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Review Article

Localised non-viral delivery of nucleic acids for nerve regeneration in injured nervous systems

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ABSTRACT

Axons damaged by traumatic injuries are often unable to spontaneously regenerate in the adult central nervous system (CNS). Although the peripheral nervous system (PNS) has some regenerative capacity, its ability to regrow remains limited across large lesion gaps due to scar tissue formation. Nucleic acid therapy holds the potential of improving regeneration by enhancing the intrinsic growth ability of neurons and overcoming the inhibitory environment that prevents neurite outgrowth. Nucleic acids modulate gene expression by over-expression of neuronal growth factor or silencing growth-inhibitory molecules. Although *in vitro* outcomes appear promising, the lack of efficient non-viral nucleic acid delivery methods to the nervous system has limited the application of nucleic acid therapeutics to patients. Here, we review the recent development of efficient non-viral nucleic acid delivery platforms, as applied to the nervous system, including the transfection vectors and carriers used, as well as matrices and scaffolds that are currently used. Additionally, we will discuss possible improvements for localised nucleic acid delivery.

1. Introduction

Treatment options targeted at stimulating nerve regeneration after injuries remain limited. Hence, continuous elucidation of the molecular mechanisms that underlie such poor nerve regeneration has driven the development of treatment strategies that aim at reversing neuropathologies at the molecular level. Correspondingly, treatments with nucleic acid therapeutics have emerged as a promising approach since it addresses the molecular causes of hindered nerve regeneration by manipulating gene expression profiles in targeted cells within the nervous system. In general, there are two main nucleic acid-based therapeutic approaches – gene therapy and gene silencing (Tinsley and Eriksson, 2004; Blits and Bunge, 2006; Bowers et al., 1997). Gene therapy for nerve regeneration is typically accomplished by introducing genes that encode for neurotrophic growth factors or corrective enzymes to injured neurons. Both pathological and functional outcomes have been observed through the use of such strategies in animal models (Hong et al., 2006; Choi-Lundberg et al., 1998; Franich et al., 2008). On the other hand, the implementation of gene silencing methods, such as RNA interference (RNAi), has witnessed the reduction in toxic protein expression levels and the minimization of growth inhibitory signals at nerve injury sites (Forte et al., 2005; Davidson and Paulson, 2004; Rodriguez-Lebron and Gonzalez-Alegre, 2006).

Nucleic acid-based therapy has seen significant advancement in various tissue repair applications, ranging from wound healing in skin (Chandler et al., 2000a; Tyrone et al., 2000; Doukas et al., 2001; Chandler et al., 2000b), to bone regeneration (Fang et al., 1996; Bonadio et al., 1999), muscle repair (Doukas et al., 2002) and optic nerve repair (Berry et al., 2001). However, due to the extreme difficulty in transfecting mature post mitotic neurons with genetic materials (Karra and Dahm, 2010), it has become crucial to use highly efficient gene vectors and carriers for effective transfection to occur within the nervous system. Hence, nucleic acids packaged in viral vectors such as the adeno-associated virus or herpes simplex virus remain the leading candidate for neuron-targeted gene therapy as they have high transfection efficiencies and enable long-term gene expression, which brings about functional recovery in various animal model (Costantini et al., 1999; Kay et al., 2000; Kordower et al., 2000; Naldini et al., 1996). However, significant safety issues and complications have also arisen out of such viral delivery strategies. In particular, viral vectors often lead to unwanted immune responses, increased risk of insertional mutagenesis, and face difficulties in storage, which are critical problems

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that limit their clinical applications (Baum et al., 2003; Li et al., 2002; Kafri et al., 1998; Thomas et al., 2001). Even though viral vectors can be altered to remove viral components that trigger the immune response, the modified viruses are often difficult to produce with substantial yield and efficacy (Daya and Berns, 2008; Samulski and Muzyczka, 2014). On the other hand, non-viral nucleic acid delivery strategies offer improved safety profiles and are promising alternatives. Unfortunately, the limited transfection efficiencies of non-viral delivery platforms must first be addressed before achieving functional nerve regeneration outcomes.

In order to improve the transfection efficiency of non-viral nucleic acid delivery platforms, it is crucial to understand the molecular structures of these nucleic acids while dwelling into the recent strategies that have been employed by the neural tissue engineering field in order to deliver these molecules non-virally. Therefore, this review will begin by looking into various types of therapeutic nucleic acids that have been used in tissue engineering. Following that, we will highlight the available delivery and transfection methodologies that are specific to neurons. We will also discuss the application of these methods to promote nerve regeneration in the injured nervous systems. Finally, we will focus on the delivery of nucleic acids via scaffolds to achieve localized and sustained therapeutic outcomes. Design considerations for better control over the delivery and uptake of nucleic acids by injured neurons will be discussed as future strategies to enhance nerve regeneration by nucleic acid therapeutics.

