplantation [217]. Although DSC have demonstrated a significant therapeutic potential for nursing SCI, further preclinical evaluation is highly encouraged to explore their natural adroitness in healing the nervous system injuries.

Dental stem cells from apical papillae (SCAP)

SCAP are a unique MSC population observed in the apical papilla, a soft tissue at the apices of developing permanent human teeth [205]. The apical papilla is an anatomically distinct area, and the cell-rich zone extends between apical papilla and pulp [205,207]. SCAP have shown to exhibit a greater proliferation rate, the number of population doublings and higher expression of proteins such as survivin and telomerase that are crucial for cell proliferation, compared to DPSC [191,207,220]. Akin to DFPC, the SCAP have expected to show greater plasticity than other DSC due to their origin from a developing tissue [205]. Importantly, SCAP expanded *ex-vivo* have demonstrated positive staining for various neural markers without neurogenic induction [191,207]. Following neurogenic stimulation these cells have shown to express additional neural markers including nestin, neuronal nuclear antigen (NeuN), neurofilament-M (NFM), neuron-specific enolase (NSE), glutamic acid decarboxylase (GAD), glial markers 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and proto-oncogene c Kit (c-Kit; progenitor cells) [221,220]. Apart from the initiation of pro-angiogenic processes and sustained production of VEGFa, SCAP subjected to hypoxia (1%) has shown to express neural stem cell markers or even potentially to induce transdifferentiation to neural lineages [221]. In an interesting study performed in our laboratory whereby transplantation of entire human apical papilla in hemisected SCI rats demonstrated, a significant decrease in glial reactivity with an improved gait, compared to the human SCAP within fibrin hydrogels [222]. The study highlighted the importance of delivering SCAP in a 3D organisation similar to their original niche and the encompassing

microenvironment [222]. Collectively, these results support the opinion that SCAP might be effective in improving the adverse microenvironment following SCI.

4.1.2. Limitations of cellular transplantations

Although, regenerative strategy via cellular transplantation is one of the promising approaches to stimulate SCI repair, the significant challenges that limit clinical efficacy include (i) requirement of a vast number of cells, (ii) cell survival post-transplantation, (iii) cell distribution and (iv) weak cell-host tissue integration [223]. Exogenous transplantation of cells using saline or media is not recommended due to the uneven cell distribution that would lead to a weaker cell activity [223,224]. Also, the shear forces experienced by the cells during injection through a thin needle and critically hostile microenvironment can significantly diminish the survival rate of transplanted cells [223–225]. Additionally, transplanted cells are often dispersed at the target site and cleared rapidly by immune cells, limiting their integration into the host system [224].

Nevertheless, increasing evidence from the pre-clinical experiments and recent developments have prompted the clinical evaluation of cellular transplantations. The cell types currently under human clinical trials are listed in Table 7.

Table 7: Current SCI clinical trials of cellular therapies to improve neurological and related functional outcomes. Source: ASIA clinical trial- Spinal Cord Outcomes Partnership Endeavour [145]

ID	Approach	Treatment	Phase	Primary outcome
NCT01676441	Intrathecal transplantation (surgical) of Bone Marrow-derived autologous mesenchymal stem cells and directly into SCI following laminectomy; + 4 weeks of rehabilitation. Total= 32 subjects	12 months ≤ Chronic SCI Follow up= 12 months post-surgery	Phase II/III Single group Open label	Efficacy/Safety, ASIA Motor Score, ASIA Sensory Score, EMG, Neurophysiology, MRI, Adverse events
NCT01833975	Intrathecal transplantation of Bone Marrow-derived autologous stem cell; 100 millioncells/dose; 3 doses over 10 days 10 subjects. Total + 50 subjects	Timing not specified Follow up= 6 months	Phase I/II Single group Open Label	Safety/Efficacy, Improvement in Frankel score, Improvement in ASIA (ISNCSCI

NCT02570932	Lumbar puncture intra thecal administration of expanded autologous adult bone marrow mesenchymal stem cells; 3 doses—one dose every 3 months. Total= 10 subjects	Chronic SCI Stable neuro≥6m Follow up= not specified	Phase II Single group Open label	ANR-SCIFRS, Penn Spasm Frequency, VAS Pain Outcome, Neurotrophic Factors in CSF, Adverse Events
NCT02096913	Transcutaneous injection of Autologous Mesenchymal Cells (Bone Marrow) transplanted into the spinal cord. (location not specified.) Total= 5 subjects	Chronic SCI>6m Follow up= 6m	Phase I/II Single group Open label	MRI, Sensory/motor examination, LEMS/AIS change, Urodynamics, Small pilot trial to study percutaneous Spinal Cord injection of MSC.
NCT02687672	Transplantation into the spinal cord of autologous bone marrow- vs. leukapheresis (from a sample of WBC)-derived stem cells. Total = 50 subjects	Chronic SCI SCI≥ 6m, ≤ 60m Follow up= not specified	Phase I/II RCT Parallel group Open label	ISNCSCI, Urine & Stool Incontinence, QoL, Independence Questionnaire, Safety (not specified)

NCT02574585	Autologous MSC transplantation. Two percutaneous injections (location not specified) of mesenchymal stem cells, with a 3-month interval between the injections; vs. randomly assigned control group without any specific intervention. Total= 40 subjects	chronic SCI≥12m Follow up= 12 month	Phase II RCT Parallel group Open label	AE assessed by spinal cord MRI, AIS, Sensory Mapping, Neuropathic Pain.
NCT02481440	Intrathecal administration of up to 1x 10^6 umbilical cord mesenchymal stem cells per kg, every month for 4 months. Total= 44 subjects	Acute-Chronic SCI 2w-1yr post-SCI Follow up 24 months	Phase III Single group Open label	ISNCSCI ASIA score change, EMG, Electroneurophysiology, Adverse Event

4.2. NEUROTROPHIC FACTOR THERAPY

Neurotrophic factors are pivotal regulatory proteins in the nervous system, known for their key roles in neuronal survival, alteration of glia phenotype, axonal growth as well as the maintenance of the neuronal function in both peripheral nervous system (PNS) and CNS [226]. Additionally, the neurotrophins are capable of modulating synaptic plasticity and neurotransmission [227]. Growing evidences from experimental studies indicate that neurotrophic factors are effective in eliciting renewed axonal growth after traumatic SCI [228]. Also, the expanding repertoire of axonal markers and tracers have assisted in identifying the diversity in the response of specific axonal populations (e.g., sensory, motor, and short-range intersegmental circuits) and glial cells to distinct neurotrophic factors [229,230].

Several neurotrophic factors are capable of eliciting a chemotropic effect, guiding the extension of the axonal populations to the region with the highest concentration of growth factors [228]. Therefore, targeting these axonal populations and glial cells might result in functional improvement, reduction of inflammation and scarring, and enhanced remyelination after SCI. Hence, neurotrophins are an attractive therapeutic tool for SCI repair [230]. The route, mode and period of time for neurotrophin delivery have extensively reviewed elsewhere [229,230]. To date, provision of neurotrophic support following SCI involves largely the replenishing of neurotrophic factors including the "classic" neurotrophin family and other growth factors. Among the neurotrophic factors investigated for neurological disorders, GDNF is well-known to be one of the most potent neuroprotective factors that have an effect on various neuronal cell types including dopaminergic, motoneurones, peripheral sensory and sympathetic neurones [231].

4.2.1. Glial cell-derived neurotrophic factor

GDNF belongs to the TGF-β super- family and is broadly expressed in both developing and adult CNS [227]. GDNF shows high affinity toward and signals through GDNF- family receptor-α1 (GFR-α1) and receptor tyrosine kinase encoding proto-oncogene (c-RET). GDNF binding to its receptor stimulates the trans auto phosphorylation of RET leading to the activation of multiple pathways such as MAPK/ERK, Akt/PI3K and PLC- pathways that contribute to the neuronal survival, neuritogenesis and neurotransmission [232]. Although a

broad expression of GFR-α1 and c-RET receptors has seen specifically in the ventral horn and moderately in the dorsal horn, the GDNF mRNA expression is scarce in the normal spinal cord [233]. Subsequent to traumatic injury, a remarkable up-regulation of GFR α-1 mRNA has been reported in DRG neurones, Schwann cells of nerve roots, astrocytes, and meningeal cells adjacent to the injury [234]. Moreover, an enhanced expression of c-Ret has observed in the neurones after ischaemic-reperfusion injury [234,235].

GDNF plays a vital role in the maintenance of neural function and nerve reconstruction post-trauma [235]. In preclinical settings, GDNF significantly promoted corticospinal motor neuron survival with a concentration 10-fold lower than what would be required for CNTF and Neurotrophin (NT)-4 [236]. Studies, whereby administration of GDNF into hemisected SCI rats have demonstrated, increased amount of axonal regeneration, remyelination and functional recovery [231,237]. It is interesting to note that, besides resourcing more axons by regeneration and/or sprouting, GDNF treatment also appeared to enhance the diameter of regenerated axons [237]. GDNF administration exerted an anti-excitotoxic effect by diminishing intracellular Ca^{2+} influx because of the NMDA induction *in vitro*. This suggests that GDNF application altered the intracellular signalling to modulate NMDA receptor function and thus, inhibited neuronal death [238]. Following neuronal injury, GDNF administration suppressed NOS activation and limited the deleterious effects caused by nitric oxide toxicity [239]. Interestingly, an experimental study in contusive SCI in rats regarding direct GDNF administration to the lesion epicentre demonstrated a better preservation of neuronal fibres, neurofilaments and GAP43 compared to controls [240]. Moreover, GDNF injection transiently stimulated MAPK pathway and upregulated anti-apoptotic Bcl-2 levels, leading to increased neuronal survival and improved locomotor function [240]. Mills *et al.* demonstrated that exogenous GDNF delivery to DRG cell bodies was induced axonal regeneration beyond the lesion site in a concentration-dependent manner [241].

Chang *et al*. reported the potential regulatory effect of GDNF on microglial activities [242]. GDNF delivery inhibited iNOS production, increased superoxide dismutase activity, enhanced phagocytic activity and regulated ICAM-1 mRNA expression as well as integrin α5 expression in primary rat microglia [242]. However, GDNF had no effect on the microglia-mediated pro-inflammatory cytokines release [242]. An increased up-regulation of GDNF mRNA expression has observed in 8 h-12 h post-SCI and then downregulated [243].

An intrathecal delivery of GDNF up to 28 days in moderate contusive SCI rats have shown increased white matter sparing, proprio- and supraspinal axons at the lesion [234]. Therefore, the provision of GDNF during the later phases of SCI when endogenous GDNF is unavailable might enhance the repair processes. Taking into account of these findings, external GDNF supply may be a beneficial strategy to improve the tissue microenvironment after traumatic SCI.

4.2.2. Limitations of neurotrophic-based therapy

Several neurotrophic factors offer promise as therapeutics for SCI. However, in contrast to the low molecular weight non-peptide pharmaceutical agents, they have high clearance and very short plasma half-lives, due to their rapid degradation by extracellular peptidases or distinct receptor-mediated clearance [244,245]. Furthermore, the rate of diffusion of many proteins into the tissue interstitial space is far lower than the rate of clearance through the lymphatic system, making it arduous to attain a similar therapeutic concentration in both plasma and target tissue following systemic administration [244]. Heparin sulphates can potentially segregate neurotrophic factors (e.g. FGF, PDGF and EGF) factors into the tissue extracellular matrix due to their affinity towards growth and modify the protein's biodistribution and signalling [246]. Another significant issue with the protein therapeutics is the risk of engendering an immune response (hypersensitivity reactions and anaphylactic shocks) that can be attributed to the formation of protein-antibody complexes; impacting protein metabolism and facilitating its elimination [245].

4.3. BIOMATERIALS-BASED THERAPY

Despite the fact that CNS neurones are intrinsically competent to regenerate damaged axons, they fail due to the structural and chemical impediment in the damaged tissue following traumatic SCI [247,248]. The complexity of the sequelae in the spinal cord, hostile environment within and beyond the lesion as well as the limitation of drug/growth factor diffusion across BSCB through standard delivery methods (oral and parenteral) have prevented cell transplantation and trophic factor delivery strategies from achieving appreciable functional reconstruction [223]. Furthermore, in the case of chronic SCI with the development of cystic cavity, cell transplantation alone might not satisfactorily promote the

functional recovery [249]. Therefore, it requires a matrix that potentially bridges the lesion, fills the tissue gap, provides a permissive environment, and concurrently act as a structural support for axonal regrowth as well as functional reconnection [249]. Scaffolds made of biomaterials are useful tools to deliver cells and bioactive molecules. They allow the combination of topographical cues with cells and/or biologically active molecules [250]. As per the European Society for Biomaterials (ESB), the definition of biomaterial is "a *material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body*" [251,252].

For achieving optimal effects, some general concerns must be taken into account while choosing or designing a biomaterial [251,253]:

a) Biocompatibility, to depreciate the adverse reactions *in vivo*;
b) Topographic signals for an aligned tissue integration and cellular invigoration;
c) Cytocompatibility for facilitating cell adhesion, migration and axon projection;
d) Physical characteristics such as elasticity, strength, tenacity that are compatible with the host tissue;
e) Biodegradability with non-toxic by-products.

The biomaterial must be essentially designed in such a way that its physicochemical and biochemical cues would contribute to the guidance of axonal growth. The objective would be to cross the lesion site towards functionally appropriate targets, while supporting the substrate remodelling [250]. Herein, the most important criteria and characteristics that impact the potential of scaffolds for spinal cord repair have reviewed.

4.3.1. Choice of biomaterials

Most if not all the biomaterials appraised for their potency to repair SCI are polymers. Typically, polymers are two or three-dimensional molecules built up principally from a vast number of similar units bonded together; and can be either natural or synthetic in origin [254–256]. Natural polymers (e.g. Collagen, Fibrin, Alginate, Chitosan etc.) are widely available, easy to harvest and well-known for their physical and biological characteristics [250]. However, decreased reproducibility due to great batch to batch variability is one of the major disadvantages of natural polymers [250]. Other potential disadvantages include difficulty in

sterilising, difficulty in controlling degradation properties and possibility to provoke the immune response through contaminated molecules [253,254,257]. Synthetic materials (e.g. PCL, PLA, PLGA etc.) can be further divided into degradable and non-degradable biomaterials [250]. They are easily sterilised and contain negligible impurities [250]. Furthermore, synthetic polymers can be precisely tuned their mechanical properties such as porosity, stiffness, architecture and degradation rate according to the desired application [250]. Nevertheless, poor biocompatibility due to the lack of cell recognition factors and the presence of unreacted monomers as well as other excipients is the inherent disadvantage of synthetic materials [250]. Moreover, the non-degradable materials can be trapped in the tissues over the time, limiting their utility [249,250]. In the case of biodegradable synthetic polymers, the by-products might induce an immunological reaction [250]. Polymers based scaffolds that present, among other things, topographical cues can facilitate cell adhesion and decrease the cytotoxic effect of the material [249,253]. The major natural and synthetic polymers used for spinal cord repair are listed in Table 8.

4.3.2. The role of substrate's topographical and mechanical cues on cell behaviour

Stem cell differentiation has been described to rely on biochemical supplements including growth factors, nucleic acids and animal products [261]. Yet, properties of materials i.e. stiffness, topography, molecular flexibility, binding affinity, degradability/degradation, by-products, adhesive property and chemical functionality can influence, or reasonably even induce, lineage specific stem cell differentiation [261,262]. Stem cells lineage specification is associated with the cell ability to sense the matrix elasticity, which involves pulling against the matrix and generation of signals based on the force that the cell requires deforming the matrix [262]. The elastic modulus of mammalian soft neural tissue is reported to be +/- 100 Pa [262–264]. Naïve MSC cultured on soft collagen hydrogels with a low modulus (0.1 kPa-1 kPa) demonstrated rapid adhesion, increased filopodia-rich morphology and presented a 5-fold upregulation of neurogenic transcripts compared to moderately stiff collagen matrices with higher elasticity (11 kPa) [262]. Although, stiffer hydrogels (>7 kPa) have favoured oligodendrocyte differentiation, hydrogels with a modulus <1 kPa facilitated expression of myelin oligodendrocyte glycoprotein (MOG) [265]. Studies have also focused on the influence of substrate geometry, in particular, fibre diameter. Smaller diameters (5–30 μm) provided greater curvature and promoted more significant oriented axonal outgrowth than

Table 8: Materials investigated for spinal cord regeneration [258–260]**.**

Natural and degradable materials	
Polymer	**Description**
Collagen	An extracellular matrix (ECM) molecule and one of the abundant proteins in the mammalian system, accounting 25-30% of total protein in human body.
Fibronectin	An insoluble glycoprotein present in the ECM which serves as a linker for cell adhesion. It also exists in the soluble form in plasma and is involved in many cellular processes.
Hyaluronic acid	An anionic, non-sulphated glycosaminoglycans composed of glucuronic acid and *N*-acetyl glucosamine. A chief component of ECM.
Fibrin	A fibrous, non-globular protein produced by the action of thrombin and fibrinogen during the clotting of blood.
Fibroin	An insoluble protein rich in glycine amino acid present in the silk produced by insects such as spiders, *Bombyx mori*, and moths.
Poly- β-hydroxybutyrate (PHB)	Bio-polyesters are produced by microorganisms including *Ralstonia eutrophus*, *Methylbacterium rhodesianum* and *Bacillus megaterium*

Alginate	An anionic linear-polysaccharides comprised of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues. Mainly found in the cells walls of brown algae.
Agarose	A linear polysaccharides composed of repeating units of agarobiose, extracted from seaweed.
Chitosan	The second most abundant polysaccharide in nature, composed of randomly distributed D-glucosamine and *N*-Acetyl-D-glucosamine. Found chiefly in the shells of crustaceans.
Synthetic and degradable materials	
Poly carbonate	Thermoplastics containing carbonate groups generally used to prepared strong and transparent materials.
Poly- lactide-co-glycolide (PLGA), Poly (lactide) (PLA), Poly-ε- caprolactone (PCL)	A member of α-hydroxy acids prepared via ring opening polymerisation in the presence of catalyst stannous octoate. Biocompatible polymers with controllable mechanical properties. Their degradation facilitated by hydrolysis of ester linkages. PLGA and PCL are approved by FDA for therapeutic application.
Poly-ethylene glycol (PEG)	A hydrophilic polymer with cell adhesion and low protein adsorption properties.
Peptide amphiphiles	peptide-based molecules consisting of a hydrophilic peptide sequence that can likely self-assemble into structures such as nanofibers.

Poly-trimethylene carbonate-co-ecaprolactone (P(TMC-CL)	A copolymer of caprolactone and trimethylene carbonate that can be processed form flexible, porous as well as tough materials with controllable degradation rate.
Synthetic and non-degradable materials	
Poly-2-hydroxyethyl methacrylate (pHEMA)	A biocompatible, non-toxic polymer produced by solution polymerization of 2-hydroxyethyl methacrylate (HEMA) and functions as a hydrogel with 40% water retention.
Poly [N-(2-hydroxypropyl) methacrylamide] (PHPMA) or Neurogel ™	A highly hydrophilic linear, cross-linked polymer generated from the monomer N-(2 Hydroxypropyl) methacrylamide. Non-immunogenic and non-toxic material with water-swelling properties.
Poly-acrylonitrile-co-vinylchloride (PAN-VC)	A non-toxic and structurally stable copolymer of polyacrylonitrile and polyvinylchloride.

larger fibre diameter (up to 500 μm) [260,266]. Rat NSPC embedded in laminin coated electrospun polyethersulfone (PES) fibres of diameters 283nm showed moderate cell spreading with spherical morphology and higher migratory activity than cells cultured on larger fibres (749nm and 1452nm) [267].

In contrast, Herbert *et al.* reported a decrease in the neurite extension in DRG cells corresponding to the increase of fibrinogen concentrations in fibrin hydrogel [268]. The study also proposed that the matrix degradability plays a pivotal role in determining neurite growth and length than fibre diameter [268]. An ideal permissive structure for cell growth should contain interconnected pores allowing cell colonisation, nutrient diffusion and removal of the waste products [269]. Increasing the polymer concentration in agarose gel reduced pore size and increased stiffness, significantly impacting neurite outgrowth in DRG cells [270,271]. PLGA and PLL scaffolds of pore size (250μm- 500μm) modified with fibronectin or matrigel have shown to support attachment and ingrowth of human ES cells [272]. Furthermore, the transplantation of these human ES-loaded scaffolds into SCID mice demonstrated larger and more adequately organised neural structures compared to the control [272].

Similarly, stem cell fate also can have influenced by ligand identity and presentation whereby cell-substrate interaction occurs [273]. Synthetic peptides grafted within engineered biomaterials have shown to improve cell-adhesion, enzymatic degradability, and growth factor-binding properties of the material [223,250,274,275]. The short amino acid sequences that promoted cell adhesion and neurite outgrowth include laminin-derived tyrosine-isoleucine-glycine-serine-arginine (YIGSR) as well as isoleucine-lysine-valine-alanine-valine (IKVAV) and neural cell adhesion molecules (NCAM) derived EVYVVAENQQGKSKA [223,250,275,274]. In contrast, DRG neurite outgrowth was inversely proportional to the arginine-glycine-aspartic acid (RGD) peptide concentration when cultured in fibrin matrices containing fibronectin-derived RGD peptides [276]. Recent development in conjugating growth factors on the surface of biomaterials has demonstrated beneficial outcomes. PDGF-A incorporation to the agarose gel and hyaluronic acid-methylcellulose (HAMC) gel through biotin-streptavidin mediated conjugation induced rat NSPC to differentiate into oligodendrocytes [223,277,278]. Furthermore, bioconjugation of interferon-γ to chitosan hydrogels promoted neuronal differentiation of NSPC [279].