2. Platforms for non-viral delivery of nucleic acids and their applications in the nervous system

Nucleic acids have been used to enhance or inhibit gene expression at transcription and post-transcriptional levels to direct tissue regrowth (Yan, 2004). The use of these nucleic acids has been explored in many tissue regeneration approaches, such as bone (Kasper et al., 2006; Itaka et al., 2007; Elangovan et al., 2014), skin (Thomas-Virnig et al., 2009; He et al., 2012; Guo et al., 2011), ligaments and tendons (Goh et al., 2003), cartilage (Odabas et al., 2013; Lu et al., 2013), cardiac (Marsano et al., 2013) and hepatic tissues (Chien et al., 2015). On the contrary, such use of nucleic acid-based therapeutics is significantly less reported for nerve regeneration. This phenomenon may be attributed to the challenges in neuronal cell transfection. The central nervous system (CNS) is protected by a barrier system that is composed of tight vascular junctions and glial elements, which forms the blood-brain barrier that prevents the access of therapeutics (Barchet and Amiji, 2009; Banks, 2016). Besides that, non-viral nucleic acid delivery systems should be designed to target and transfect specific neuronal populations while ensuring that the nucleic acids bind to these cells before being washed out of the nervous system (Hanz and Fainzilber, 2004; Von Bartheld, 2004; Syková and Nicholson, 2008). Furthermore, the design of an efficient non-viral delivery platform is dependent on the type of nucleic acid used.

An extensive literature search revealed that therapeutic nucleic acids can be broadly categorized by either gene overexpression or gene silencing. Plasmids and messenger RNAs (mRNAs) are the two most commonly used nucleic acids for overexpressing genes (Morgan et al., 1987; Deodato et al., 2002; Liechty et al., 1999; Lin et al., 2006) while antisense oligonucleotides (AS ODNs) (Liang and Pan, 2002; Shohami et al., 2000), short interfering RNAs (siRNAs) (Low et al., 2015; Rungta et al., 2013) and microRNAs (miRs) (Kosik, 2006; Khudayberdiev et al., 2009)are most commonly involved in gene silencing for neural tissue engineering. Notably, the mechanisms of how these nucleic acids modulate gene expression are different. Hence, delivery considerations will vary from one type of nucleic acid to another. An overview of these nucleic acids, along with some of the important properties that should be considered when designing non-viral platforms for the delivery of therapeutic nucleic acids to the nervous system are listed in Tables 1A and 1B.

2.1. In vitro studies on transfection of neurons

Stimulating the intrinsic growth ability of neurons is crucial to achieve the desired regeneration outcomes after injuries in the nervous system. Nucleic acid therapeutics have emerged as promising approaches since they can potentially be used to downregulate growth inhibitory molecules (eg. Nogo, OMgp and MAG) or upregulate growth promoting factors. However, the application of nucleic acid therapy, through non-viral delivery methods, on neurons requires rigorous optimisation since neurons are especially sensitive to physical stress, temperature alterations, pH shifts and changes in osmolarity(Karra and Dahm, 2010). Despite these constraints, numerous non-viral methods of gene delivery, such as chemical transfection, electrical transfection and physical transfection have been established to deliver nucleic acids to neurons *in vitro* with impressive outcomes(Wiesenhofer and Humpel, 2000; Malamas et al., 2013; Jiang and Chen, 2006). Table 2 provides an overview of studies that have been carried out on neuronal cell transfection, including the transfection approaches, vectors used and their respective transfection efficiencies.

2.2. Chemical transfection methods

2.2.1. Calcium-phosphate/DNA co-precipitation

Calcium phosphate transfection remains a convenient and economical method for transfecting foreign genes into neurons. Specifically, transfection is performed by mixing calcium chloride with recombinant DNA in a phosphate buffer and allowing the formation of DNA/calcium phosphate precipitates. These precipitates are then added into the cell culture medium and administered to the cells, where they are then endocytosed and shuttled into the nucleus(Karra and Dahm, 2010). Notably, this method can be applied to neurons at all stages of its cell cycle, including those that have already formed neuronal networks (Sun et al., 2013). Generally, the transfection efficiency obtained using calcium phosphate as the carrier ranged between 0.5-5%(Alavian et al., 2011). However, with further optimisations, it is possible to reach 50% transfection efficiency(Sun et al., 2013).