4.3.3. Design of biomaterials

The biomaterial devices used in the spinal cord therapy fall into three broad categories: scaffolds with guidance channels, micro/nanoparticle-based delivery systems and hydrogels [280]. They can either be administered alone as guidance structures to cells and regenerative processes or combined with therapeutic molecules such as drugs, neurotrophic factors and specific cell types [250,258,260,280].

Guidance channels

Hollow conduits or nerve guides are one of the simple physical guides for nerve repair used in experimental SCI. They aimed to bridge the gap between two severed ends of a nerve in a hollow tube for a guided axonal regrowth towards the distal end [260,280]. The concept of utilising hollow conduits for filling the nerve recesses dated back to the late 1800s, when Gluck suggested using a demineralised bone tube to span the nerve gaps [281]. The important criteria that nerve conduits should meet are tractability, sterility and gaseous exchange as well as permeability [250,260,280]. Furthermore, the structure should not cause irritation or any mechanical damage to the adjacent spinal cord tissues [250,260]. The key advantages of this system include the capability to load therapeutic elements such as drugs, growth factors, peripheral glia and MSC [260,282–284]. Hollow conduits are a successful strategy in peripheral nerve bridging. When considered for SCI, they might be beneficial for transected spinal cord lesion which leaves a discrete nerve projection [280]. Unfortunately, most of the SCI are incomplete contusive types with irregularly shaped gaps restricting effective utilisation of nerve guide [280]. Furthermore, the need for a surgical procedure to implant the devices limits their practicality [280].

Micro/nanoparticle based delivery systems

Polymeric micro/nanoparticles have extensively been used for encapsulation and delivery of growth factors, biomolecular drugs and inhibitory substances in a myriad of drug delivery applications for the repair of multiple tissues. These are attractive for spinal cord repair as they can locally deliver various therapeutic agents via minimally invasive techniques without systemic toxicity [280]. Also, a combinatorial neuroprotective strategy via administration of a sequence of growth factors and various antagonistic agents to modify the lesion

microenvironment can be achieved by blending distinct micro or nanoparticle formulations [280,285]. However, the key challenges for micro/nanoparticle formulation development for spinal cord regeneration are the preservation of growth factor activity and the biomolecule accumulation at the target site [286]. Although natural polymers such as chitosan have been used to prepare microparticles, most of the drug delivery systems used in CNS regeneration are mainly composed of the synthetic polymer PLGA.

Being a saturated poly (α-hydroxy esters), the hydrolytic degradation of PLGA is facilitated by de-esterification and give rise to polymeric monomers PLA and poly (glycolic acid) (PGA). These monomers are then eliminated through natural pathways [287]. In addition to its biodegradability, PLGA has also confirmed to be biocompatible therefore approved for clinical applications by U.S Food and Drug Administration (FDA) and European Medicine Agency (EMA) [286,288]. Furthermore, the feasibility to adjust surface properties to render a better interaction with biological materials, ability to encapsulate a broad variety of bioactive molecules as well as therapeutic agents, and protect these molecules from degradation have considerably amplified the appeal of PLGA for CNS regeneration [288,289].

Administration of Sonic hedgehog (Shh) encapsulated in PLGA microparticles to two different mouse injury models (contusion and dorsal hemioversection) reduced astroglial scar at the lesion site, oligodendrocyte lineage cell proliferation, CST axonal sprouting at the injury site [290]. In an another experimental study, PLGA nanoparticles loaded with fluorescence-labelled bovine serum albumin was showed significant absorption by neurones and glia *in vitro* [291]. Subsequently, when administrated via the intraspinal route following contusive SCI in rats, GDNF loaded PLGA nanoparticles demonstrated remarkable neuronal preservation leading to hindlimb recovery [291]. A sustained co-release of three angiogenic factors such as VEGF, angiopoietin-1 and bFGF encapsulated in three distinct PLGA microspheres demonstrated significantly enhanced angiogenesis, neurogenesis and neurological recovery following administration into the epicentre of contusive SCI rat model [285].

Pharmacologically activated PLGA microspheres have demonstrated their ability to encapsulate and provide a controlled release of a variety of growth factors including NT-3, BDNF, HGF and IGF-1 [292–294]. Further, PLGA with bioactive surfaces has demonstrated

to facilitate adhesion of human NSC and neuronal differentiation [292,295]. The sustained release of flavopiridol, a cell-cycle inhibitor, from PLGA nanoparticles into a hemisected rat spinal cord reduced the adverse effects of the drug, enhanced neuronal survival and decreased the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) while supporting IL-10 expression [296]. Moreover, the formulation also inhibited astrocyte proliferation, glial scarring and decreased cavitation volume leading to improved motor restoration in the injured animals [296]. Recently, sustained release of GDNF from PLGA microparticles has shown to promote restoration of dopaminergic functions [297]. The study reported few adverse side effects being caused by GDNF-PLGA microparticles confirming the system as a safe and efficient carrier of the neurotrophic factor [297].

Hydrogels

Hydrogels are a 3D structure composed of hydrophilic polymers linked together by either strong chemical linkage (covalent bond, ionic bond) or weak cohesive forces (e.g., hydrogen bond, van der Waals interactions) and are used to mimic the physical attributes of the host environment [298,299]. These structures can swell while conserving their initial shape to develop into elastic gels and retain water up to 95% of their total weight. Importantly, hydrogels can span the irregular lesion site and gradually degrade in the physiological environment allowing the host tissue to replace the formerly filled space [269,298]. Moreover, the minimally invasive implantation and their ability to gel *in situ* to create a growth permissive 3D network have made them an appealing system for spinal cord repair [280]. Based on their degradation properties hydrogels are grouped into resorbable hydrogels or stable hydrogels [299,300]. A resorbable hydrogel can degrade according to its polymer chain degradation reaction via either hydrolysis or proteolysis into smaller molecules, whereas stable hydrogels cannot degrade [299,300].

The principal features of hydrogels used for spinal cord regeneration include (a) tissue-like physicomechanical properties, (b) porous structure supporting cell survival, proliferation, host cell infiltration and axon outgrowth, (c) ability to encapsulate therapeutic agents e.g. biomolecules and gene/vectors for a precise *in situ* delivery [298,301,302]. Regarding the origin of molecules being used, hydrogels can classify into two broad categories: synthetic and natural. Synthetic hydrogels are currently unable to recapitulate the complexity of the

tissue structure, and bioactive motifs observed in the biological extracellular matrix (ECM) [269]. On the other hand, natural polymers contain essential amino acid motifs for cell adhesion and are readily degradable by the body [303]. Gels formed of biopolymers simulate the morphology and mechanical characteristics of the biological ECM and may contribute to neuronal and axonal growth [258,269].

Injectable hydrogels can be physical hydrogels (gelation is independent of chemical reaction) or chemical hydrogels (chemically cross-linked gels) [300].

(i) Injectable chemical gels

Chemical gels are irreversible cross-linked systems where a reactive moiety can either be conjugated to the prepolymers or added as small molecules/enzyme to generate a free radical to form covalent bonding between adjoining polymer chains [269,304]. In contrast to the preformed implantable scaffolds, crosslinkers or unreacted monomers incorporated in these hydrogels cannot be washed away or quenched before implantation. Hence the reactants employed for the synthesis of chemical gels should be non-cytotoxic and necessarily be in the approved concentration range [304]. NT3 encapsulated in an acrylic PLA-PEG-PLA-derived hydrogel, a synthetic polymer containing degradable PLA and non-degradable PEG units, was released over 14 days *in vitro*. Injected in an adult rat SCI, the NT 3 loaded hydrogel exhibited greater axonal growth in both CST and raphespinal tracts (RST), leading to an improved open-field Basso, Beattie and Bresnahan (BBB) motor score in comparison with the control animals injected with hydrogel alone [305].

(ii) Injectable physical gels

Gelation induced by the addition of crosslinking agents can cause additional damage at the site of injection from unreacted monomers, initiators or exothermic reactions [306]. Physical gels are reversible cross-linked systems and are able to transit from fluid to gel with a change in the environmental conditions, including temperature, ionic concentration or pH [304,307]. In physical gels the gelation reaction is mild, making the system compatible with cells and sensitive therapeutic agents. Also, the reaction rate can be tuned to occur post-haste upon administration to the body [269]. A synthetic reversed-gelling diblock copolymer, Poly(N-isopropylacrylamide)-g-polyethylene glycol (PNiPAAm-PEG) hydrogel has been used to

deliver NT3 and BDNF to the injured spinal cord. This system gels above the lower critical solution temperature (LCST) (29–32 ^{0}C) [308]. The PNiPAAm-PEG hydrogel (compressive modulus of 3-5 kPa) has demonstrated a sustained release of biologically active NT3 and BDNF over four weeks [308].

An experimental study by Jain *et al.* reported that *in situ* gelling agarose hydrogel containing BDNF loaded lipid microtubules supported and promoted axonal sprouting in a hemisection model in adult rats [309]. The study also reported a reduction in the reactive astrocytes, CSPG molecules and improved axonal regrowth beyond the injury site in the group treated with BDNF/agarose hydrogel [309]. However, the regenerated axons did not cross to the distal cord [309]. It is interesting to note that the control group treated with agarose hydrogel alone displayed significantly higher reactive astrocytes, CSPG and axonal bulbing at the edges of the graft. This was the opposite of what was expected considering the proprieties of the material [309].

In physiological conditions, cells are encompassed by ECM as a supporting and regulating substance for cellular functions, including cell survival, proliferation, morphogenesis differentiation, and modulation of signal transduction activated by several bioactive molecules such as growth factors and cytokines [310–313]. Based on the particular functions of the tissue, the ECM molecules and network vary in their composition, structure and biomechanical properties [253,314]. Therefore, a 3D network derived of or functionalised with ECM should play an influential role in promoting regeneration of tissues. Alternatives to the whole ECM are (i) hydrogels composed of purified crucial ECM molecules (e.g. fibrin, collagen, fibronectin, hyaluronic acid etc.), (ii) hydrogels composed of complex mixtures of ECM proteins (e.g. Matrigel™) and (iii) hydrogels derived from decellularised mammalian tissue [315].

A triple-helical protein, collagen is a fundamental component in all ECM networks of the mammalian body. This US FDA approved biopolymer has been studied extensively for its ability to support the growth and differentiation of neurones *in vitro* and has been often used as a hydrogel scaffold *in vivo* [316–321]. Interestingly, collagen suppresses glial proliferation which might subdue the glial scarring following spinal cord injury [314]. Collagen gelation is temperature dependent (above a certain concentration) and occurs via fibre assembly under physiological pH and ionic strength [254,280]. An injection of *in situ* gelling collagen

hydrogel containing NT3-loaded collagen microspheres into hemisection SCI rat demonstrated a significant reduction in the glial scarring and microglia activation at 1 and six weeks post injury compared to the non-treated animals [322]. The study reported an increased axonal growth and reduction in collagen deposition in NT3-loaded hydrogel versus hydrogel alone [322]. Nevertheless, the beneficial effects of NT3 and collagen did not contribute to the functional recovery of the animals. The proposed hypothesis was that in the absence of a guidance cue, the randomly oriented collagen fibres might have miswired the sprouted axons [322].

Hyaluronic acid is an anionic glycosaminoglycan found in the ECM of connective, epithelial and neural tissues [323]. These non-immunogenic ECM molecules are non-adhesive on their own, but, in conjunction with other natural polymers such as methyl cellulose, hyaluronic acid can create an ECM-like network [321]. An intrathecal delivery of HAMC hydrogel into contusive SCI rats showed mild neuroprotection over a period of one month, which led to a significant improvement in the BBB score in the injured rats compared to controls [324]. HAMC was also effective in reducing subarachnoid inflammation. An injection of HAMC into a rodent model of post-traumatic syringomyelia (PTS), closely associated with traumatic SCI, reduced the extent of post-traumatic parenchymal fibrous scarring. The beneficial effect of HAMC was linked to the reduction of CSPG deposition and IL-1α secretion [325]. Baumann *et al.* developed a hydrogel-nanoparticle blend composed of HAMC and PLGA that facilitated attained of various release profiles [326]. The neuroprotective molecules NBQX and FGF-2 were released for 1 and 4-days respectively, from the composite hydrogel [326]. Whereas the neurodegenerative molecules dbcAMP, EGF, and model proteins such as alpha-chymotrypsin and IgG (for NT3 and anti-Nogo-A, respectively) were released over a period of 28 days from PLGA nanoparticles [326].

Matrigel™ is a well-characterised, thermosensitive, injectable material has been widely used *in vitro* and *in vivo* to study the proliferation and differentiation of various cell types [254,327]. Patel *et al*. compared three different matrices such as methylcellulose, matrigel and laminin:collagen gel to deliver Schwann cells [328]. The injection of Schwann cells suspended in these three matrices into a chronic contusive SCI rat model induced an increased cell number, axonal growth and angiogenesis in matrigel and laminin:collagen gel [328]. The methylcellulose gel exhibited less promising results, this may be due to its non-

adhesive nature and inability to support angiogenesis [328]. Furthermore, the study reported a significant increase in the Schwann cell survival, lesion revascularisation, axonal growth leading to an improvement in the motor function recovery in the injured rats, with Schwann cell- matrigel than Schwann cell-laminin:collagen gels [328]. Although Matrigel™ is promising and easy to obtain, its preparation from mouse tumour cell-derived ECM proteins makes it less than ideal for clinical translation [254,327].

The decellularisation of mammalian tissue dates back to 1973 where it utilised as a method to preserve tissue for its application in burns treatment [329]. Later the ECM produced by decellularisation of porcine small intestine mucosa (SIS-ECM) was considered for a variety of tissue reconstruction (e.g. urinary bladder, intestine, urethra, diaphragm, peripheral nerve etc.) [330,331]. Studies have reported that ECM material harvested by decellularisation of mammalian tissue retains the biochemical complexity, bio-inductive properties, and nanostructure of the native matrix [315]. Also, they have shown to support the *in vivo* creation of site-specific, functional tissue [315]. ECM material is FDA approved and SIS-ECM has been implanted in millions of people to date [330,332]. Some of the clinically available products composed of ECM material are Lyoplant® (Dura mater), NeoForm™ (Breast), GraftJacket®, OrthAdapt®, Unite® (soft tissue and chronic wounds), IOPatch (Ophthalmic use), CopiOs® (Dentistry) and Prima™ Plus (Valve replacement) [333].

Hydrogels derived from ECM material (ECM hydrogel; ECMh) have shown to retain the intrinsic bioactivity of the native matrix to promote heterologous tissue remodelling applications [331]. The regenerative mechanism whereby ECM hydrogel modulates cell behaviour has not been understood fully yet. However, it likely involves the degradation mediated release of matrix bound growth factors, cytokines and chemokines, presentation of chemotactic cryptic peptides, and through embedded matrix-bound nanovesicles (MBV) [315,331,334,335].

Formation of homogeneous ECM hydrogel is a collagen based self-assembly process regulated in part by ECM proteins, glycosaminoglycans and proteoglycans [336]. The key steps involved are i) enzymatic digestion of the ECM material into protein monomeric components, and ii) induction of spontaneous intramolecular bond reformation in the monomers by temperature and/or pH controlled neutralisation [315]. In practice, the digested

ECM material is neutralised to physiological pH as well as ionic strength and maintained at low-temperature < 22^0 C, until the application intended for temperature controlled gelation (e.g. catheter or needle injection to gel *in situ*). The ability of digested ECM material to gel *in situ* permits the hydrogels to adapt the shape of the defect [337].

It has been reported that ECM hydrogels allow a rapid infiltration of pan (CD68+) macrophages which in turn contribute to hydrogel degradation [338]. Also, these hydrogels have shown to promote mononuclear cell response that quickly transitions from an early M1 pro-inflammatory phenotype to an M2 immunomodulatory, pro-remodelling phenotype [331,339]. These effects can likely promote recruitment of endogenous stem cell/progenitor cells and stimulate neo-matrix deposition at the lesion site [331]. Zhang *et al.* suggested that muscle-derived ECM hydrogel injected into hemisected adult rat was well- integrated with the host tissue [340]. The hydrogel stimulated axonal ingrowth within and beyond, revascularisation at the lesion site and enhanced neuronal survival [340]. The study reported the formation of very few myelinated axons with no functional recovery [340]. Of interest, the ECM hydrogels derived from CNS tissue have recently been described for retaining growth factors such as bFGF, VEGF and NGF [341]. These acellular hydrogels have shown to induce assertive mitogenic, and chemotactic effects without altering the viability of incorporated cells [341–343]. Further, CNS tissue derived scaffolds have demonstrated a potential tissue-specific effect as indicated by the stimulation of neurite outgrowth and differentiation of NSCs into neurones [342,343].

4.3.4. Combinatorial approaches

It is highly improbable that a single treatment will be able to counteract damage in the spinal cord. An ideal treatment should be a multifaceted approach which targets directly and/or indirectly multiple events. In this regard, treatments that combine cell transplantation, neuroprotection, activation of regeneration stimulating pathways and/or repressing growth attenuation signals have been widely investigated.

Many studies have demonstrated synergistic responses achieved through the combination of different therapies [344–347]. Fibrin scaffolds supplemented with PLGA microspheres and lipid microtubules able to release NEP1-40 peptide (myelin-associated inhibitor) and chondroitinase ABC (chABC, CSPG inhibitor) improved axonal growth while decreasing

CSPG deposition and glial scar development [344]. Adult hemisected SCI rats receiving chABC and lithium chloride (LiCl) individually demonstrated poor anatomical outcomes [345]. A delayed administration of adeno-associated virus encoding the L1 cell adhesion molecules (AAV-L1) and chABC in compression SCI in mice exhibited synergistic effects. This system improved motor recovery and increased the density of cholinergic and GABAergic terminals at motoneuronal cell bodies [346].

The use of cells in conjunction with other potential molecules with/without a material platform has attracted a great deal of interest, wherein Schwann cells, OEC, NSC/NPC and BMSC are the most frequently exploited cell types. Pearse *et al.* demonstrated that transplantation of Schwann cells, combined with rolipram in contusive SCI rats supported a remarkable myelination and axonal sparing [348]. Moreover, addition of db-cAMP to the combination of rolipram and Schwann cells prevented the cAMP depletion following SCI, resulting in stimulated serotonergic fibres ingrowth and beyond the grafts. This led to a significant functional recovery [348]. Intriguingly, transplantation of neonatal mouse lamina propria-derived OEC combined with db-cAMP into the injured rat spinal cord increased rubrospinal axons but failed to stimulate their regeneration both within and beyond the graft [349].

Transplantation of a combined system, composed of a fibrin scaffold with heparin binding sites, embryonic stem cell-derived neural progenitor cells (ESNPC) and growth factors (NT3 and PDGF-AA) showed robust cell survival and proliferation in hemisected rats [350]. Also, the combined system stimulated the differentiation of NPC to bipolar neurons [350]. In a subsequent study, nestin-positive ESNPC and fibrin scaffold containing growth factors (NT3 and PDGF-AA) with or without heparin-binding domains were transplanted into hemisected rats [351]. The combination of growth factors and fibrin without heparin-binding domain improved NPC cell survival and increased the number of NeuN positive neurones at eight weeks post-injury [351]. All the groups treated with embryonic stem cell-derived NPC demonstrated functional recovery at four-weeks post-transplantation, but some of the animals showed over proliferation of NPC indicating the need to purify the cell population before its injection in the spinal cord [351]. The synergistic effects of self-assembled peptide K2(QL)6K2(QL6) and NPC injected into compressive SCI rats reduced lesion volume and

cystic cavitation [352]. Furthermore, the combination preserved neurones reduced perilesional inflammation and promoted forelimb neurobehavioural recovery [352].

A promising combinatorial approach is to rescue the residual demyelinated yet non-interrupted axons and to stimulate endogenous repair following SCI. In a recent experimental study, Yokota *et al*. elucidated the direct contribution of the propriospinal circuits reorganisation to the functional recovery, which involved synapse formation between the survived host neurones and grafted NSPCs following SCI [353]. The study demonstrated that severe SCI abolished the regenerative potential of grafted NSPC albeit its efficacy in mild and moderate SCI. They proposed that neuroprotection provided by NSPC and neurotrophic factors of the host neurones and grafted cells could efficiently stimulate functional recovery [353]. Similarly, OPC expressing CNTF transplanted in the injured spinal cord of rats differentiated toward APC+ oligodendrocytes [354]. This facilitated partial electrophysiological function and enhanced hindlimb locomotor function in the injured rats [354]. The benefit of a combination strategy aimed at neuroprotection and activation of myelin-forming cells was also strengthened by the studies performed by Girard *et al* [355]. They demonstrated that transplantation of Schwann cells, infected with human immunodeficiency virus (HIV)-derived vectors inducing BDNF and NT3 secretion, into demyelinated mice spinal cord promoted functional recovery [355]. Furthermore, these cells provided neuroprotection via reducing astrogliosis [355].

4.3.5. Biomaterials and combinatorial approaches currently in clinical trials

Currently undergoing clinical trials that are based on biomaterials and combinatorial approaches are listed in table 9. Despite the enormous research effort involving various strategies, there is no treatment that can assure a complete functional restoration after SCI so far. Furthermore, most of the successful procedures including cell replacement, neuroprotective, and remyelination strategies involve either genetically modified stem cells/progenitor cells or embryonic cells. Thus, these systems are difficult to translate to the clinic due to ethical and safety concerns. Therefore, it is important to explore the possibility of changing the SCI-microenvironment, utilising cell types that can be easily isolated and autologously transplanted, coupled with appropriate growth molecules and biomaterials for achieving spinal cord repair.

The present doctoral research hypothesises that implantation of human dental stem cells from the apical papilla (SCAP) in injectable drug delivery systems may promote recovery of damaged spinal cord by multiple regenerative mechanisms.

Table 9: Current SCI clinical trials of biomaterial and combinatorial strategies to improve neurological and related functional outcomes. Source: ASIA clinical trial- Spinal Cord Outcomes Partnership Endeavour [145]

ID	Approach	Treatment	Phase	Primary outcome
NCT02510365	Collagen scaffold transplanted into spinal cord after acute spinal cord injury. Total= 10 subjects	Acute SCI SCI≤ 21d Follow up= 12	Phase I Single group Open label	AIS, SSEP, MEP. Adverse events. Functional neural regeneration collagen scaffold transplantation in complete acute SCI.
NCT02688049	Surgical implantation of NeuroRegen scaffold with either 10^7 MSC or 10^7 NSC into the spinal cord in patients with chronic spinal cord injury. All patients have surgical removal of spinal cord scar tissue, and post-operative comprehensive rehabilitation. Total= 30 subjects	Chronic SCI Timing after SCI not specified Follow up= 6 months	Phase I/II RCT Parallel group Double blind	SSEP/MEP, FIM, MRI, Bladder/Bowel Function Safety/Tolerability/Adverse events/ Efficacy.

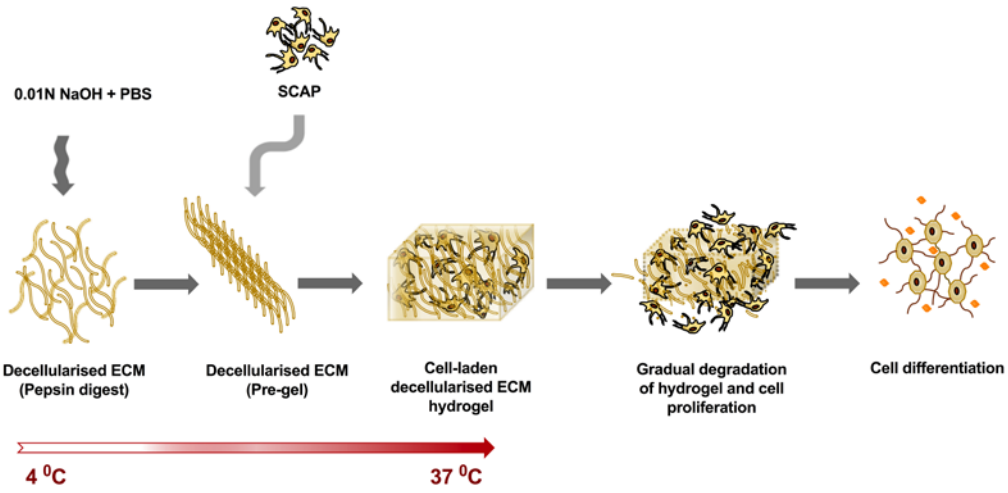
NCT02688062	NeuroRegen Scaffold™ with bone marrow mononuclear cell transplantation vs. intradural decompression and adhesiolysis in persons with chronic SCI. Total = 22 subjects	Chronic SCI Timing after SCI not specified Follow up= 24 months	Phase I/II RCT Parallel group Double blind	SSEP/MEP, FIM, MRI, Bladder/Bowel Function Safety/Tolerability/Adverse events/ Efficacy.
NCT02096913	Surgical Implantation of PLGA Poly-L-Lysine Scaffold (Neuro-Spinal Scaffold) into the injured spinal cord in subjects with complete thoracic AIS-A spinal cord injury. Total = 20 subjects	Acute SCI Able to receive implant ≤96h after SCI Follow up= 6m	Phase III Single group Open label	Scores/Sensory Scores, SCIM III, Bowel/Bladder Function, Incidence of adverse events, Humanitarian Device Exemption (HDE) Probable Benefit Study to demonstrate safety and probable benefit in support of future studies and an HDE application with subsequent FDA approval

5. AIM OF THE THESIS

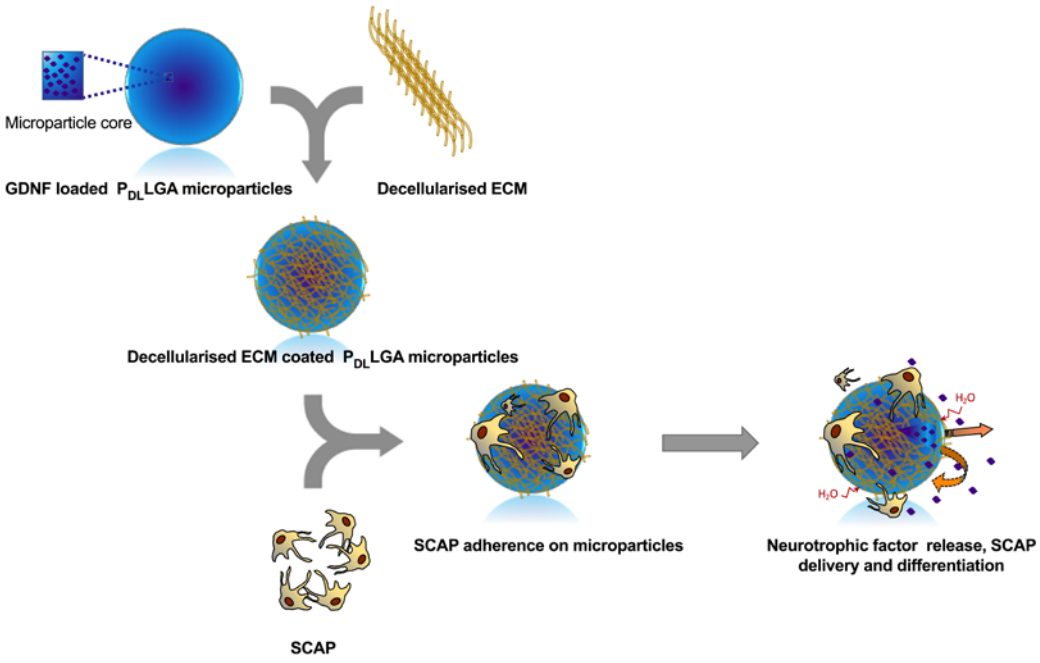
The overall aim of the thesis was to explore the potential of dental stem cells from apical papillae (SCAP), as a suitable source of autologous stem cells, to promote the repair process of injured spinal cord. Two discrete injectable matrices: decellularised mammalian tissue derived ECM hydrogels and GDNF-loaded *poly*(Lactide-*co*-Glycolide) (PLGA) microparticles were devised for delivering dental stem cells, to provide cell replacement and/or promote the neuroprotection, following traumatic SCI.

Specific aims:

A. Develop different decellularised ECM derived hydrogels from different mammalian tissue sources and evaluate their suitability to support SCAP viability as well as differentiation.



B. Evaluate the appropriateness of PLGA microparticles loaded with GDNF to promote the SCAP survival, by facilitating cell adherence while providing a controlled release of GDNF.



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CHAPTER II.

EXTRACELLULAR MATRIX-DERIVED HYDROGELS FOR DENTAL STEM CELL DELIVERY

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ABSTRACT

Decellularised mammalian extracellular matrices (ECM) have been widely accepted as an ideal substrate for repair and remodelling of numerous tissues in clinical and pre-clinical studies. Recent studies have demonstrated the ability of ECM scaffolds derived from site-specific homologous tissues to direct cell differentiation. The present study investigated the suitability of hydrogels derived from different source tissues: bone, spinal cord and dentine, as suitable carriers to deliver human apical papilla derived mesenchymal stem cells (SCAP) for spinal cord regeneration. Bone, spinal cord and dentine ECM hydrogels exhibited distinct structural, mechanical and biological characteristics. All three hydrogels supported SCAP viability and proliferation. However, only spinal cord and bone derived hydrogels promoted the expression of neural lineage markers. The specific environment of ECM scaffolds significantly affected the differentiation of SCAP to a neural lineage, with stronger responses observed with spinal cord ECM hydrogels, suggesting that site-specific tissues are more likely to facilitate optimal stem cell behaviour for constructive spinal cord regeneration.

Key words

ECM hydrogels, Dental stem cell delivery, Spinal cord, Bone, Dentine

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1. INTRODUCTION

Traumatic spinal cord injuries (SCI) cause devastating neurological deficits and disabilities. Initial trauma leads to immediate disruption of neural tissue by axon shearing, blood vessel rupture and cell death. Subsequently a cascade of secondary events occurs composed of ischemic injury, inflammation, cell death, demyelination of axonal tracts and the creation of a glial scar, forming a physical and chemical barrier [1]. Treatment options for SCI have been limited in part due to this complex pathophysiology [2]. Cell transplantation to replace damaged cells, provide trophic support and promote functional recovery is a favourable strategy for SCI [3].

Stem cells derived from dental tissues are an attractive source for cell transplantation in the central nervous system (CNS) due to accessible supply, high proliferation rate and the potential for autologous transplantation [4]. Human dental stem cells are of neural crest origin, display neural stem cell properties [5], and have recently been shown to induce functional recovery in SCI repair [6]. Whilst several different populations of dental stem cells have been identified, stem cells of the apical papilla (SCAP) have been shown to possess greater proliferation potential than dental pulp stem cells [7] and express a variety of neural markers including βIII tubulin, NeuN, nestin, neurofilament M and glial fibrillary acidic protein (GFAP) [5, 8]. Recently, whole human apical papillae implanted into rat hemisected spinal cords induced a significant improvement of rat motor function [4]. However, SCAP cells transplanted in a fibrin hydrogel in the same study did not have the same effect upon motor function [4], highlighting the importance of delivering SCAPs in their original niche or a similarly supportive medium. The goal of the present work was thus to identify a suitable hydrogel to support SCAP delivery for SCI.

Biologic scaffolds composed of extracellular matrix (ECM) produced by decellularisation of mammalian tissues largely retain the functional complexity of their tissues of origin [9]. Hydrogel forms of ECM do not retain the three dimensional ultrastructure of the native tissue but still maintain in vitro and in vivo biologic activity, including differentiation stimuli for neural stem cells [9, 10] and the promotion of an M2 macrophage phenotype [11, 12]. In addition, ECM hydrogels may supply signalling molecules and growth factors [13, 14] required to support delivered cells in the CNS [15]. Tissue-specific ECM scaffolds, originating from a tissue type homologous to the repair site, have previously shown the ability

to enhance site appropriate cell phenotypes compared to ECM scaffolds derived from non-homologous tissues for a range of complex tissues [16-18].

The objectives of the present study were to evaluate the suitability of different ECM derived hydrogels to support SCAPs for delivery into SCI. Previously developed protocols were utilised to produce bone and spinal cord ECM hydrogels (bECMh and scECMh, respectively) and a new method was developed to create a hydrogel form of human dentine ECM (dECMh). The biomolecular composition, mechanical properties, and in vitro cytocompatibility of the hydrogels were evaluated. We hypothesised that the different ECM hydrogels would influence SCAP viability, proliferation and gene expression.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

The Quant-iT™ Picogreen® assay kit and the Presto Blue kit were from Life Technologies (Carlsbad, US). Bovine serum, L-glutamine, penicillin and streptomycin were from Gemini Bio-Products (Sera Laboratories International, Bolney, UK). All other reagents were purchased from Sigma-Aldrich (Poole, UK).

2.2. ECM BIOLOGIC SCAFFOLD PRODUCTION

Porcine spinal cord and bovine cancellous bone were obtained from market weight animals. Human wisdom teeth were collected from healthy individuals aged between 16 and 18 years. An informed consent was obtained from all donors (Approved by the Commission d'Ethique Biomédicale Hospitalo-Facultaire of UCL, 2012/14JUN/283). Female and male donors and animals have been used indiscriminately. Decellularisation of bone (bECM) [19] and spinal cord (scECM) [20] were performed as described previously. Extraction of ECM from human dentine (dECM) was performed using the protocol developed for bECM preparation with modifications. Briefly, teeth were frozen in liquid nitrogen and broken with a Carver press (Carver, #3850, Wabash, US) and the pulp was removed. The enamel was destroyed by sonication in 10% HCl for 20 min [21]. Acid neutralisation was performed by immersing the tissue in 2 volumes of water containing penicillin-streptomycin (0.5% w/v) for 1 hr.

Demineralisation was performed by incubating tooth fragments in a 0.5M HCl solution under agitation (300 rpm) for 24 hrs at room temperature. The resultant material was washed thoroughly with deionised water and incubated in a 0.05% trypsin-0.025% EDTA solution for 24 hrs at 37°C at 300rpm for enzymatic decellularisation. Finally, the material was washed with 2 volumes of PBS and freeze dried.

2.3. ECM DIGESTION AND SOLUBILISATION

Pepsin digestion and solubilisation was used to obtained pre-gel solutions [22, 23]. Lyophilized ECM powders were added to 1mg/ml of pepsin in 0.01 N HCl for a final concentration of 10 mg ECM/ml. The suspension was stirred at room temperature for 48 hrs for spinal cord ECM, 72 hrs for dentine ECM and 84 hrs for bone ECM. The resultant pepsin digests were aliquoted and stored at -20^{0}C. Gelation of pepsin digests was induced by neutralisation of salt concentration and pH at 4°C followed by warming to 37°C. Briefly, pepsin digests were mixed with one tenth volume of 0.01N NaOH, one ninth volume of 10X PBS and made up to desired volume with 1X PBS. 8 mg/ml of pre-gel solutions were prepared and incubated for 1 hr at 37°C to obtain spinal cord, bone and dentine hydrogels, denoted scECMh, bECMh and dECMh.

2.4. DNA, COLLAGEN AND SGAG QUANTIFICATION

Quantitative assessment of DNA in pepsin digests was conducted by an adaptation of previously published method [23]. DNA was extracted from pepsin digests by addition of an equal volume of 25:24:1 (v:v:v) phenol/chloroform/isoamyl to 1ml of sample (10 mg/ml). Phenol was removed using an equal volume of 24:1 (v:v) chloroform/isoamyl alcohol. DNA was precipitated from the aqueous phase by the addition of 2 volumes of ice-cold ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) and was frozen at -80^{0}C for 30 min. The frozen DNA was then centrifuged at 13,000 rpm for 10 min and washed with ethanol, dried at room temperature and resuspended in 0.1 ml of TE buffer. DNA concentration was measured using a Quant-iT™ Picogreen® assay kit following supplier instructions. A standard curve was prepared with known DNA concentrations from 0 – 100 ng/ml and samples were read using a Tecan Infinite M200 plate reader (Tecan UK, Reading, UK).

Collagen content of pepsin digests was measured by quantifying hydroxyproline [24]. Briefly, pepsin digests and a blank pepsin solution were hydrolysed with 1 volume of concentrated HCl (12N) overnight at 120°C. The samples were dried by incubation at 90°C for 2 hrs followed by the addition of 4 volumes of 0.25 M sodium phosphate buffer (pH 6.5). The hydrolysed blank pepsin solution served as a negative control as well as diluent for the assay. The samples were oxidised by incubation with 1 volume of 3-Chloramine T solution (0.07M) at room temperature for 20 min followed by the addition of 4- p-dimethylaminobenzaldehyde (p-DAB) solution [1.16M p-DAB in 60% perchloric acid (6.5ml) and Isopropanol (15ml)] and incubation at 60^0C for 30 min. A standard curve was prepared using known concentrations of hydroxyproline ranging from 0 – 100 µg/ml and the absorbance was measured at 540nm with a Tecan Infinite M200 plate reader. Collagen content was determined using the relationship that hydroxyproline forms 14.3% of total amount of collagen [24].

The sulphated glycosaminoglycan (sGAG) content in pepsin digests was determined using the 1, 9–Dimethyl methylene blue assay (DMMB) [25]. One ml of digest was incubated with 0.1mg/ml of Proteinase K (>600U/ml) overnight at 37°C. A solution of blank pepsin was used as negative control as well as diluent for the assay. 50µl of sample was mixed with 200 µl of DMMB solution (0.03 M sodium formate, 0.046M DMMB, ethanol (0.5% v/v) and formic acid (0.2% v/v) in a 96 well plate. A standard curve was constructed using known concentrations of Chondroitin-4-sulphate from 0 – 75 µg/ml. The absorbance was measured immediately at 525nm.

2.5. ECM-BASED HYDROGEL GELATION KINETICS

Turbidimetric gelation kinetics of ECM-based hydrogels was determined as previously described [26]. Normalised Absorbance (NA) was calculated as shown in Eq. (1).

$$NA= (A-A0)/(Amax-Ao) \quad (1)$$

Were A is absorbance at given time, A0 is the initial absorbance and Amax is the maximum absorbance attained by the sample after inducing gelation. Normalised gelation was used to calculate the time at which 50% of gelation ($t_{1/2}$) and 95% of gelation (t_{95}) occurred. The lag

time, t_{lag}, was defined as the intercept of the linear region of the gelation with 0% absorbance [19]. Speed of gelation (S) was determined by calculating the slope of the curve (n=8).

2.6. RHEOLOGICAL CHARACTERISTICS

bECMh, dECMh, and scECMh rheological characteristics were determined as described previously [19] using a Malvern Kinexus ultra+ rheometer (Malvern Kinexus Pro rotational rheometer and rSpace software, Malvern Instruments, Worcestershire, UK). Cold pre-gel solutions were placed between two pre-cooled (4°C) 25 mm parallel plates separated by a gap of 0.3 mm. For each run, the plates were warmed to reach 37°C (2°C/sec) and the sample was exposed to 1% oscillatory strain with a constant angular frequency of 2 rad/sec. Data were recorded every 30 sec (n=3).

2.7. SCANNING ELECTRON MICROSCOPY (SEM)

Gel specimens (300µl per well) were fixed in 5 % glutaraldehyde at room temperature. Samples were dehydrated in a graded series of ethanol (25-100%), dried using a critical point dryer (CPD, Balzers Union, Balzers, FL) and sputter coated with gold using a Cressington 208HR metalizer (ELOISE s.a.r.l., Tremblay, FR). Images were acquired using a JSM-7600F (JEOL, Zaventem, BE) scanning electron microscope at a voltage of 15 kV.

2.8. SCAP CULTURE

Previously characterised human stem cells of the apical papilla (SCAP) (RP89 cells) [27] were used between passages 6 and 9. SCAP were grown at 37°C in 5% CO2 in Minimum Essential Medium (Sigma-Aldrich) supplemented by 10% bovine serum (Gemini Bio-Products), 1% of L-glutamine (Gemini) and 1% of penicillin and streptomycin (Gemini) until they reached ~80% confluence. SCAP were then incorporated in the pre-gel solutions, placed in 96 well plates and incubated at 37°C for 1 hr.

2.9. SCAP VIABILITY

Pre- gel solution (10µl) containing 500,000 cells/ml were loaded into µ-Slide Angiogenesis ibiTreat microscopy chamber (Ibidi, Proxylab, Beloeil, BE) and were allowed to gel (n=3,

N=3). After 2 days, a Live/Dead assay was performed. Pictures (2 fields/sample, Z-stack: 1 picture every 3µm, for a total of 210 pictures/stack) were then acquired with a confocal microscope (LSM700, Zeiss, Zaventem, BE). All the pictures were merged to count dead and living cells. Cell viability was expressed as a percentage of the number of living cells compared to the total number of cells.

2.10. SCAP PROLIFERATION

The effect of ECM origin on SCAP proliferation was analysed when cells were encapsulated in the hydrogels (3D culture) or grown on the surface of hydrogel (2D culture).

2.10.1. SCAP proliferation in 3D culture

Pre-gel solutions containing 500,000 SCAP/ml were placed in 96 well plates (N=3, n=6) and incubated for 1hr at 37°C to initiate gel formation. Pre-gel solution (100µl) without cells was used as a control. Independent samples were prepared for each time point. After 2, 7 and 14 days, cell proliferation was analysed using a Presto Blue kit. The fluorescence intensities (590 nm) were measured 2 hours after the addition of the Presto Blue reagent. Values obtained for hydrogels without cells were used as background and subtracted from values obtained for hydrogels with cells. A standard curve (from 13,000 to 250,000 cells) was used to correlate the fluorescence intensity with cell number. Cell number fold increase was calculated by dividing the number of cells at day 2, 7 or 14 by the initial number of seeded cells. Samples were collected and frozen at -80°C for further processing for PCR analysis.

2.10.2. SCAP proliferation in 2D culture

Pre-gel solutions (20µl) were placed in 96 well plates and allowed to gel for 1 hr at 37°C. Two thousand cells were seeded onto the surface of the hydrogels (N=3, n=6). Cell proliferation was evaluated as described for 2.10.1.

2.11. SCAP GENE EXPRESSION

Total mRNA was extracted using the phenol/chloroform extraction method. One microgram of mRNA was reverse transcribed using the Reverse Transcription System kit (Promega, Madison, USA) (n=3). The resulting cDNA was used as template for 30 cycles of semi-

quantitative polymerase chain reaction in a T100 TM thermo cycler (Bio-Rad, BE). Primer sequences are summarized in Table 1. The PCR products were pooled and subjected to electrophoresis on Syber™Safe (Invitrogen, BE) stained 2% agarose gel. mRNA relative expression of tested genes was normalised with GAPDH.

2.12. STATISTICAL ANALYSIS

Statistical analyses were performed using PRISM (GraphPad software, CA, USA) and JMP10 (SAS, NC, USA). Two-way ANOVA with post hoc Bonferroni's multiple comparison tests as well as Tukey's HSD were performed with a P-value of 0.05. Errors bars represent the standard deviation (SD) in all figures.

3. RESULTS

3.1. INFLUENCE OF ECM ORIGIN ON CELLULAR, COLLAGEN AND SGAG CONTENT

Determination of decellularisation efficiency was based upon quantification of dsDNA. Regardless of origin, all three ECMs contained less than 50 ng/mg of dsDNA (Figure 1A). Concentrations of nucleic acid of bECM, dECM and scECM were significantly different with the lowest quantity detected in scECM; bECM, dECM and scECM contained 17.8±1.5 ng of DNA/mg, 13.56±0.5 ng of DNA/mg and 6.8±0.17 ng of DNA/mg, respectively. No significant difference was observed between the collagen content of the three ECMs, although scECM tended to contain less collagen (Figure 1B); bECM, dECM and scECM contained 84.28±10.20%, 83.16±5.55 % and 67.41±23.16 % of collagen, respectively. Significantly more sGAG was detected in scECM (0.65 % of dry weight) compared to bECM and dECM (0.3 % and 0%, respectively) (Figure 1C).