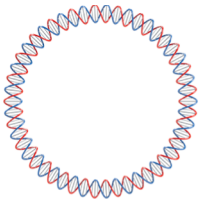
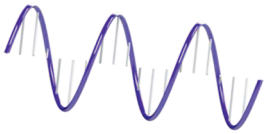
2.2.2. Lipofection

Lipid-mediated gene delivery platforms work through the effects of cationic lipid molecules. These lipid molecules contain a positively charged head group, which can interact with the negatively charged nucleic acids to form complexes. The lipid-nucleic acid complexes can then fuse with the cell membrane(Sirna, 2004), and deliver the nucleic acids into the cells effectively. To further facilitate the fusion of the complexes with the cell membrane, the cationic lipid molecules are often combined with a neutral co-lipid (helper lipid). Besides being used for transfecting large nucleic acids (i.e. DNA and mRNA), lipid-based vectors have also been utilized for the delivery of small oligonucleotides due to their high transfection efficiencies.

An example of cationic lipofection reagent that works well in neuron cultures is Lipofectamine® RNAiMAX(Han et al., 2015). For this transfection reagent, the transfection efficiency was found to be affected by the culture medium as well as the volume ratio between the transfection reagent and the nucleic acids. By simply using Neurobasal-A instead of DMEM for transfecting miR-21 into cortical neurons, higher amounts of miR-21 could be detected within the cells. Furthermore, the transfection efficiency peaked when the volume ratio of Lipofectamine® RNAiMAX:miR-21 was 3:5(Han et al., 2015).

Similar to Lipofectamine® RNAiMAX that is commonly used to deliver siRNA and miRNA, Lipofectamine® 2000 is another cationic lipid reagent that is widely utilized for the delivery of nucleic acids with larger number of base pairs (i.e. DNA, mRNA)(Scientific, 2013). When Lipofectamine® 2000 was utilized for the delivery of mRNAs into DRG neurons, a transfection efficiency as high as 25% was observed (based on EGFP mRNA transfection)(Williams et al., 2010). Besides that, further analysis validating the expression of several heterologous proteins

Table 1A
A summary of therapeutic nucleic acids for gene overexpression and some design considerations for development of non-viral delivery systems.

Gene overexpression		
Properties	***Plasmids***	***mRNAs***
Structure	 Several kilo base pairs Double stranded DNA constructs	 Long single stranded RNA up to 130 nucleotides in length
Charge	Negatively charged due to phosphate backbone	
Place of action	Nucleus	Cytoplasm
Duration of gene regulation	Long-term or permanent depending on site of integration within host genome	Transient
Transfection barriers	Cell membrane and nuclear membrane	Cell membrane
References	[56],[57]	[58–63]

namely, a cannabinoid receptor (CB1R), a G protein inwardly rectifying K+ channel (GIRK4) and a dominant-negative G protein α-subunit mutant, suggested successful mRNA transfection (Williams et al., 2010). As an improved version of Lipofectamine 2000, Lipofectamine 3000 has also been widely used for neuronal cell transfection(Han et al., 2017; Chen et al., 2015; Zhu et al., 2016). However, these studies did not report their transfection efficiency. As such, it is difficult to directly compare with other delivery vectors.

Overall, attributing to the ease of use, lipid-based carriers have been widely utilized for the delivery of both large (i.e. plasmid DNA, mRNA) and small nucleic acids (i.e. siRNA, miRNA) (Kaech et al., 1996; Tonges et al., 2006) *in vitro*. Additionally, liposomes do not induce strong toxicity and are highly reproducible when used for transfecting various neurons(Han et al., 2015; Tonges et al., 2006). Compared to viral delivery methods, there is also a lower risk of mutation and immune-related issues(Zhdanov et al., 2002). Expanding from the success of *in vitro* neuronal cell transfection, lipid-based vectors have also been widely used for *in vivo* studies, as highlighted in the subsequent section.

2.3. Electrical transfection methods

Electroporation is a technique that enables the cellular plasma membrane to be transiently permeable to its surrounding materials and was shown to work well for both embryos and dissociated neurons(Dib-Hajj et al., 2009). By exposing neurons to a voltage pulse, the nucleic acids can then enter the cytoplasm via the pores that were formed in the cell membrane(Washbourne and McAllister, 2002). Generally, the transfection efficiency of neurons through electroporation is relatively low (0.5–3%)(Dib-Hajj et al., 2009). However, higher transfection efficiency can be achieved by sacrificing cell viability(Dib-Hajj et al., 2009).

Buchser *et al.* used electroporation to transfect primary mouse cerebellar granule neurons (CGNs) and rat hippocampus neurons(Buchser et al., 2006). According to them, increasing voltages gave higher transfection efficiency while resulting in lower cell viability. Moreover, a calcium-free intracellular buffer(Majoul et al., 2001) provided significantly better transfection efficiency than standard extracellular buffers or media. With the necessary optimizations, the average transfection efficiency of mouse CGNs and hippocampal neurons reached up to ~ 26.8% ± 8.6% and ~ 17.3% ± 3.2%, respectively, as evaluated by GFP expression changes(Buchser et al., 2006).