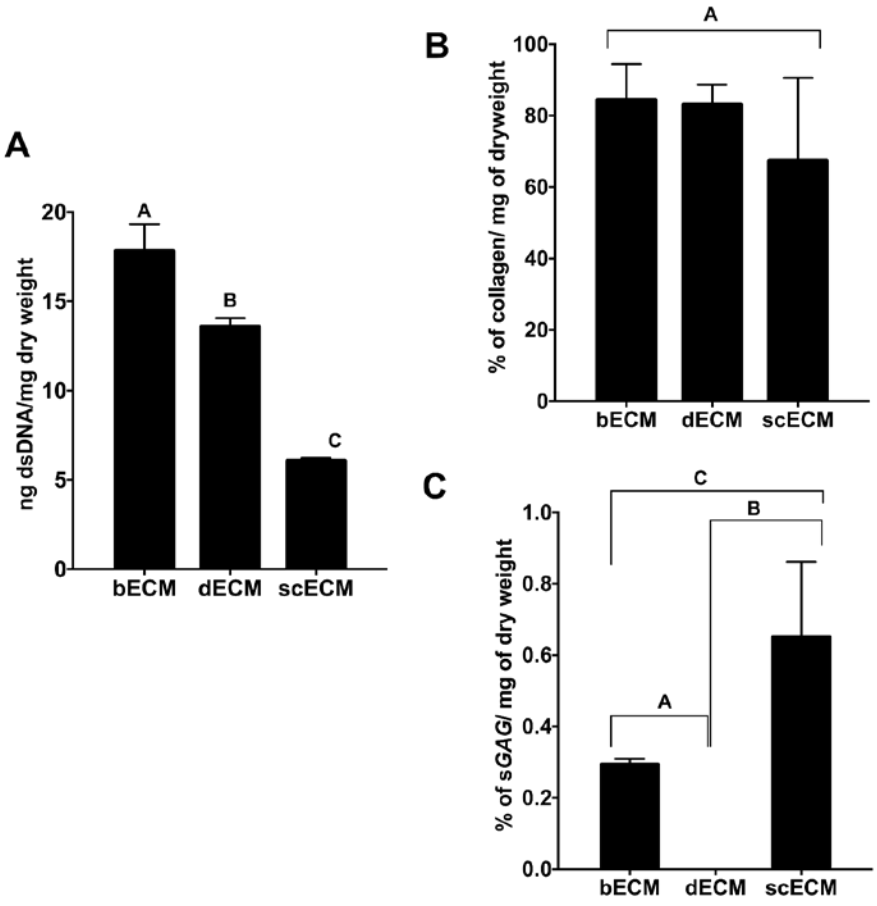

Figure 1: Influence of ECM origin on cellular, collagen and sGAG content. (A) Efficiency of decellularisation was assessed by quantifying the amount of dsDNA/mg dry weight. B) Collagen content and (C) sGAG content of ECM scaffolds were evaluated by quantifying hydroxyproline and using the 1, 9 –Dimethyl methylene blue assay, respectively. Data is presented as Mean ± standard deviation (n=3). Conditions not linked by the same letter are significantly different ($p<0.05$).

Table 1: Human genes and primers used for RT-PCR analysis

Symbol	***Gene name***	***Primer Sequence (5'->3')***	***Amplicon length (bp)***
GAPDH	*GAPDH = Glyceraldehyde 3-phosphate dehydrogenase*	F : GATCAACTCACCGCCAACA R : CCAGCGACTCAATCTTCCTC	452
SNAIL	*Zinc finger protein SNAIL*	F : CTAGGCCCTGGCTGCTACAA R :CATCTGAGTGGGTCTGGAGGT	129
BIII Tubulin		F : GGGCCTTTGGACATCTCTTC R : GGATACTCCTCACGCACCTT	244
PDGFRa	*Platelet-Derived Growth Factor Receptor, Alpha Polypeptide*	F : AGCTTGAAGGCAGGCACA R :GCGACAAGGTATAATGGCAGA	122
VEGFA	*Vascular endothelial growth factor A*	F : CATCTTCAAGCCATCCTGTG R : CAACGCGAGTCTGTGTTTT	303,357, 375,426
GDNF	*Glial cell derived neurotrophic factor*	F : CAGACATCTGATCCCCTTCC R : GCTGGGGTTTGTCACTGTTT	172

3.2. INFLUENCE OF ECM ORIGIN ON HYDROGEL GELATION KINETICS

The gelation kinetics of scECMh, bECMh and dECMh were sigmoidal, and gelation occurred within 40 min (Figure 2). The tlag and $t_{1/2}$ was 1.40-1.53 fold lower for dECMh compared to sc ECMh and bECMh. However, the time required to reach 95% of the final turbidity is 0.75-0.84 fold lower for scECMh with respect to bECMh and dECMh. The gelation speed was 1.3 and 2.1 fold faster for scECMh compared to bECMh, dECMh respectively (Table 2).

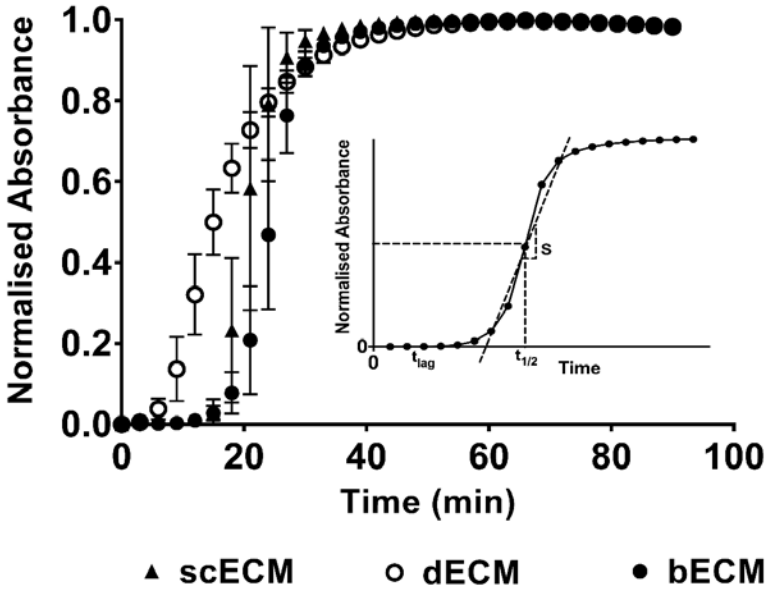

Figure 2: Influence of ECM origin on hydrogel gelation kinetics. Ice-cold neutralised pre-gel solutions were placed into a 96 well plate and incubated at 37°C. Absorbance was measured at 405nm and every 3 min. Data were normalised, 0 being the initial absorbance and 1 the maximum absorbance (n=8). Inset: diagrammatic representation of the parameters determined from normalised gelation kinetics.

Table 2: Gelation kinetics for scECM, bECM and dECM hydrogels

Turbidity (Mean± SD)	**scECM (Mean± SD)**	**bECM (Mean± SD)**	**dECM (Mean± SD)**
Lag phase (t_{lag}: min)	11.998 ± 1.90	11.25 ± 1.4	7.8 ± 1.55
50% gelation ($t_{½}$: min)	20.582 ± 2.42	21.06 ± 3.2	14.60 ± 1.24
95% gelation (t_{95}: min)	30.248 ± 2.91	36.00± 0.00	39.85 ± 3.34
Speed (S, min^{-1})	0.074 ± 0.009	0.057 ± 0.003	0.035 ± 0.004

n=8, $p<0.05$

3.3. INFLUENCE OF ECM ORIGIN ON HYDROGEL MECHANICAL PROPERTIES

Storage modulus (G´) and loss modulus (G˝) of all the pre-gel solutions increased 5 min after pH neutralisation and temperature elevation and reached a maximum after 1 hr (Figure 3). G' was consistently higher than G", reflecting the formation of a solid hydrogel. Regardless of origin, hydrogels showed similar gelation profiles, although bECMh, dECMh and scECMh final moduli were significantly different (Table 3). bECMh had the highest storage modulus (287.17 ± 8.43 Pa); while scECMh and dECMh were significantly lower (19.35 ± 1.16 and 4.79 ± 0.33 Pa, respectively).

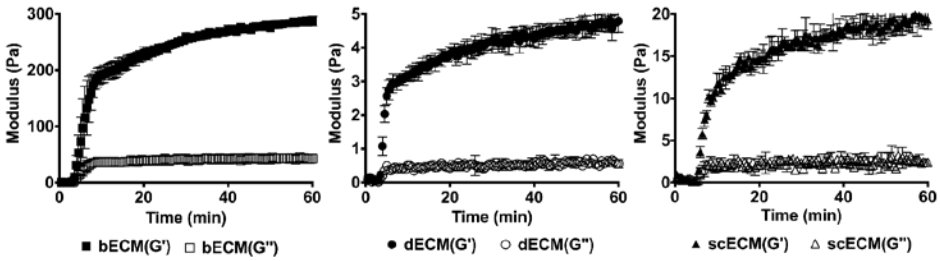

Figure 3: Influence of ECM origin on hydrogel mechanical properties. Rheological properties of hydrogels were investigated by small amplitude oscillatory shear rheology. The storage (G′) and loss moduli (G″) were measured using a Kinexus Pro rotational rheometer and rSpace software (n=3).

Table 3: Influence of ECM origin on hydrogel final moduli

Material	Storage modulus (G′, Pa)	Loss modulus (G′′, Pa)
bECM (8mg/ml)	287.17 ± 8.43^{A}	42.61 ± 3.72^{A}
dECM (8mg/ml)	4.79 ± 0.33^{B}	0.56 ± 0.128^{B}
scECM (8mg/ml)	19.35 ± 1.16^{C}	2.40 ± 0.385^{C}

ECM not related by the same letter are significantly different (G' and G'' separately) ($p<0.05$; n=3).

3.4. INFLUENCE OF ECM ORIGIN ON HYDROGEL MICROSTRUCTURE

The origin of hydrogel markedly influenced the hydrogel morphology. While bECMh and dECMh exhibited a fibrillary structure with disorganised thin fibres (Figure 4A and B); scECMh showed a honeycomb structure with thick walls and large pores (Figure 4C). dECMh appeared to be composed of thicker fibres and smaller pores in comparison to bECMh.

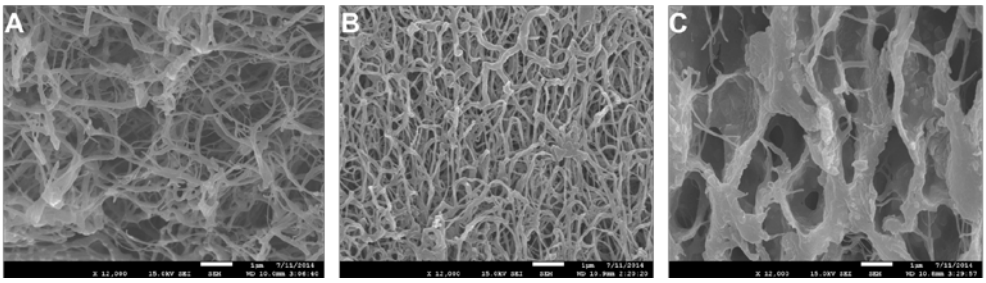

Figure 4: Influence of ECM origin on the hydrogel microstructure. ECM hydrogels were formed and processed for SEM analysis, scale bar = 1 μm with (A) bECM, (B) dECM and (C) scECM hydrogels. Magnification: 12,000x.

3.5. INFLUENCE OF ECM ORIGIN ON DENTAL STEM CELL PROLIFERATION

Regardless of the ECM origin, SCAP proliferated when incorporated in the ECM hydrogels. When cultured in 3D, the number of SCAP increased between 0.36 and 1.47-fold 7 days post seeding and 2 to 4.78-fold 14 days post seeding compared to the initial seeding density (Figure 5A). No significant difference was observed between day 2 and 7 for any of the hydrogels, whilst SCAP proliferation significantly increased between day 7 and day 14 for all of the hydrogels. The SCAP proliferation was 1.8 to 2.5-fold higher in scECMh compared to the dECMh and bECMh on day 14, respectively.

When grown on the hydrogels (2D culture), the SCAP proliferation on various ECM hydrogels increased between 0.48 to 1-fold 7 days of post seeding and between 12.6 to 60-folds 14 days post seeding (Figure 5B). SCAP proliferation on tissue culture treated plastic

(TCP) was much higher than either on 2D or in 3D hydrogels with a fold increases of 16.28 and 205.7 at day 7 and 14, respectively. No significant difference was observed between Day 2 and 7.

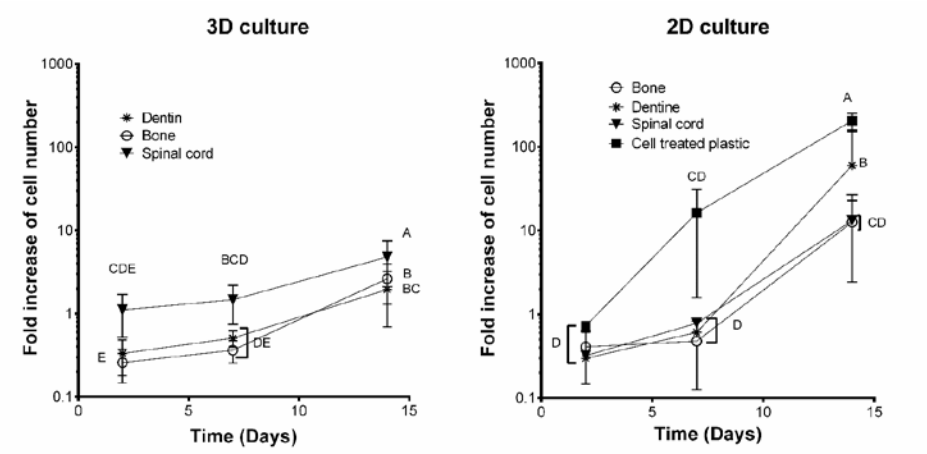

Figure 5: Influence of ECM origin on SCAP proliferation. A- 500,000 SCAP/ml were incorporated in different ECM hydrogels and incubated at 37°C. Presto blue test was performed at different time points (2, 7 and 14 days). Fluorescence intensities were measured at 590 nm (n=6, N=3). B- Pre-gel solutions were allowed to gel at 37°C. Then 2000 SCAP were seeded on the ECM. After 2, 7 and 14 days, cell proliferation was analysed using a Presto Blue kit (n=6, N=3). As a positive control, the same amount of cells was seeded on tissue culture plastic. Conditions not related by the same letter are significantly different ($p < 0.05$).

3.6. INFLUENCE OF SCAP INCORPORATION INTO DIFFERENT ECM ON CELL VIABILITY

SCAP viability 2 days after incorporation in all ECM hydrogels was above 65 % (Figure 6B). Viability was significantly higher in bECMh than in dECMh and scECMh (85 %, 65 % and 68 %, respectively). In general, SCAP morphology was elongated in all three ECM hydrogels, however some rounded cells were also observed in bECMh (Figure 6A).

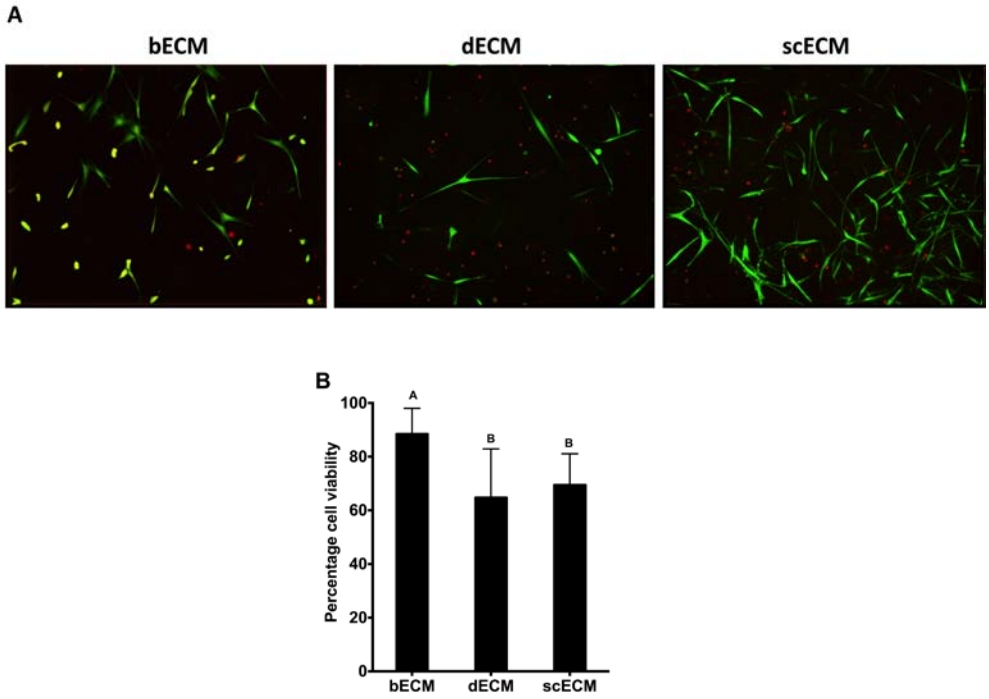

Figure 6: Influence of ECM origin on SCAP viability. 500,000 SCAP/ml were incorporated in different ECM hydrogels and incubated for 2 days. A Live/Dead assay was performed. Living and dead cells were observed by confocal microscopy (488 and 568 nm, respectively) (n=3, N=3). (A) Live cells were stained with Calcein (green) and dead cells were stained with ethidium homodimer-I (red). (B) The images were processed to quantify the number of live cells and dead cells using ImageJ (n = 3–5 acquisitions/sample). Conditions not related by the same letter are significantly different ($p < 0.05$).

3.7. INFLUENCE OF ECM ORIGIN ON SCAP GENE EXPRESSION

The expression of genes related to the neural lineage (SNAIL, βIIITubulin, GDNF, VEGFA, PDGFRa, and GAPDH as a housekeeping gene) were analysed to determine whether ECM origin would elicit or support SCAP orientation. When SCAP were cultured routinely (Figure 7), GDNF and PDGFRa were slightly expressed but no expression of SNAIL, βIIITubulin or VEGFA was detected. When SCAP were incorporated in bECM, no expression of the gene of interest was detected at Day 2 and Day 7. SNAIL, βIIITubulin, GDNF, VEGFA and PDGFRa were expressed after 14 days of incubation in the hydrogel. SCAP incorporated in dECM showed no expression of the genes of interest. Only when SCAP were grown in scECM a marked expression of all the tested genes was detected after 7 days.

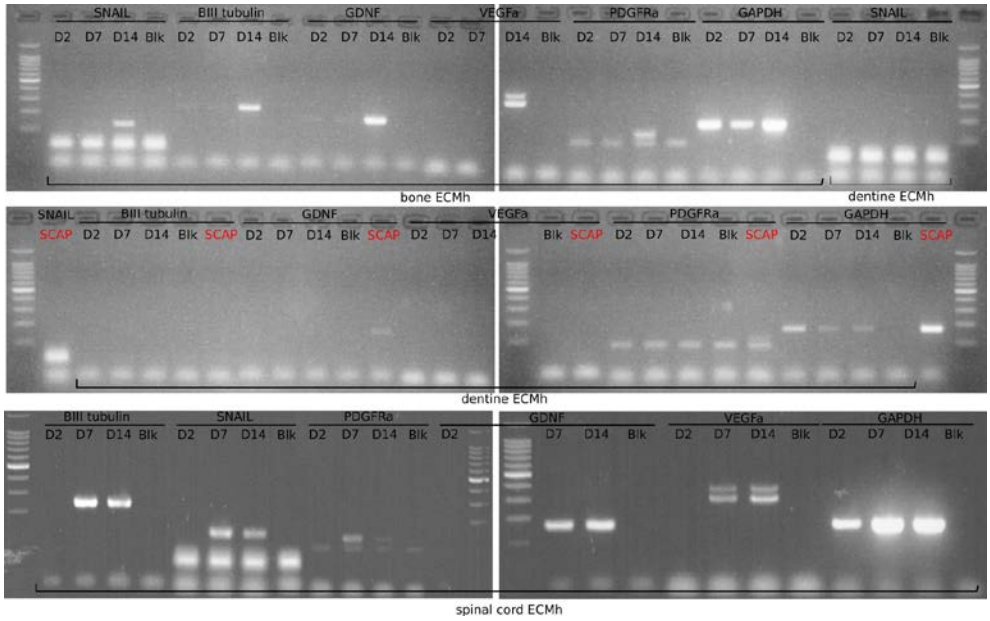

Figure 7: Influence of ECM origin on SCAP gene expression. 500,000 SCAP/ml were incorporated in different ECM hydrogels and incubated for 2, 7 and 14 days (D2, D7 and D14). mRNA was extracted and gene expression was analysed by RT-PCR (n=3, N=3). Blanks (Blk) were obtained by replacing mRNA by RNAse free water. Gene expression of SCAP grown in 2D without ECM hydrogel (SCAP) was also analysed

4. DISCUSSION

The present study illustrates the influence of biological scaffolds derived from homologous and non-homologous tissue on the gene expression profile of dental stem cells from apical papilla (SCAP). Previously published methods were employed to prepare ECM scaffolds derived from spinal cord (scECM) [20] and bone (bECM) [19]. A new decellularisation method was developed for the preparation of dentine ECM scaffolds (dECM) based on the material characteristics and its similarity towards the bone tissue.

The acellular nature of bECM, dECM, and scECM was determined by quantifying residual dsDNA content. The amount of dsDNA per mg of dry ECM present in bECM, dECM and scECM was considerably lower than the <50 ng threshold previously determined as effective criteria for decellularisation [28]. The presence of DNA and other cellular material, either xenogeneic or allogeneic in nature, within in the ECM scaffold due to inefficient decellularisation could potentially elicit host immunological response. However, it is unlikely that complete removal of cells will be obtained even with harsh decellularisation procedures [29].

The percentage of collagen and glycosaminoglycan in the ECM scaffolds derived from bone, spinal cord and dentine varied depending upon the function and nature of tissue source. In addition to the intrinsic ECM composition influencing sGAG content, the higher sGAG content in scECMh could be due to the decellularisation process. It has been demonstrated previously that small intestinal mucosa-based ECM matrix sterilised with peracetic acid induced a greater loss of cellular components such as lipids, nucleic acids, and other undesirable elements ensuing more sGAG and FGF-2 compared to ECM non-treated with peracetic acid. We postulate that the higher sGAG content in scECM with respect to bECM and dECM might be due to the peracetic acid treatment during decellularisation [30]. Also, we hypothesise that the differences observed between ECM of different origins might be due to i) either different amounts of sGAG in the tissue before treatment or ii) to the different treatments used to obtain each ECM [30, 31].

The gelation kinetics based on turbidimetry demonstrated that scECM, bECM and dECM hydrogels (Figure 2) exhibited sigmoidal gelation kinetics. Although the speed to complete gelation as well as t_{95} were greater for scECMh and bECMh, the lag time as well as $t_{1/2}$ of

dECMh was considerably lower with respect to bECMh; this is likely to be the effect of glycosaminoglycan presence during the in vitro self-assembly of collagen fibres [32].

It is noteworthy that the individual ECMs demonstrate distinct morphologies when analysed via scanning electron microscopy. The basal lamina of the spinal cord is comprised predominantly of collagen IV though types I and III are also present [33] whereas bone and dentine are composed primarily of collagen type I, with traces of type III, V, IX, XII, XIV, XIX, and XXI [34]. We hypothesize that scECMh contains Type IV collagen that tends to form a flexible triple helix that self-assembles to incorporate structural glycoproteins [35], resulting in the sheet-like structure observed in Figure 4C. However, in bECMh and dECMh collagen XII, XIV, XIX, and XXI serve as molecular bridges between collagen I monomers to produce fibrils.

Rheology was used to study the viscoelastic properties of ECM hydrogels. It is interesting to note that regardless of the tissue origin, hydrogels exhibited similar sigmoidal shaped gelation profiles. However, the storage modulus for dECMh is considerably lower than scECMh and bECMh. We postulate that this difference is due to the disparity of source of tissue as well as the decellularisation processes. The potential impact of hydrogel solid content has been evaluated but no difference was visible between hydrogels of different origins (Appendix I: Figure 1). We hypothesise that the difference in composition and architecture of different ECM hydrogels could be responsible for the difference of moduli, particularly when considering the different morphologies observed with SEM [36, 37]. Although the final moduli of dECM and scECM are lower than 0.1kPa, the final modulus of bECMh was in the range of 0.1-1.0kPa. Hydrogels with elastic moduli within this range have been reported to have a positive influence on the probability of neuronal marker expression and neuronal differentiation [38].

Several authors have emphasised the intrinsic ability of ECM derived scaffolds to aid viability, migration and proliferation of numerous cell types [9, 39]. Nevertheless, the present study is the first time ECM scaffolds from decellularised bone, dentine and spinal cord have been used as a 3D culture system for apical papilla stem cells. High cell viability (Figure 6) was observed in bECMh, scECMh and dECMh, confirming the suitability of these scaffolds as delivery vehicles for SCAP. Greater cell viability was observed in bECMh compared to

dECMh and scECMh. The choice of detergents used for the decellularisation procedure could have a marked deleterious effect up on the ECM [40]. We hypothesise that the lower cell viability observed in scECMh may be due to traces of residual detergent present after decellularisation. This is the first time that dentine has been decellularised and further studies may be needed to optimise the duration as well as the concentration of HCl solution used in the enamel removal step. We postulate that too harsh treatment applied during decellularisation procedure may have led to the lower cell viability observed in dECMh

In both 2D and 3D culture environment, SCAP proliferated till day 7 irrespective of the culture conditions (Figure 5). However, in the 2D environment, there was a remarkable increase in the proliferation between day 7 and day 14 for SCAP grown on TCP and dECMh. We postulate that after the initial phase of cell and ECM hydrogel interaction, the surface with higher elasticity (dECMh) facilitated quicker cell migration and proliferation compared to the more rigid surfaces of bECMh and scECMh. SCAP grown in 2D culture proliferated faster than SCAP grown in 3D. Cell movement on 2D environment is relatively unrestricted; whereas in a 3D environment, cells need to degrade their adjacent material enzymatically in order to migrate [41]. Furthermore, in 2D culture, the cells are exposed to a homogenous concentration of nutrients which influence their proliferation and differentiation in comparison to the gradient pattern observed in 3D environment [42]. We also postulate that the porous structure of scECMh (Figure 4C) facilitated greater proliferation rate in 3D environment compared to bECMh and dECMh.

A slight expression of some neural lineage markers by non-differentiated SCAP, attributed to their neural crest origin, was reported previously [43]. When SCAP were embedded into scECMh, there was a remarkable expression of key neural lineage markers on day 7 and day 14 (Figure 7). We hypothesise that the potential neurotrophic factors as well as proteoglycans embedded within scECMh have induced a neural-like phenotype. ECM scaffolds derived from CNS tissues have already been reported to induce neuronal cell differentiation of neuronal progenitor cells [9, 20, 23]. Furthermore, decellularisation using peracetic acid might have resulted in a higher concentration of FGF-2 as well as sGAG [44], which in turn enhanced the gene expression of neuronal lineage markers in scECMh. On day 14, SCAP embedded in bECMh also showed an expression of neural lineage genes. We hypothesise

that the by-products of ECM degradation including soluble biomolecules in conjunction with scaffold elasticity may influence SCAP differentiation towards neural phenotype.

5. CONCLUSION

Solubilised forms of decellularised ECM from spinal cord, dentine and bone tissues were prepared by a combination of enzymatic and chemical processes and induced to form hydrogels. These acellular ECM hydrogels had discrete structural, mechanical and biological characteristics. Human stem cells from apical papilla exhibited a strong positive response to scECMh by proliferating within and on the hydrogel and by expressing neural genes; substantiating the influence of neurotrophic factors present in decellularized spinal cord. SCAP exhibited a greater proliferation rate in 2D in dECMh, compared to bECMh and scECMh, however no neuronal differentiation occurred. Neuronal lineage markers were expressed by SCAP when cultured with bECMh and it is hypothesized that the hydrogel mechanical properties combined with active biomolecules provided the stimulus for this behaviour. The results of the present study show that decellularised ECM hydrogels facilitate SCAP viability and proliferation. However, ECM hydrogels from site-specific tissues demonstrated are more likely to facilitate optimal stem cell behaviour for constructive spinal cord regeneration.

APPENDIX I

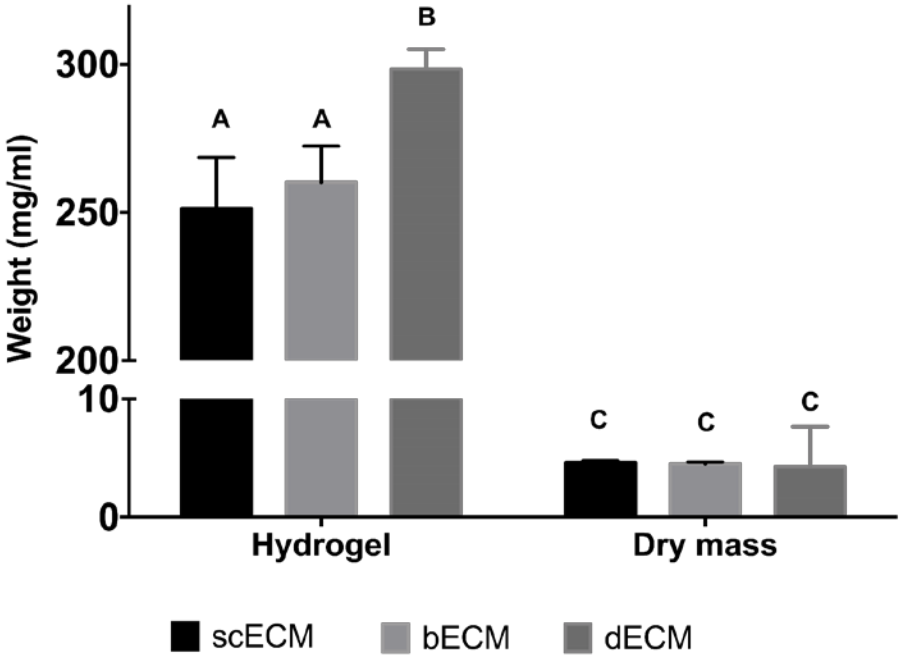

Figure 1. Influence of ECM origin on hydrogel solid content. The hydrogel masses were compared at 8mg/ml concentration before and after dehydration process. A significantly higher water retaining capacity was observed with dECM in the hydrogel form compared to scECM and bECM. There was no significant difference observed in the dry mass of scECM, bECM and dECM. Data represent mean± SD (n=3). Conditions not linked by same letter are significantly different ($p<0.05$).

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CHAPTER III.

SALT CONTAINING, SURFACE COATED MICROPARTICLES FOR GDNF AND DENTAL STEM CELL DELIVERY

TABLE OF CONTENTS

ABSTRACT

Transplantation of stem cells combined with biomolecules is a promising strategy in tissue regeneration, particularly in the treatment of traumatic spinal cord injury (SCI) which leads to the permanent loss of sensory function. In spite of extensive research, cell survival, integration to the host tissue, differentiation, safety and efficacy of biomolecules still remain major concerns. Drug formulation systems such as microparticles may aid to overcome these issues by providing structural support for transplanted cells and controlled release of growth factors at the site of repair. The present study investigated fabrication of 20% w/w PLGA-PEG-PLGA triblock containing 50:50 P_{DL}LGA microparticles as a suitable substratum for delivering human dental stem cell from apical papillae (SCAP) and glial cell-derived neurotrophic factor (GDNF) for spinal cord repair. The P_{DL}LGA- 20% TB microparticles containing sodium acetate assisted a successful encapsulation and controlled release of bioactive GDNF. The microparticles were surface modified with laminin, water and different decellularised mammalian extracellular matrices derived from spinal cord and bone. ToF-SIMS data exhibited an increased protein adsorption on microparticle surfaces coated with scECM and bECM. All the surface modified microparticles supported SCAP adhesion and survival. P_{DL}LGA- 20% w/w TB microparticles are therefore an efficient formulation system to provide GDNF and may facilitate the delivery of SCAP to stimulate spinal cord regeneration

Key words

PLGA microparticles, decellularised matrix, GDNF, SCAP, bone extracellular matrix, spinal cord extracellular matrix, extracellular matrix hydrogels, spinal cord regeneration.

1. INTRODUCTION

The prognosis of a complete recovery of neuronal function after traumatic spinal cord injury (SCI) is poor. The regenerative capacity of the damaged spinal cord is hampered by instant cellular, vascular and ischemic damage, exacerbating the inflammation, necrosis, and apoptosis of neurons and oligodendrocytes [1]. These events can, in turn, contribute to the development of glial scar [1]. Treatment strategies such as early surgical decompression, stabilisation of vertebrae and drug therapies are focused on preventing secondary complications, but, exhibit limited effect on locomotor functions [2,3]. Cellular transplantation to replace lost functional cells and to diminish the establishment of cystic cavity, has shown promise as a strategy for spinal cord repair [4].

Several cell types including Schwann cells, olfactory ensheathing cells (OECs), embryonic stem cells (ES), neural progenitor/stem cells (NPSC), induced pluripotent cells (iPSCs) and bone marrow derived stromal cells (BMSC) have successfully demonstrated their potential to enhance the neural function and axonal regeneration [5–11]. Mesenchymal stem cells derived from dental tissues have a significant advantage for their use in central nervous system (CNS) regeneration, as they exhibit neural crest phenotype, constitutively express neural progenitor markers and promote neural cell survival [12,13]. Additionally, human dental stem cells have been a favourable source for cellular transplantation due to their accessibility, rapid proliferation rate and possibility of autologous transplantation [14,15]. Although, various dental stem cell populations have demonstrated preclinical potential for CNS repair [16], dental stem cells from apical papillae (SCAP) are derived from developing tissue that might display early stem cell/progenitor population and can be a superior cell source for tissue regeneration [17]. Furthermore, SCAP express distinct neural markers including glutamic acid decarboxylase (GAD), beta III tubulin, neuronal nuclear antigen (NeuN), neurofilament M (NFM), neuron-specific enolase (NSE), and glial markers 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPas) upon neural induction [18]. In addition, SCAP transplanted within a niche or supporting environment have shown improved locomotor function, cell proliferation and viability in SCI models [14,19].

Alongside cell transplantation, delivery of active biomolecules, particularly neurotrophic factors to elicit desired cellular responses has been a key strategy for CNS regeneration [20,21]. Whilst various neurotrophic factors have been identified, GDNF is considered to be

a significant neuroprotective and survival factor in neuronal regeneration [22–24]. A short in vivo half-life of approximately 34h for GDNF in cerebrospinal fluid as well as the side effects due to a high concentration dosage via systemic route have emphasised the need for a controlled and sustained release of GDNF [25].

Microparticles have gained great interest in tissue engineering due to their potential to deliver various therapeutics agents, biomolecules and cells [26–28]. Poly (lactic acid-co-glycolic acid) (PLGA) is one of the widely accepted biodegradable polymers with successful application in drug delivery products such as Zoladex® (AstraZeneca), Profact® Depot or Suprefact® Depot (Aventis Pharma), Sandostatine LAR® (Novartis). Recently, administration of GDNF loaded PLGA microparticles in non-human primates exhibited sustained release of the growth factor to significantly improve dopaminergic restoration and motor improvement [29]. PLGA microparticles have also been utilised as a structural support for human neural stem cells (hNSC) to facilitate neuronal differentiation [30].

A constructive approach to address problems such as cell death after transplantation, improper cell distribution and differentiation at the site of injury, is the provision of a structural support for SCAP with tailored neurotrophic factor release. Increased cell attachment to the microparticles can be achieved by surface modification with ECM macromolecules such as laminin, fibronectin etc. [27,31]. Extracellular matrices derived from decellularised porcine spinal cord tissue retain tissue specific growth factors and signalling molecules which might enhance the ability of transplanted cells to differentiate into neural like phenotypes [32,33]. Recently, SCAP cells cultured within spinal cord ECM hydrogels (scECMh) showed significant expression of neural lineage markers [34].

Herein, we describe a system comprised of microparticles (<50µm) fabricated from poly (DL- lactic acid-co-glycolic acid) (PDLLGA) and triblock copolymer, PLGA- Poly (ethylene glycol) (PEG)- PLGA for regulated release of growth factors. The objective of the present study is to develop a suitable substratum using surface coated PDLLGA: PLGA-PEG-PLGA microparticles capable of providing structural support to SCAP, with controlled and tunable release of GDNF to promote cell survival and differentiation. Previously demonstrated protocols were utilised to manufacture microparticles and spinal cord ECM (scECM) [33–35]. The surface coated microparticles were characterised in terms of their size, morphology, growth factor entrapment and in vitro cell attachment.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

P_{DL}LGA polymer 50:50 (54 kDa, Lakeshore Biomaterials, Inc., USA) , dichloromethane (DCM, Fisher Chemicals, UK), pierce™ BCA Protein Assay Kit, GDNF ELISA kit and hank's balanced salt Solution (HBSS) were purchased from Thermofisher Scientific (UK), Quant-iT ™ Picogreen®assay kit, natural mouse laminin, nuclease free water and Poly D Lysine, neurobasal A, B27, glutamax , penicillin/streptomycin and proteinase K were from Life technologies (Invitrogen, UK), recombinant human GDNF (Peprotech, UK), pan neurofilaments (Covance, Brussels, Belgium, SMI-32R), anti–immunoglobulin BV 480, 4',6-diamidino-2-phenylindole (DAPI), L-glutamine, antibiotic/antimycotic were from Gemini Bio-Products (Sera Laboratories International, Bolney, UK), All other reagents were purchased from Sigma Aldrich (Poole, UK).

2.2. MICROPARTICLE PREPARATION.

Synthesis of PLGA- PEG- PLGA Triblock co polymers (TB) were performed as reported previously [35]. The polymeric microparticles (MP) were prepared using 'water in oil in water'(w/o/w) emulsion method [36]. Briefly, an aqueous solution containing molecule of interest (Human Serum Albumin or GDNF) was added to a solution of P_{DL}LGA and triblock copolymer (P_{DL}LGA–TB) in dichloromethane. The polymer solution was then subjected to high speed homogenisation for 2 minutes at 4000 rpm in an Ultra-Turrax, T25 Basic IKA homogeniser. This primary emulsion was then transferred into 0.3% w/v Poly Vinyl Alcohol (PVA) and was homogenised for a second time at 7000 rpm for 2 minutes in L5M Silverson homogeniser. The resultant emulsion was left stirring at 300 rpm to facilitate DCM evaporation and hardening of MP. The microparticles were then washed and lyophilised (ModulyoD, Thermoelectron corporation, UK) until dry.

Different weight percentages (10% $^{w}/_{w}$, 20% $^{w}/_{w}$, 30% $^{w}/_{w}$) Triblock copolymers were added P_{DL}LGA (50:50) with a total polymer mass of 1g in 5ml of Dichloromethane. The subsequent MP were referred to as P_{DL}LGA-10%TB microparticles, P_{DL}LGA-20%TB microparticles and P_{DL}LGA-30%TB microparticles, respectively. Control microparticles were prepared using 100µl of deionised water in the primary emulsion.

2.3. SALT MODIFICATION OF MICROPARTICLES

All commercial preparations of GDNF being stabilised in salt, a modified protocol was required to prepare microparticles. A mimic of commercially available GDNF was prepared via dissolving 1mg of HSA in 1ml of (i) 10mM monosodium citrate and 150mM Sodium Chloride, (ii) 10mM trisodium citrate and 150mM Sodium chloride and (iii)10mM sodium acetate solutions. All the formulations were freeze dried and stored at -20^0 C.

An aliquot of HSA- 10mM sodium acetate powder (0.5mg) was mixed with 9.5mg of HSA and dissolved in 100µl of deionised water. The aqueous solution was then added to a solution of P_{DL}LGA-20%TB in dichloromethane. These phases were homogenised using high speed homogenisation for 2 minutes at 4000 rpm in an Ultra-Turrax, T25 Basic IKA homogeniser. The primary emulsion was then added to 200ml 0.3% w/v Poly Vinyl Alcohol (PVA) and homogenised for a second time at 7000 rpm for 2 minutes in L5M Silverson homogeniser. The subsequent double emulsion was left for stirring for 4hours at 300rpm for DCM evaporation. The sodium acetate-P_{DL}LGA-20%TB microparticles, referred to as SA-P_{DL}LGA-20% TB microparticles were then washed, lyophilised and stored at -20^0 C.

2.3.1. GDNF salt replacement

The salt replacement in commercially available recombinant human GDNF was performed using Bio-Spin® Polyacrylamide P-30 columns (Bio-Rad, UK). In brief, the Bio-Spin® P 30 columns were inverted sharply to resuspend the settled gel. The tip of the column was snapped off which allowed the packing buffer to drain into a 2ml micro centrifuge tube under gravity. The column was centrifuged at 1,000x *g* for 2minutes for a complete removal of packing buffer. 10mM sodium acetate (pH 4.0) was applied to the columns in 500µl aliquots. After each application, the buffer was allowed to drain out by gravity initially, followed by centrifugation at 1,000x *g* for 2minutes. The process was repeated for three times before applying sample to the column.

The columns were equilibrated with 5ml of sodium acetate (10mM, pH 4.0) solution in 500µl aliquots and allowed to drain out by gravity. A clean 2ml micro centrifuge was placed under the column for the collection of salt replaced GDNF. An aliquot of GDNF (0.5mg) was dissolved in 100µl nuclease free water and loaded directly to the centre of the gel matrices

and centrifuged at 1000x g for 4minutes. The resultant solution was mixed with 9.5mg of HSA and lyophilised until dry.

2.3.2. GDNF microparticle preparation

The sodium acetate containing GDNF (0.5mg)-HSA (9.5mg) powder was dissolved in 100µl of deionised water to achieve a 1% $^{w}/_{w}$ loading in the microparticles. The GDNF microparticles were manufactured adapting the SA-P_{DL}LGA-20%TB microparticles described above.

2.4. MICROPARTICLE CHARACTERISATION

Fabricated microparticles (10mg/ml) were subjected to mean diameter analysis and particle size distribution using Coulter LS230 particle size analyser (Beckman, UK). The particle size distribution was determined as a function of diffraction and plotted as a function of percentage volume. Surface morphology of microparticles was examined via scanning electron microscopy. Briefly, microparticles were loaded onto carbon discs and mounted on aluminium stubs (Agar scientific, UK). The microparticles were gold- coated using Balzers SCD 178 030 gold sputter coater (Balzers Union Ltd., Leichtenstein) and imaged using JEOL-JSM 6060L V scanning electron microscope imaging system (JEOL Ltd, Hertforshire, UK) at 10kV ionisation radiation.

2.5. PROTEIN RELEASE FROM MICROPARTICLES

10mg aliquots of microparticles in triplicates were suspended in 1ml phosphate buffered saline (PBS, pH 7.4). The samples were kept in low protein binding Eppendorf tubes under humidified incubator at 37^0 C with gentle rocking movement at 5 rpm (Gyrotwister. Fisher Scientific Ltd, UK). At defined time intervals, all the PBS was collected without disturbing particles and centrifuged at 10,000rpm for 2minutes. The supernatants were collected, stored at -20^0 C and analysed for total protein content using Micro BCA assay kit following supplier instructions, with a standard curve of HSA (0-40µg/ml). 1 ml of fresh PBS was added to the collection tubes to resuspend the particles, if any, and replenished to the samples.

2.6. GDNF ELISA

The frozen GDNF released supernatant was first brought to room temperature and then quantified using Human GDNF ELISA kit (Thermoscientific, UK). Briefly, 100µl of released samples (1/1000) were added to anti-human GDNF pre-coated 96 well strip plate (Thermoscientific, UK) and incubated for 2.5 hours at room temperature with gentle shaking. The wells were washed 4 times thoroughly with 300µl of wash buffer and blotted against clean tissue paper before adding 100µl of 1x biotinylated antibody reagent. The plate was then incubated at room temperature for 1 hour with gentle shaking and washed thoroughly as mentioned earlier. A 100µl of streptavidin-HRP solution was added to the wells followed by incubation for 45 minutes at room temperature with gentle shaking. The solution was discarded and the wells were washed thoroughly before the addition of 100µl stop solution TMB substrate. The plate incubation was performed at room temperature, in the dark with gentle shaking for 30minutes. Optical density (OD) was measured at 450-550nm using a multimode microplate reader (Tecan infinite M200, Reading, UK). A standard curve was prepared using human GDNF (0-2000 pg/ml).

2.7. SURFACE MODIFICATION OF MICROPARTICLES

Previously described methods were employed to obtain scECM and bECM [34,37,38]. Aliquots (250mg) of SA-P_{DL}LGA-20%TB microparticles and were suspended in 5ml of antibiotic-antimycotic solution. The samples were agitated at 60rpm for 2 hours, followed by washing with deionised water. A solution of pepsin digested scECM (100ug/ml), bECM (100ug/ml) and mouse laminin (10ug/ml) were prepared in nuclease free water. 50 milligrams of sterilised microparticles were suspended in 5ml of different protein solutions and incubated overnight under stirring for 60 rpm at 37^0 C. The microparticles were then washed with deionised water, freeze dried (ModulyoD, Thermoelectron corporation, UK) and stored at -20^0 C.

In order to determine if surface coating could be improved, a non-polymerising plasma treatment with oxygen was performed on the surface of microparticles using automated plasma polymerisation chamber (Germany). In brief, 100 milligrams of sterilised microparticles particles in glass scintillation vials were transferred into plasma polymerisation chamber. Under close monitoring, the chamber was evacuated followed by

exposing the samples to the plasma state oxygen with a gentle rotation for 5min. The resultant surface etched samples (50mg) were immediately transferred to various protein solutions (5ml) prepared as mentioned above. The particles in protein solution were incubated for overnight at 37^0 C with a stirring speed of 60 rpm. The resultant material was washed with deionised water and lyophilised until dry (ModulyoD, Thermoelectron corporation, UK).

2.8. TIME-OF-FLIGHT SECONDARY ION MASS SPECTROSCOPY

The surface modified microparticles were loaded onto double sided sticking tape and mounted on aluminium stubs. The excess material was removed using nitrogen pump. The samples were then transferred into a flat mount stage and held in place with metal clips, prior to insertion into the instrument.

ToF-SIMS spectra were collected using a ToF-SIMS IV instrument (M€unster, Germany) equipped with an argon cluster sputter gun and a bismuth liquid metal ion gun with an energy of 25keV. Spectra of positive ions were collected over a mass range of m/z 0-800. Negative ion spectra were not collected due to lower quality of information acquired from proteinaceous surfaces. The positive ion spectra were calibrated against peaks for CH_2, CH_3, C_3H_2 before analysis.

2.9. SCAP CULTURE

The previously characterised SCAP (RP89 cells) [39] between passages 6 and 8 were used. SCAP were cultured routinely at 37^0 C with 5% CO2 in minimum essential medium (MEM-Sigma Aldrich) supplemented with 10% foetal bovine serum (Gemini Bio-products), 1% L-glutamine (Gemini Bio-products) and 1% Antibiotic/Antimycotic solution (Gemini Bio products). The cells were harvested at ~80% confluency.

2.10. SCAP ATTACHMENT AND PROLIFERATION ON MICROPARTICLES

Aliquots of surface modified/non-modified freeze dried microparticles (1mg) were suspended in 1ml of serum free MEM for 1hour at room temperature. The samples were then centrifuged at 3000rpm for 3 minutes and the media carefully removed without disturbing the microparticles. A solution of 10,000cells/ml was added to all the samples and mixed

gently. The cell-microparticle solution was transferred to 6 well cell repellent tissue culture treated plastic (TCP) (Greiner Bio-One, UK) and incubated at 37^0 C with 5% CO2 with a gentle rocking movement at 20rpm (Gyro rocker, UK) for 4 hours followed by 40 rpm for 7days (N=3, n=3). Independent samples were prepared for each time point (day 1, day 3 and day 7). A cell solution of 10,000 cells/ml without microparticles was cultured in 6 well cell repellent TCP (Greiner Bio-One, UK) and used as control.

After 1,3 and 7 days the cell-microparticle complexes were harvested and cell proliferation was quantified using Quant-iT ™ Picogreen®assay kit. In brief, cell-microparticle complex was mixed with lysis buffer composed of 10mM Tris-HCl, 100mM NaCl, 10mM EDTA, 0.5% SDS and 20µg/ml Proteinase K and incubated for 24 hours at 37^0 C. The DNA isolation from the lysate was performed by adapting a previously published method [34]. An equal volume of 25:24:1 (v:v:v) phenol/cholorofom/isoamyl alcohol was added to the cell-microparticle complex lysate, followed by by the addition of equal volume of 24:1 (v:v) cholorofom/isoamyl alcohol. DNA was extracted from the aqueous phase by salting out method using two volumes of ice-cold ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) and frozen at -20^0 C for overnight. The resultant DNA was then centrifuged at 13,000 rpm for 10 min and washed with 70% ethanol, dried at room temperature and suspended in 0.1 mL of TE buffer. The supplier instructions for Quant-iT ™ Picogreen®assay kit were followed to quantify the DNA. A standard curve was prepared with known DNA concentrations from 0 to 100 ng/mL and samples were measured for excitation:480 and emission:520nm using a Tecan Infinite M200 plate reader (Tecan, Reading, UK). A standard curve (2x 106 to 0.0019 x 106 cells) was used to correlate amount of DNA with cell number. Fold increase in cell number was calculated by dividing the number of cells at day 2, 3, or 7 by the initial number of seeded cells.

2.11. NEURITE OUTGROWTH ASSAY

Neurite outgrowth assay using murine dorsal root ganglion (DRG) cells was performed by adapting a well-documented protocol [40]. Rat pups (Sprague-Dawley) of age P3-P5 were decapitated and DRG cells were collected. The cells were then transferred to digestion media containing collagenase in HBSS (2mg/ml) and incubated at 37^0 C water bath for 30min with gentle inversion at defined time intervals. The cells were centrifuged at 20x *g* for 30sec and

supernatant was discarded. The pellet was then re suspended in neural basal media followed by mechanical trituration with a 1000µl pipette tip. The cells were further centrifuged at 10x *g* for 30sec and plated in 24 well Poly D Lysine coated TCP at a seeding density of 2000 cells/ well. The culture medium for DRG cells was neurobasal A containing supplement B27 without Vitamin A (1%), glutamax (1.3%) and pencillin/streptomycin (1%).

1mg aliquots of microparticles in triplicates were suspended in neural basal media (1ml) containing 15% BSA. The supernatant was collected after 24hours and transferred to the 24 well plates containing previously plated DRG cells. A positive control was created by adding 100ng/ml exogenous human recombinant GDNF and the cells were cultured for 5days. DRG cultured routinely in poly D Lysine coated TCP functioned as a negative control for the experiment.

2.12. FIXATION AND IMMUNOSTAINING

The DRG plates were washed with PBS followed by fixation using paraformaldehyde (4%) for 15min at room temperature. The wells were then washed twice with PBS and filled with blocking solution containing 10% BSA and 1% goat serum in PBS. The samples were incubated in blocking solution for 1h at room temperature. Immediately after blocking step, Anti-pan neurofilaments antibody (1/1000) in blocking solution was added to the samples and incubated for 1hour at room temperature. The samples were then washed twice with PBS and secondary antibody, Anti Ig BV480 (1/250) in 10% BSA solution was added. The samples were incubated at room temperature for 1hour and carefully mounted onto microscopic slides. The cell nucleus was staining using DAPI and images were acquired with an immunofluorescent microscope (Zaventam, BE).

2.13. STATISTICAL ANALYSIS

Statistical analyses were performed using PRISM (GraphPad software, CA). The Two-way ANOVA with Tukey's HSD were considered to be significantly different if $p<0.05$. Errors bars represent the standard deviation (SD) in all figures.

3. RESULTS

3.1. INFLUENCE OF TRIBLOCK COPOLYMER RATIO ON MICROPARTICLES CHARACTERISTICS.

Each of the three formulations showed spherical, smooth and non-porous surface microparticle (Appendix III, Figure 1). The mean diameter of all three P_{DL}LGA- TB microparticles with encapsulated HSA was observed to be less < 50µm. However, the entrapment efficiency of P_{DL}LGA- 30% TB microparticles was significantly lower (53.0 ± 4.1 %) compared to P_{DL}LGA- 10% TB microparticles (92.5 ± 8.4 %) and P_{DL}LGA- 20% TB microparticles (90.5 ± 6.2 %) (Table 1).

The cumulative protein release from each of the P_{DL}LGA- TB microparticle formulations is depicted in Figure 1. The incorporation of triblock co polymer modulated the initial burst and facilitated the sustained release of protein as reported previously [35,41]. An increased protein release was observed in the P_{DL}LGA- 30% TB microparticles with respect to P_{DL}LGA- 20% and 10%TB microparticles; resulting 85.67± 7.7%, 17.5± 5.9% and 4.07± 2.7% of total protein release during a period of 30 days respectively.

Analysis of average HSA daily release from each formulation demonstrated P_{DL}LGA- 20%TB microparticles had optimal release pattern reflecting GDNF release required i.e. 100ng/day.

Table 1: Size distribution and entrapment efficiency

Formulation	Mean diameter	Entrapment
P_{DL}LGA- 10% TB	15.18 ± 9.21µm^{A}	92.5 ± 8.4 %^{A}
P_{DL}LGA- 20% TB	13.19 ± 7.53µm^{A}	90.5 ± 6.2 %^{A}
P_{DL}LGA- 30% TB	25.11 ± 20.10µm^{B}	53.0 ± 4.1 %^{B}

N=2, n=3. Conditions that are not linked by the same letter as significantly different

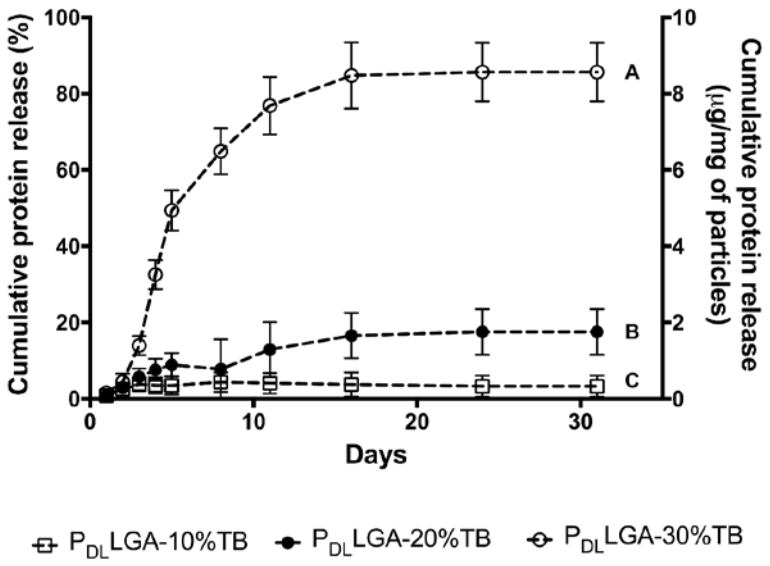

Figure 1: Influence of triblock co polymer ratio on the release profile of microparticles, Cumulative release of HSA from microparticles over 30 days. Data represents mean± SD (n=3, N=2). Conditions that are not related by the same letter are significantly different with respect to the overall protein release in each formulation ($P<0.05$).

3.2. INFLUENCE OF SODIUM ACETATE ON P_{DL}LGA-TRIBLOCK MICROPARTICLES AND SCAP ATTACHMENT

All commercially available GDNF contains salt. Thus, microparticle preparation had to be developed to incorporate commercially available GDNF and tested by mimicking the growth factor formulation using HSA containing three different combinations of salts. Microparticles encapsulated with HSA containing monosodium citrate, trisodium citrate and sodium chloride were exhibited to be distorted and porous morphology with less than 20% entrapment efficiency (Appendix III, Figure 2). However, the inclusion of sodium acetate in the microparticles did not impact morphology, entrapment efficiency or release pattern (Figure 2A, 2B, and 2C). Henceforth, all studies were done with sodium acetate-P_{DL}LGA-20%TB (SA- P_{DL}LGA-20%TB) microparticles. SCAP were allowed to grow on sodium acetate containing as well as regular P_{DL}LGA-20%TB microparticles and viability was

evaluated for 7 days. The cells attached on the surfaces of both microparticles regardless of the salt modification. The number of SCAP increased 1.4 fold in SA-P_{DL}LGA-20%TB and 1.2 fold in regular P_{DL}LGA-20%TB microparticles compared to the cells cultured on cell repellent TCP on day 1. However, a remarkable decrease in the SCAP number was observed at 3 days and 7 days post seeding (Figure 2D).

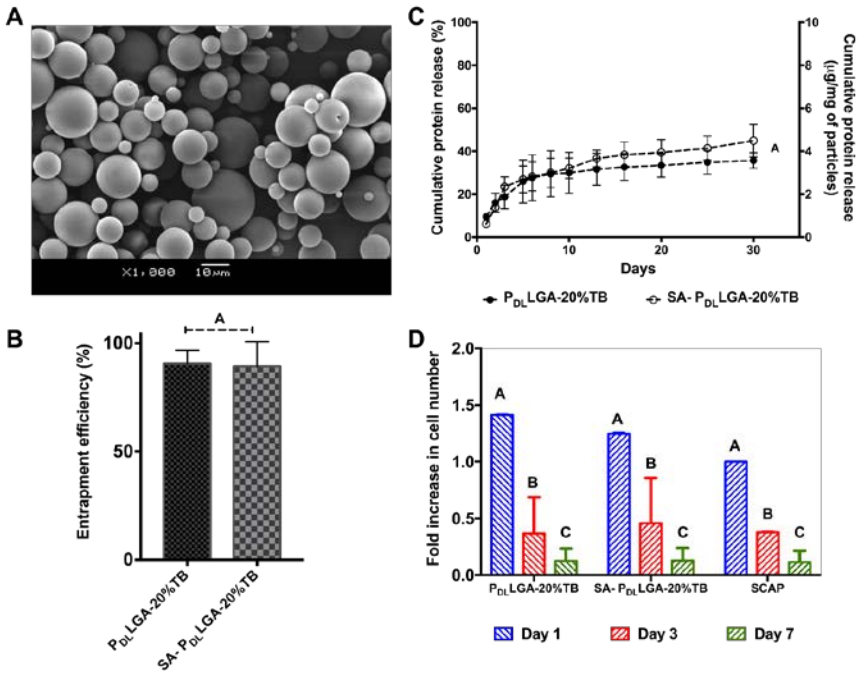

Figure 2: Influence of sodium acetate on microparticles morphology, entrapment efficiency, release profile and SCAP attachment. (A) morphology of SA-P_{DL}LGA-20%TB microparticles, (B and C) entrapment efficiency and cumulative release of HSA from P_{DL}LGA-20%TB and SA-P_{DL}LGA-20%TB microparticles, (D) SCAP attachment was analysed by culturing 10,000 SCAP/mg of microparticles at 37°C for 7 days. The cell number in each condition was normalised against SCAP seeded on cell repellent TCP after 24hours (Day 1) to compare the viability of SCAP after attachment on microparticles. Data represent mean± SD (n=3, N=3). Conditions that are not related by the same letter are significantly different ($p<0.05$).

3.3. EFFICIENCY OF SURFACE MODIFICATION ON SA-P_{DL}LGA- 20% TRIBLOCK MICROPARTICLES

Representative images of normalized ion intensities for protein adhesion on the surfaces and underlying P_{DL}LGA is depicted in Figure 3A and 3B. A significant regression was observed in P_{DL}LGA characteristics peaks including C2H3O2- (m/z 59), C3H3O2- (m/z 71) and C2H3O3- (m/z 75) on scECM and bECM coated microparticles compared to the laminin and non-coated microparticles. Correspondingly, the higher intensity of CN- (m/z 26) and CNO- (m/z 41.99) ions representing peptide backbone in proteins reflected the increased adherence of ECM proteins on the surface of microparticles. An enhanced and homogenous adherence of scECM and bECM proteins was observed in the positive ion intensity map compared to the laminin coated microparticles (Figure 3C)

Although, non-polymerising plasma treatment with oxygen was undertaken in order to activate the surfaces of microparticles to improve protein adhesion; no significant enhancement in the ion intensities was observed (Appendix III, Figure 3A and 3B). However, an improved but heterogeneous adherence of laminin on plasma treated microparticle surfaces was observed in the positive ion intensity map (Appendix III, Figure 3C).

3.4. SCAP PROLIFERATION ON DIFFERENT SURFACE MODIFIED SA-P_{DL}LGA-20% TRIBLOCK MICROPARTICLES

Regardless of the type of surface modification, SCAP proliferated when cultured on the SA-P_{DL}LGA- Triblock microparticles (Figure 3D). The number of SCAP increased between 0.6 - 1 fold on 1-day post seeding, 0.2 fold - 0.7 fold on 3 days post seeding and 0.07 fold – 1.2 fold on 7 days post seeding compared the initial seeding density. On day 1 post seeding, a significant difference was observed in SCAP proliferation on scECM coated microparticles with respect to the water treated and SCAP cultured alone. No significant difference was observed in SCAP proliferation on laminin and bECM coated microparticles with respect to other conditions on day 1 post seeding. No significant difference occurred in SCAP proliferation on scECM, bECM, laminin and water coated microparticles after 3-day post seeding. However, SCAP cultured alone in cell repellent TCP demonstrated a significant reduction with respect to SCAP cultured on surface modified microparticles on 3 and 7-day post seeding. After 7-day post seeding, SCAP cultured on laminin and bECM coated

microparticles showed a significant increase in cell number with respect to the scECM and water treated microparticles. Whereas, no significant increase was observed in SCAP cultured on scECM and water treated microparticles between day 3 and day 7.

3.5. EVALUATION OF GDNF RELEASE AND BIOLOGICAL ACTIVITY

The HSA-GDNF loaded SA-P_{DL}LGA- 20%TB microparticles exhibited controlled release of protein (Figure 4A). An increased GDNF release during the burst phase, with 7% of total protein was observed after 24 hours and 80% of total protein was released over a period of 15 days.

Murine DRG cells, which possess a high sensitivity to neurotrophins, were used to assess the biological activity of released GDNF. When cultured routinely for 5 days, the DRG cells did not show neurite growth (Figure 4B). Whereas, DRG cells cultured with exogenous GDNF (100ng/ml) as well as 24h released GDNF displayed extensive neurite outgrowth (Figure 4C, 4D). Although, DRG cell population treated with released GDNF exhibited neurite extension, some cells displayed round morphology without extensions. The cell nucleus of each cells was labelled with DAPI and the neurites were stained against pan- neurofilaments [42]

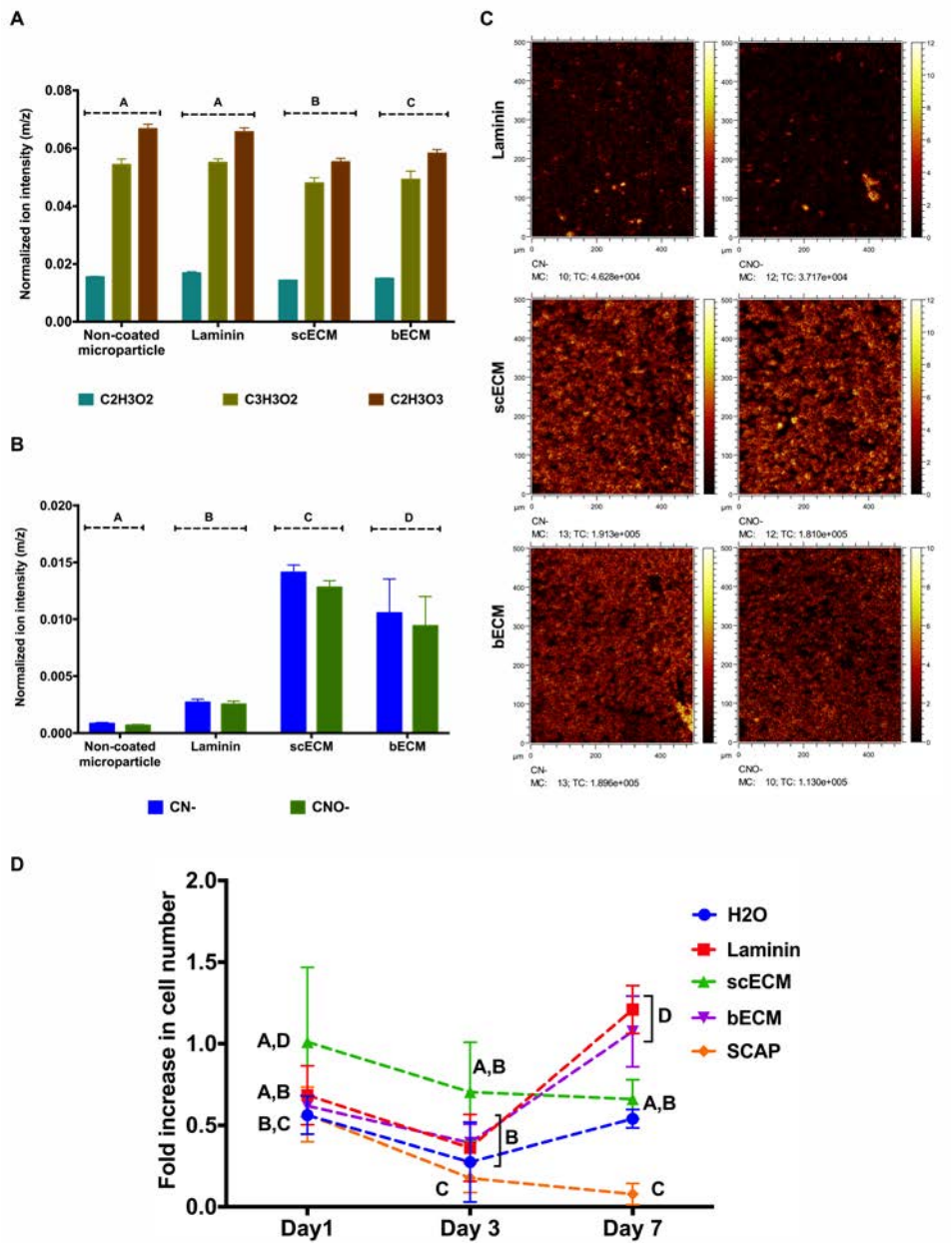

Figure 3: Influence of microparticle surface modification with ECM and laminin. (A) Normalised ion intensity (m/z) for PLGA, (B) Normalised ion intensity (m/z) for peptide backbone of proteins and (C) Positive ion intensity map of peptide backbone showed an homogenous scECM and bECM adhesion (D) SCAP proliferation after the attachment on various surface coated microparticles over 7 days. Data represents mean± SD (n=3,N=3). Conditions that are not related by the same letter are significantly different, ($P<0.05$)

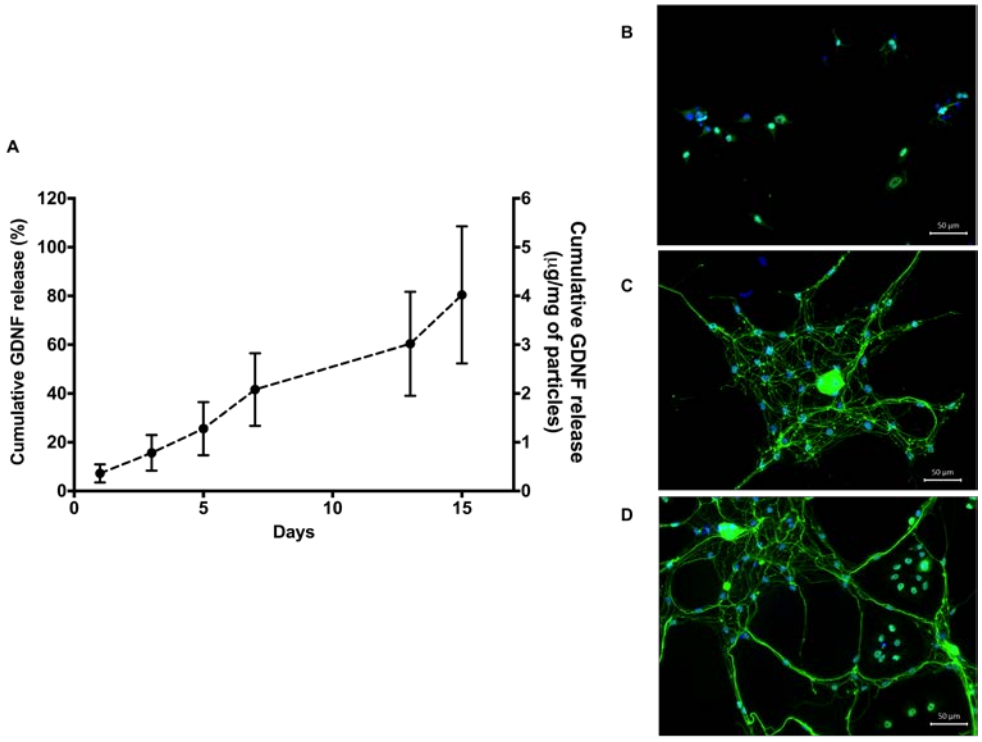

Figure 4: Representative images of GDNF release profile and biological activity of GDNF. (A) Cumulative release of GDNF from SA-20%TB PLGA microparticles over 15 days. (B) murine dorsal root ganglion (DRG) cells cultured in the absence of GDNF, (C) DRG cells treated with exogenous GDNF (100ng/ml), (D) DRG cells treated with GDNF released after 24 hours. The cells were cultured for 5 days and stained against pan- neuronal neurofilaments (green) and nucleus (blue). (N=2, n=3, $P<0.05$)

4. DISCUSSION

Although many polymeric scaffolds and delivery vehicles are capable of exhibiting a spatiotemporal controlled release of biomolecules, providing a structural support for cells along with a sustained release of protein/growth factor of interest for a constructive tissue repair remains a challenge [43]. The present study illustrated the development of P_{DL}LGA: triblock copolymer microparticles as a constructive substratum for dental stem cells from apical papillae (SCAP) and providing appropriate release of GDNF and to promote cell survival and proliferation for spinal cord tissue repair.

It has been demonstrated previously that inclusion of a hydrophilic-hydrophobic block copolymer to protein delivery systems can modulate the release kinetics by influencing the polymer degradation rate [44]. PLGA-PEG-PLGA co-polymers have been shown to enforce rapid swelling rate and structural erosion to create a hydrogel-like nature in microparticles facilitating controlled release of proteins and growth factors [45]. Moreover, poly-(ethylene glycol) (PEG) is non-toxic, FDA approved hydrophilic polymer that is known for its stabilising effect on proteins [46].

In the present study, the incorporation of different weight percentages of PLGA-PEG-PLGA triblock co polymers did not affect the morphology and size of the resultant microparticles considerably. However, the protein release profile of the three formulations showed a significant difference in the amount of HSA release, confirming the influence of triblock copolymers as release modifier. For P_{DL}LGA- 30% TB, a quick release of encapsulated HSA was observed over a period of 30 days. A moderate and sustained release of HSA was observed in P_{DL}LGA- 20%TB and a very slow but controlled release of protein was seen in P_{DL}LGA- 10% TB. The release profile of P_{DL}LGA- TB microparticles illustrated herein is in line with the previous studies [35]. However, White et al. suggested a higher amount of protein release through triblock incorporated P_{DL}LGA microparticles. We assume that the difference in molecular weight of P_{DL}LGA and triblock copolymers has influenced the concentration of protein release from the microparticles [45].

The potential impact of salts on PLGA microparticles has been utilised by several researchers to successfully create porous scaffolds for tissue engineering [47–49]. Nevertheless, it is noteworthy that presence of zinc salts such as zinc oxide, zinc carbonate and zinc acetate has

enhanced the encapsulation efficiency and maintained the secondary structure of therapeutic proteins like insulin [50]. The present study depicted a porous and distorted morphology of microparticles when HSA was encapsulated with salt combinations such as monosodium citrate and sodium chloride or trisodium citrate and sodium chloride. Also, the entrapment efficiency of the formulations were 19.6± 6.0% for P_{DL}LGA-20%TB-monosodium citrate/NaCl and 12.9± 8.4% for P_{DL}LGA-20%TB-trisodium citrate/NaCl salt combinations (Appendix III, Figure 2). On the other hand, inclusion of 10mM sodium acetate to P_{DL}LGA-20%TB microparticles demonstrated a smooth and round morphology with an entrapment efficiency of 89.3± 11.4% for HSA.

The addition of sodium acetate did not appear to affect SCAP attachment as SCAP attached to the surface of P_{DL}LGA- 20% TB and SA- P_{DL}LGA- 20% TB microparticles. An increase in the SCAP was observed in both salt containing and regular P_{DL}LGA- 20% TB microparticles on day 1 compared to the SCAP cultured alone in cell repellent TCP. However, there was no remarkable proliferation observed between SCAPs grown on microparticles and in cell repellent TCP on 3 and 7 days post seeding. We assumed that a scaffold-like nature in the microparticles created by the swelling and structural erosion of triblock copolymer might have led to the initial SCAP adhesion [46]. We also hypothesised that the smooth surfaces of P_{DL}LGA- 20%TB/ SA- P_{DL}LGA- 20% TB microparticles and lack of functional cell-adhesive ligands could be non-optimal for prolonged support of SCAP [51–53]. Surface modification of microparticles using proteins such as fibronectin and laminin to facilitate cell attachment has been demonstrated previously [27,53]. In the current study, the microparticles were subjected to surface adsorption of laminin, scECM, bECM and water. ToF SIMS was used to analyse the surface of protein-coated microparticles. The difference in the amino acid ion intensity of peptide backbone in conjunction with PLGA characteristic ions demonstrated an increased adsorption of scECM and bECM to the microparticles compared to that observed with laminin. Also, the positive ion intensity maps for scECM, bECM coated microparticles demonstrated an improved as well as homogenous adherence of scECM and bECM proteins on microparticle surfaces compared to laminin-coated microparticles (Figure 3C).

Although, the individual amino acid fragments of spectra can be identified, the complexity of proteinaceous surfaces interpreted through ToF-SIMS spectra makes it difficult to identify

differences between scECM, bECM and laminin. Interestingly, a non-polymerising plasma treatment using oxygen was carried out as an extra measure to improve the surface adhesion of protein [27], but failed to enhance the peptide backbone ion intensities for protein-coated surfaces. It has been demonstrated previously that the extracellular matrices retain the proteins and active biomolecules including collagen, glycosaminoglycans, bFGF, NGF, VEGF after decellularisation [37,54]. Since decellularised ECM materials contain a cocktail of proteins rather than a single protein, we postulate that the difference in the adsorption is due to the variance in protein concentration present in the scECM, bECM and laminin.

SCAP attached and proliferated for 7-day post seeding upon all surface modified microparticles. The decline in the proliferation observed in SCAP grown on cell repellent TCP illustrated the need for suitable substrates for cell support. Microparticles coated with scECM demonstrated a considerably different initial SCAP proliferation rate with respect to bECM and water treated microparticles. However, there was a significant increase in the SCAP proliferation was observed in laminin as well as bECM coated microparticles after 7 days. It has been demonstrated previously that laminin influences cell adhesion, survival and enhances neurite growth [54,55]. Likewise, hydrogels derived from decellularised scECM and bECM have been shown to support SCAP viability and proliferation [34]. Cell attachment on non-adhesive polymers functionalised with precisely spaced adhesion peptides demonstrated the enhanced sensitivity of cells to inter-ligand spacing which in turn influenced successful cell adhesion and migration [56]. We hypothesised that SCAP attachment and proliferation were influenced by the distribution of protein ligands in scECM, bECM and laminin on microparticles. We also postulate that the water treatment on the surface of microparticles might degrade them to produce a scaffold-like structure facilitating cell adhesion [45].

A good entrapment efficiency and retention of protein bioactivity were observed in the present study. It is noteworthy that the biological activity of GDNF was preserved during encapsulation and release as illustrated by the extended and interconnected neurite growth in small diameter DRG neurons. This is likely due to the inclusion of HSA into the formulation as it prevented the loss of GDNF secondary structure at the water/organic solvent interface. It has been previously demonstrated that HSA competes for the interfacial site and effectively

keep the therapeutic proteins out of the interface during emulsion. Thus, avoid interfacial protein aggregation and conformational rearrangement of proteins [57,58].

5. CONCLUSION

Smooth, spherical and non-porous SA- P_{DL}LGA-20% TB microparticles capable of delivering a controlled and sustained release of GDNF were prepared. The surfaces of microparticles were coated with water, laminin and decellularised materials including scECM and bECM in order to facilitate SCAP adhesion and proliferation. Although, the SCAP proliferated in all the modified surfaces, laminin and bECM coated surfaces promoted a significant increase in the proliferation after 7 days. The GDNF released from the microparticles was shown to retain its biological activity and stimulated neurite growth in DRG cells. The results of the present study provide a promising system to deliver cells and growth factors for spinal cord regeneration.

APPENDIX II

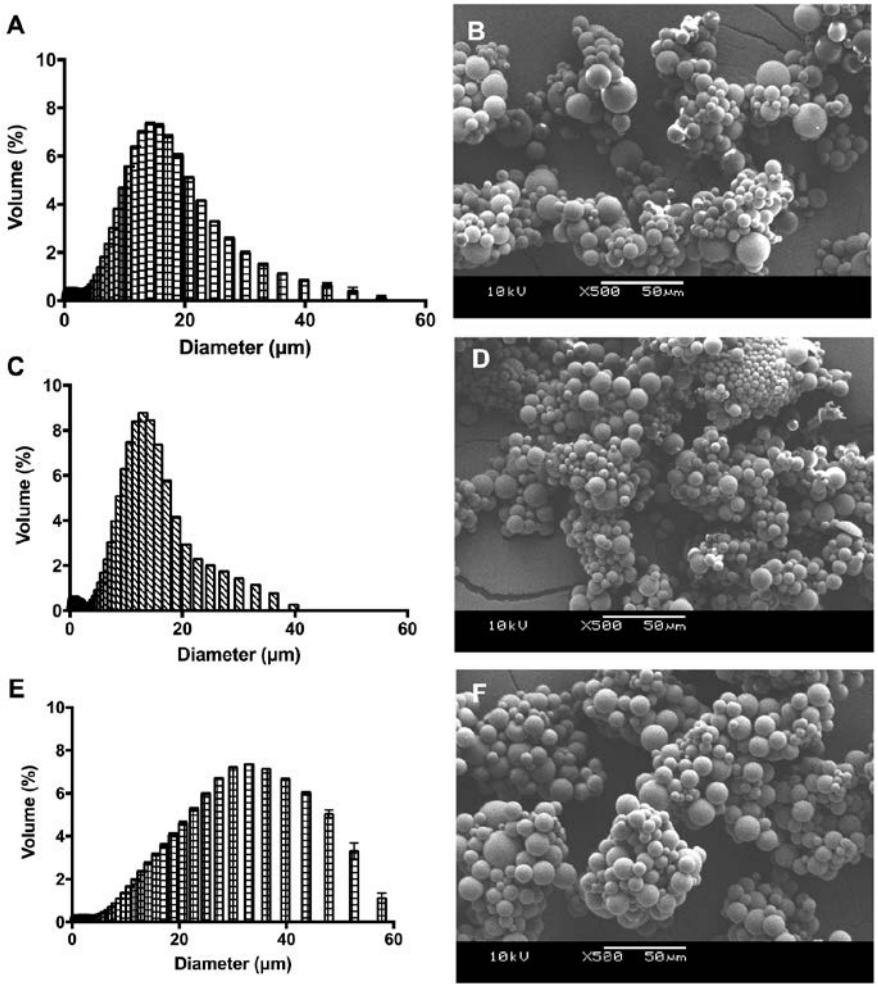

Figure 1: Influence of triblock *co*-polymer ratio on the size and morphology. Particle size distributions and SEM of P_{DL}LGA microparticles with 10% TB (A and B), 20% TB (C and D) and 30% TB (E and F). The addition of triblock co polymer increased the hygroscopic nature of the microparticles and caused aggregation as seen in the SEM images. Data represent mean± SD (n=3).

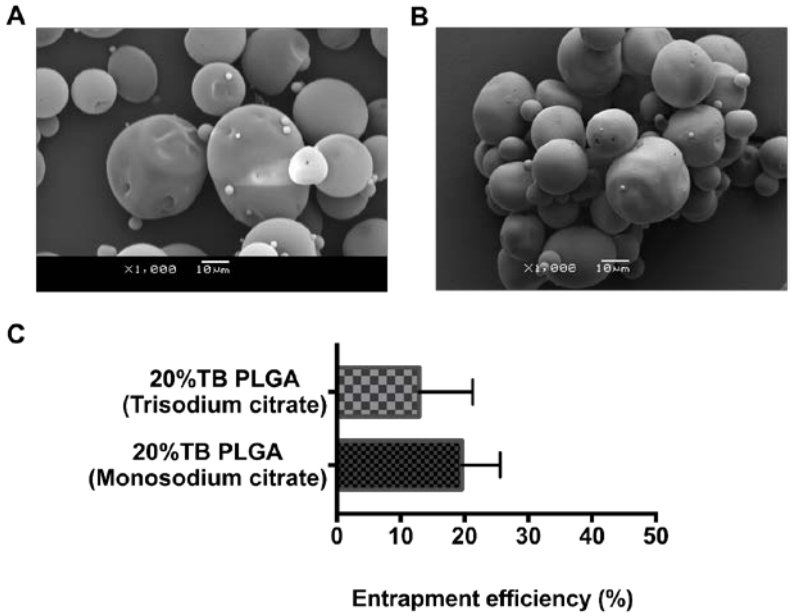

Figure 2: Influence of salt and pH on microparticles. Distorted and porous morphology was observed for P_{DL}LGA with 20% TB microparticles containing (A) 10mM monosodium citrate/150mM NaCl of pH 3.8, (B) 10mM Trisodium citrate/150mM NaCl of pH 7.3. (C) The entrapment efficiency of the formulations was observed to be less than 20%. There was no significant difference in the entrapment efficiency of the two formulations. Data represent mean± SD (n=3).

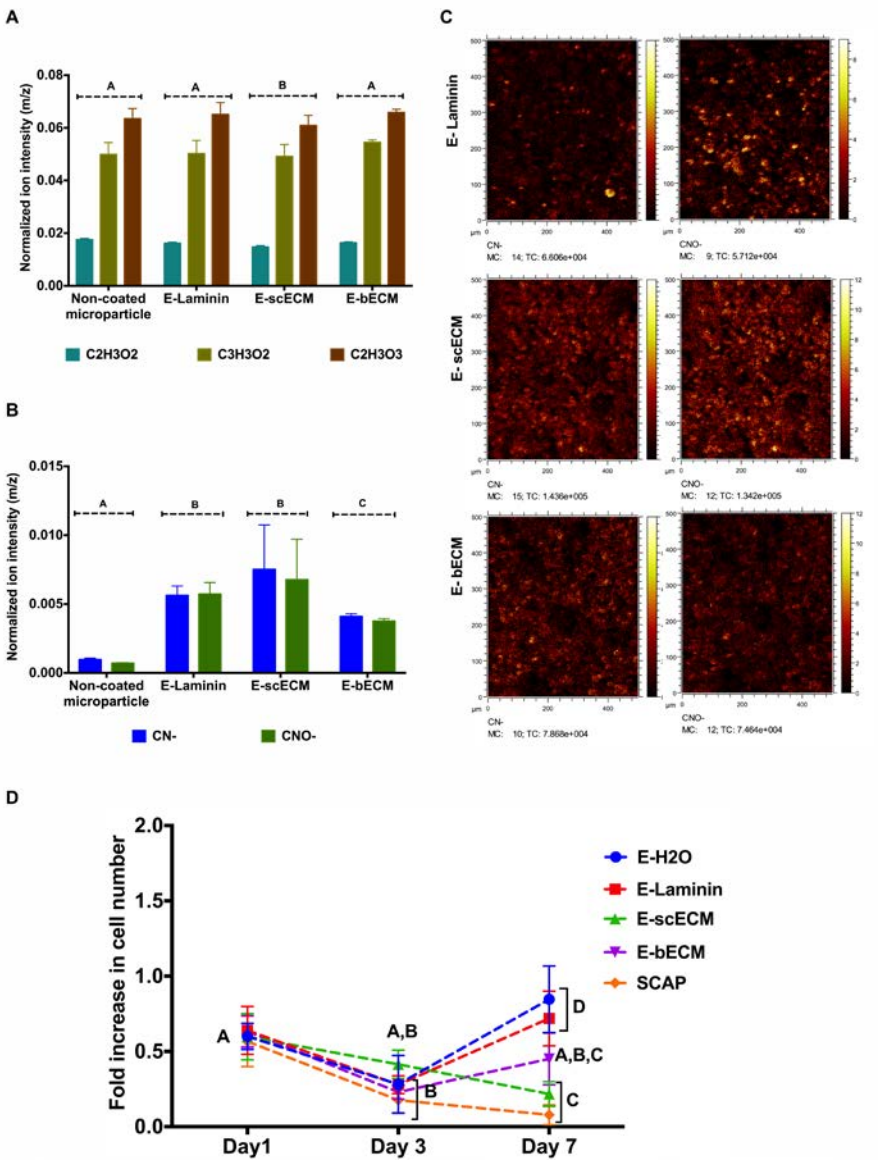

Figure 3: Influence of microparticles coating after surface etching. (A) Normalised ion intensity (m/z) of underlying $P_{DL}LGA$, (B) Normalised intensity (m/z) of ions representing peptide backbone of proteins, (C) Positive ion intensity map of peptide backbone showed an improved but heterogeneous laminin adhesion after surface etching and (D) SCAP behaviour after attachment on various etched-surface coated microparticles over 7 days. Data represent mean± SD (n=3). Conditions not linked by same letter are significantly different ($p<0.05$).

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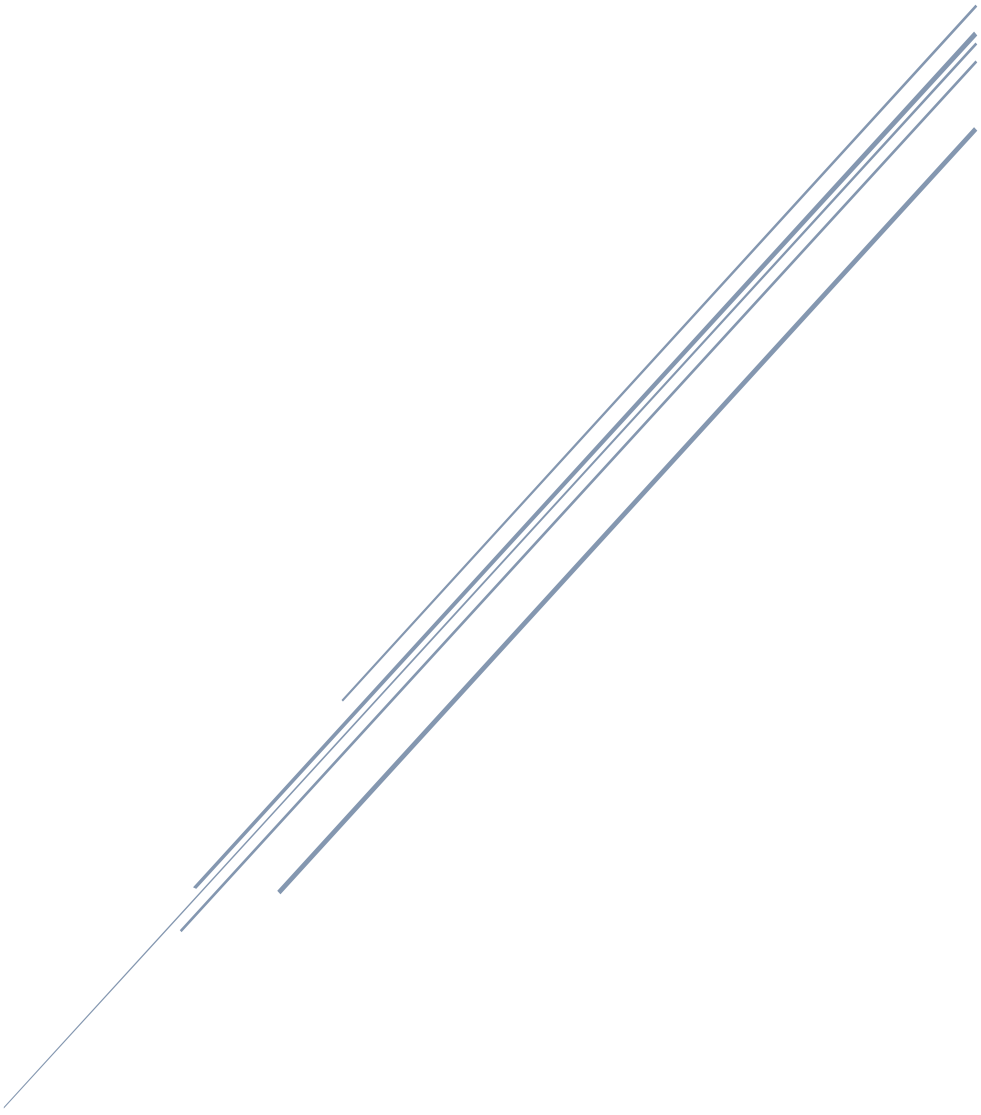
CHAPTER IV.

DISCUSSION AND FUTURE PERSPECTIVES



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1. PRIME ACHIEVEMENTS OF THE THESIS

As highlighted in the introduction (Chapter I), a single treatment will not be sufficient to curb the complex cascade of events occurring after spinal cord injury. Thus a combination strategy of biomaterial-based stem cell transplantation to stimulate the endogenous tissue repair process is imperative for the development of current and future therapies. Dental stem cells (DSC), originating from neural crest, are accessible and highly expandable. They are a promising source of autologous stem cells for transplantation. Additionally, uniting these stem cells with a biomaterial platform and a compelling growth factor may benefit the repair of complex tissue defects like spinal cord injury in humans. We hypothesised that combining DSC with decellularised mammalian tissue derived ECM hydrogels (ECM hydrogels, ECMh) or PLGA microparticles loaded with GDNF would support cell survival and neuroprotection at the lesion site.

The present work has evaluated the potential of dental stem cells from apical papillae embedded in distinct injectable matrices to sustain the endogenous repair activities of the spinal cord. The initial part of the work focused on developing different ECMh (Chapter II) for encapsulation of SCAP. This resulted in significant cell-material integration whereby SCAP exhibited a satisfactory survival, metabolic activity, cell number expansion and expression of key neural lineage markers. The remainder of the thesis dealt with the fabrication of P_{DL}LGA -triblock copolymer based microparticles (Chapter III) to provide controlled release of GDNF and SCAP attachment. To achieve a conventional microenvironment that preserves exogenous GDNF bioactivity, the P_{DL}LGA -triblock microparticles were modified with 10mM sodium acetate, leading to a successful encapsulation and sustained release of bioactive GDNF from the delivery system. Furthermore, surface coating of the P_{DL}LGA -triblock microparticles using different ECM pre-gel and laminin improved the SCAP attachment, survival and influenced their cell number increase. This work contributed to the *in vitro* information regarding stem cell based neuroprotection and remyelination for SCI repair. To the extent of our knowledge, this is the first time SCAP has been combined with ECM hydrogels and surface/salt modified GDNF-P_{DL}LGA microparticles to investigate SCAP cell behaviours that might be beneficial for SCI therapy.

The major contributions of this thesis are: (i) production and characterisation of three potent ECM hydrogels, including one novel hydrogel from decellularised dentin tissue, (ii) salt modified PLGA-triblock microparticles to provide spatiotemporal release of bioactive GDNF, (iii) surface coating of P_{DL}LGA -triblock microparticles for enhanced stem cell adhesion as well as survival, and (iv) two desirable combinations of SCAP and biomaterials that have potential as cell replacement and/or to modify the lesion microenvironment-following transplantation into an injured spinal cord.

In conclusion, the thesis demonstrated the ability of ECM hydrogels to improve as well as sustain the encapsulated SCAP survival and expansion. Furthermore, the hydrogels scECMh and bECMh upregulated the specific genes for neural lineage in SCAP. Similarly, the ECM pre-gel and laminin coated microparticles containing sodium acetate supported extended SCAP adhesion and expansion. Additionally, the microparticles have exhibited their ability to support the release of biologically active GDNF. Therefore, taking all the information presented in this dissertation together, it can be said that, the thesis demonstrated the plausibility of SCAP combined with ECM hydrogels or GDNF-P_{DL}LGA-20% TB microparticles as a reliable autologous stem cell treatment for promoting spinal cord repair and warrants further research.

2. OPEN-ENDED DISCUSSIONS AND FORETHOUGHTS

The results presented in the thesis warrant further investigation. Among the several questions or aspects to be discussed, some are described below which reflect my personal opinion supported by available literature.

2.1. IMPROVEMENT IN THE DECELLULARISATION PROTOCOLS: METHOD MODIFICATION

It is worthy to note that all the cell-removal factors and/or processes induce some extent of disruption in the ECM ultrastructure and composition [1]. In this dissertation work, different decellularisation protocols were used and optimised for production of spinal cord, bone and dentine ECM based on the individual tissue properties. Although the decellularisation protocols adopted for this thesis were the most feasible to acquire ECM with minimal structural disruption from the particular tissue sources, the decellularisation agents such as

Trypsin-EDTA, concentrated HCl and Triton-100 can potentially damage the ECM protein ultrastructure [1–5]. Therefore, future investigations should focus on eliminating and/or limiting the tissue exposure to the factors mentioned above to achieve ECM ultrastructure akin to the native tissue.

Apart from trypsin, the enzymes that are widely investigated in tissue decellularisation process are nucleases, e.g. collagenase, thermolysin, lipase, dispase and α-galactosidase [1]. However, collagenase affects the collagen retention and preservation of ECM ultrastructure significantly, and others are insufficient to act alone to produce an acceptable degree of decellularisation [6–9]. Pepsin is known to cleave conservative telopeptides regions from collagen in xenogeneic tissues without altering the collagen helical structure, making them less immunogenic for clinical use [10]. Studies have demonstrated that pepsin in combination with the low concentration of HCl can effectively decellularise the tissues hence it can be considered instead of trypsin [4]. However, the much lesser cleavage specificity of pepsin compared to trypsin can cause high fragmentation of proteins which in turn may affect the hydrogel formation [11]. Treatment of trypsin at pressurised chamber has reduced the time required to lyse the cells; leaving a better preserved ECM structure [3].

EDTA-containing solution can be a mild demineralisation agent and was shown to preserve growth factors and ECM ultrastructure in both bone and tooth [12,13]. The demineralisation of dental tissues with 4.3% EDTA showed a satisfactory preservation of the fine structures of the matrix. However, it requires a moderately long incubation time to achieve complete demineralisation [12,14]. A solution to shorten incubation times could be to increase the temperature to 42 ^{0}C, resulting in the acceleration of 0.1M EDTA-mediated demineralization process of teeth [14]. Therefore, the ECM disruption during demineralization and enamel removal can be minimised by adopting either EDTA or a lower concentration of HCl. Tissue treatment with supercritical CO_2 is a potential decellularisation technique that avoids the use of enzymes and surfactants [4]. The critical temperature (31.1 ^{0}C) and pressure (7.40Mpa) of CO_2 are approved for biological applications [15]. Furthermore, owing to the diffusivity of the gas, it can be readily released from the tissue without leaving remnants, unlike surfactants [15]. An experimental study using supercritical CO_2 with ethanol as an entrainer at gentle extraction condition of 15Mpa and 37 ^{0}C, exhibited satisfactory removal of cellular nuclei from the aortic tissue [16]. The treatment preserved the collagen and elastin content [16].

However, further investigation is required to evaluate the impact of this process on the abilities of the ECM based material to stimulate recellularisation and tissue remodelling applications.

2.2. LIMITATIONS OF THE THESIS

Although this research has been carefully designed, there are still certain limitations and shortcomings. One of the major limitations was the incomplete analysis of the ECM components following decellularisation procedure. The effort to examine the principal components using Liquid Chromatography-Mass spectrometry (LC-MS) was unsuccessful due to the detergent traces in the sample and multiple cleavages of the ECM proteins by enzymes including trypsin and pepsin. Several researchers have successfully demonstrated the reasonableness ToF-SIMS for surface characterisation of tissue or cell derived ECMs, exploring the cell-ECM interaction through cell secreted matrices (CSM) and surface molecular functionality of decellularised extracellular matrices [17–21]. Therefore, for future investigations, it would be really interesting to apply techniques such as ToF SIMS or cryosectioning of ECM hydrogels followed by specific antibody staining methods to decipher the molecules within decellularised ECM.

Another limitation was the complication to extract mRNA from the SCAP-biomaterial construct that limited the gene expression profiling using qPCR. Interestingly, the results of an investigation focused on RNA extraction from cells incorporated in PEG hydrogels demonstrated that the acidic conditions of TRIzol® (pH 4.5) can potentially accelerate the degradation of PEG gel and the resultant components affect the RNA stability in concentration dependent manner [22]. It has been shown that a stable cationic surfactant like cetyltrimethylammonium bromide (CTAB) could help to extract mRNA with significantly higher quantity and purity compared to the guanidinium thiocyanate-based methods (TRIzol®) alone [23–25]. Furthermore, treatment of CTAB in a basic pH has shown to reduce the formation of polysaccharide-nucleic acid complexes resulting in improved RNA quality from cells embedded in the hydrogels [24,25]. Recently, an investigation comparing a diverse range of RNA extraction procedures to yield high-quality RNA from human MSC grown within various hydrogels demonstrated a clear upper hand for protocol comprising micro homogenisation/enzymatic degradation using CTAB and silica membrane [24].

Taken together, future work in this direction should focus on an immediate RNA extraction using a combination of methods such as micro homogenisation followed by enzymatic degradation of the construct by CTAB treatment and finally concentration using commercially available silica membrane columns to acquire better quality mRNA for gene expression profiling.

2.3. FURTHER EVALUATION OF INJECTABLE MATRICES, SCAP AND PRE-CLINICAL DEVELOPMENT

Apart from the mechanical properties, intact growth factors that have resisted to the decellularisation procedure can contribute to the differentiation potential of ECM hydrogels. The decellularised spinal cord ECM tends to retain growth factors such as VEGF and bFGF and has the potential to promote neovascularization [26]. Furthermore, Crapo et al. demonstrated that spinal cord ECM induced a higher chemotactic response by embedded neural stem cells and promoted the neuronal differentiation, compared to brain ECM and Urinary bladder ECM within the tested gel concentration range [27]. Likewise, the deeper understanding of stem cell biology, cell-based therapies and bio-engineering have highlighted the potential of cell-secretome components as curative agents for tissue repair [28]. Taking into account of the crucial role of biomolecules in intracellular communications, it could be interesting for future investigators to explore the various intrinsic growth factors and SCAP secretome after incorporation into injectable matrices.

A variety of animal models of different species (e.g. rodents, felines and primates) with different lesion models at different locations of the spinal cord with varying severities have been used for SCI research [29]. It has been demonstrated previously that transplantation of DSC to pre-clinical SCI animal models induced positive outcomes on functional recovery [30,31]. Sakai et al. have reported that human stem cells from human exfoliated deciduous teeth (SHEDs) grafted into a completely transected spinal cord resulted in significant recovery of hind limb locomotor functions by inhibition of neural apoptosis and differentiation into mature oligodendrocytes [31]. Importantly, transplantation of whole papilla, the natural niche of SCAP, into a hemisection rat model has shown significant improvement in the recovery of hindlimb locomotor function [30]. Taken together, it would be of a real interest to test the SCAP-biomaterial combination strategy described in the

present work on *in-vivo* SCI animal models. It was shown that a better simulation of the biomechanics and neuropathology of human injury can be achieved in contusion and compression models [32]. Therefore, for future pre-clinical evaluations, the contusion/compression SCI animal models should be favoured.

3. FUTURE PERSPECTIVES

3.1. OTHER POTENTIAL CELL TYPES CAN BE ENCAPSULATED IN THE ECM HYDROGELS AND DELIVERED FOR SPINAL CORD REPAIR

As addressed in Chapter I (Section 4), many stem cell sources and types have been investigated for their regenerative potential in the pre-clinical, and recently, in the clinical settings of SCI. Among these cells, bone marrow stem cells (BMSC), dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), neural stem cells (NSC), embryonic stem cells (ES), Schwann cells (SC), olfactory ensheathing cells (OECs), activated macrophages and induced pluripotent stem cells (iPSC) have been tested [33–35]. Additionally, studies have asserted better cell behaviour and functional recovery, at least in part, when the cells mentioned above were transplanted at the lesion site through various natural and synthetic polymer derived biomaterials, functionalised with specific ECM molecules or synthetic peptide sequences from laminin and fibronectin [36,37].

In the present thesis, SCAP encapsulation within bECMh (non-homologous tissue ECM), and scECMh (site-specific tissue ECM) supported cell survival, expansion and expressed gene specific for neural lineage without specific exogenous induction. Previously, CNS tissue derived ECM bioscaffolds have been shown to retain the neurotrophic potential of the tissues of origin and supported excellent in vitro 3D neurite extension as well as preferred mitogenic effects, chemotactic effects, and differential differentiation of model neural-like cells [26,38]. The information mentioned above reinforces the idea that the inherent capability of ECM hydrogels, particularly, the scECMh, would support the recruitment of the cell populations referred earlier, and promote the preferred cell behaviours including synthesis of de novo ECM, beneficial for constructive spinal cord repair.

3.2. OTHER GROWTH MOLECULES, DRUGS AND BIOMOLECULES CAN BE ENCAPSULATED IN P_{DL}LGA-TB MICROPARTICLES

In addition to GDNF, the other most successful contributors to trophic support and neurogenesis include BDNF, NGF, NT-3, NT-4/5, ciliary neurotrophic factor (CNTF) and FGF, platelet-derived growth factor (PDGF) and Insulin-like growth factor-1 (IGF-1) [39]. Similarly, experimental investigations have described neuroprotective effects of cytokines and pharmacological agents such as IL-1 α and β, IL-6, TNF-α, erythropoietin, rolipram and riluzole [37,40]. Anti-growth inhibitory molecules including ChABC (degrades CSPG) and anti-myelin associated inhibitors (e.g., cethrin®, C3 ribosyltransferase, NEP1-40 and Y-27632) are shown to mitigate the chronic injury phase of SCI and increase locomotor recovery in vivo [41]. In the present investigation, P_{DL}LGA-TB microparticles exhibited spatio-temporal release of encapsulated GDNF in its bioactive form. It is noteworthy that the excipients such as PEG, HSA and sodium acetate in the polymeric microparticles have shown to subdue the likelihoods of protein inactivation during the manufacturing process, storage within the polymeric microenvironment and release [42–44]. Therefore, the P_{DL}LGA-TB microparticles maintained the structure and bioactivity of the biomolecules and pharmaceutical agents as specified earlier while providing sustained release at the site of trauma. Delivery of a combination of biologically active substances can be significantly more efficient in curbing the progression of secondary injury cascade, compared to single treatment after SCI [41]. Hence, the future investigations in this direction should consider exploring the possibilities of blending three separate P_{DL}LGA-TB microparticle formulations that would possess distinct release profiles for delivering an assorted range of bioactive molecules.

3.3. COMBINE SCAP, ECM HYDROGELS AND GDNF- P_{DL}LGA MICROPARTICLES FOR SPINAL CORD REPAIR

Considering the multifaceted nature of SCI, researchers have explored the potential of various combinatorial strategies to stimulate the SCI repair process. In an empirical study, Kim et al. investigated the effectiveness of embedding dibutyryl cyclic-AMP (dbcAMP) loaded PLGA microspheres, and NSPC seeded fibrin scaffolds within chitosan guidance channel [45]. When cultured in the guidance channel where microspheres provided the only

source of dbcAMP, the NSPCs preferentially differentiated into neurones in vitro [45]. Therefore, considering the promising results observed in the present thesis and the possible superior effects of a multicomponent system on SCI repair as mentioned above, an early effort has been made to analyse the combinatorial implications of the ECM hydrogels and GDNF- P_{DL}LGA -TB microparticles for delivering SCAP. The rheology data of microparticles incorporated in ECM hydrogels (Appendix III- Figure 1A) demonstrated a sigmoidal gelation similar to the original ECM hydrogels. However, a decrease in the final storage moduli and longer gelation time of microparticle- ECM hydrogel combination compared to the original ECM hydrogel indicated the possible interference of microparticles in the gelation process. Regarding SCAP response, we have used two formulations: i) scECM coated GDNF-P_{DL}LGA-TB microparticles and scECM coated P_{DL}LGA-TB microparticles embedded in scECMh (referred as scGDNF MPs-scECMh and Blank MPs-scECMh). The combinatorial system supported SCAP viability and metabolic activity (Appendix III-Figure 1B) for two weeks post encapsulation. Importantly, a significant increase in the metabolic activity of encapsulated SCAP was observed at day 14 in both combinations with the highest activity seen for scGDNF MPs-scECMh. We thus hypothesised that the significant increase of SCAP metabolic activity in scGDNF MPs-scECMh might be attributed to the combined influence of GDNF and ECM hydrogels.

In the light of these promising preliminary experimental results, future investigators are strongly encouraged to explore the effect of the microparticles and ECM hydrogels combinatorial strategy for delivering bioactive molecules and cells. Furthermore, researchers should also consider examining the cell secretome following the 3D culture of SCAP in ECM hydrogels, P_{DL}LGA-TB microparticle and the combination system to understand the changes in cell fate before proceeding towards preclinical experiments.

APPENDIX III

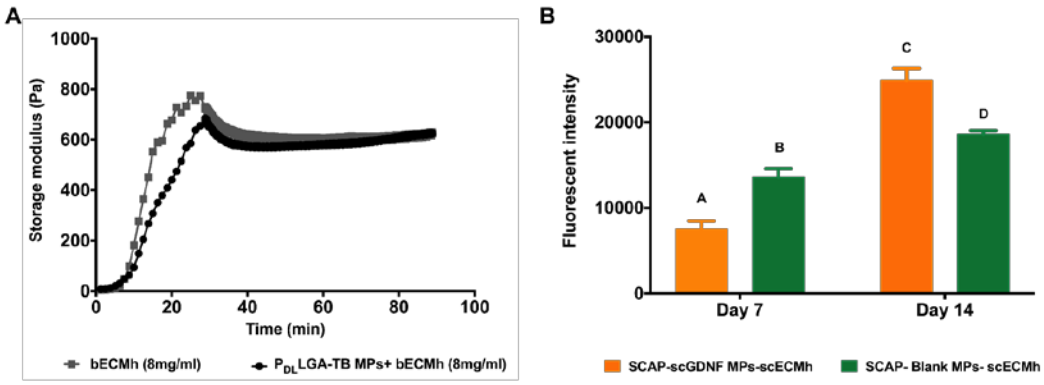

Figure 1: Rheology of combinatorial strategy and influence on SCAP metabolic activity. A) The storage modulus of 1mg P_{DL}LGA -TB microparticle blended with bECMh (8mg/ml) against original bECMh (8mg/ml) was investigated by small-amplitude oscillatory shear rheology using Physica MCR 301 rheometer (Anton Paar, Hertford, UK); following the protocol as described in Chapter III (section II.6). **B)** 250,000 cells were cultured on 2mg of both with and without GDNF loaded scECM coated P_{DL}LGA-TB microparticles for 24 hours. The cell-microparticle complex was then incorporated into 200µl scECM hydrogels and incubated at routine cell culture condition. Metabolic activity of the cells was analysed at day 7 and 14 post-encapsulation, using Presto blue assay kit (n=3). Conditions not related by the same letter are significantly different ($p<0.05$).

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