A novel micropipette electroporation technique was developed by Haas *et al.* for single cell transfection(Haas et al., 2001). Single-cell transfection enables the individual monitoring of genetic changes in a specific cell. This technique allows for genetic changes to be made in a specific single neuron, which is suitable for studying single cell behaviour during live cell imaging. Single-cell electroporation is applicable for delivering both large plasmid DNAs(Haas et al., 2001) and small oligonucleotides(Boudes et al., 2008a).

The transfection efficiency of single-cell electroporation was affected by various factors, including pulse shape, the number of pulses delivered and the voltage amplitude. However, the limitation of electroporation is the requirement of a large number of neurons to survive the electrical pulse. In general, high cell density facilitates the transfection outcomes as the firm cell-cell attachment prevents cell death. On the contrary, insufficient cells for electroporation caused higher cell death rate and unhealthy surviving cells. As compared to lipofection, electroporation was more commonly reported for the delivery of large plasmid DNA.

2.4. Physical transfection methods

2.4.1. Microinjection

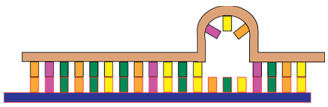
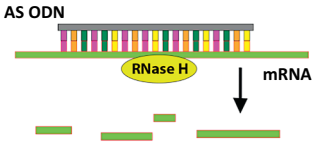
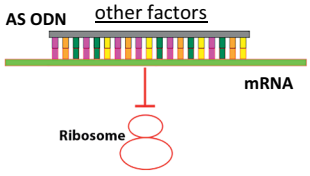
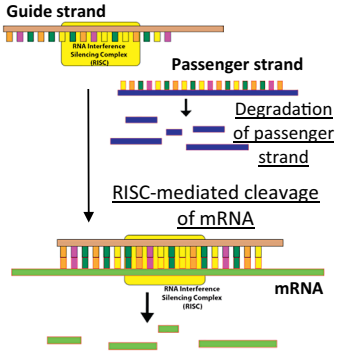
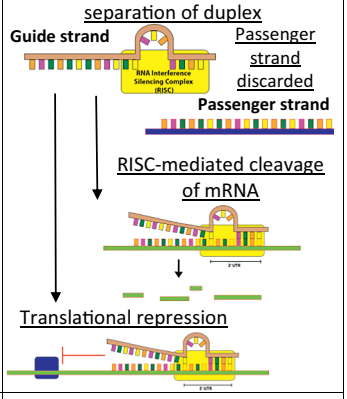
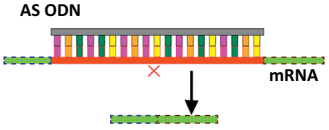
Although electroporation or chemical-mediated transfection have been widely utilized for neuronal cell transfection, the post-mitotic nature of primary neurons somehow prevents effective protein expression(Lu et al., 2009). Hence, intranuclear injections may serve as an alternative. In particular, nucleic acids can be injected into the cytoplasm or cell nucleus with fine glass capillaries, during which substantial pressure is applied to disrupt the cell plasma membrane. However, one of the main disadvantages of this approach is the low cell survival rate. Thus, this method may be more suitable for transfecting more robust neurons, such as invertebrate neurons(Karra and Dahm, 2010). In addition, in dividing neuronal cell lines, such as PC12, the injected material is often diluted during cell division, hence resulting in the loss of effects of the injected substance(Zhang and Yu, 2008).

Despite the drawbacks, microinjection provides substantial advantages. In theory, the transfection efficiency is 100%. As compared to traditional transfection or infection, single-cell microinjection allows targeted transfection of pre-defined cells of interest within a mixed culture. Although microinjection is not as efficient as other transfection methods as it needs to be done cell by cell, the delivery dosage and delivery location can be precisely controlled.

2.4.2. Biolistics (Gene gun)

Biolistic transfection is based on the injection of subcellular-sized

Table 1B
A summary of therapeutic nucleic acids for gene silencing and some design considerations for development of non-viral delivery systems.

Gene silencing			
Properties	***AS ODNs***	***siRNAs***	***miRs***
Structure	 15 to 20 nucleotides 6 to 10 kDa Single stranded DNA	 21 to 23 nucleotides 12 to 13.3 kDa Duplex RNA strand with 3' overhangs	 21 to 25 nucleotides 14 to 15 kDa Duplex RNA strand with interspersed mismatches and 3' overhangs
Charge	Negatively charged due to phosphate backbone		
Place of action	Cytoplasm		
Mechanism of gene regulation	Recruiting protein factors such as RNase H  Steric blocking of ribosomes and other factors  	Binding of siRNA to RISC facilitates separation of duplex  	Binding of miR to RISC facilitates separation of duplex 
	Modulate mRNA splicing  		
Complementary to mRNA	Completely complementary to mRNA	Completely complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region (UTR) of mRNA
Number of mRNA targets	One	One	Multiple
Duration of gene regulation	Transient		
Transfection barriers	Cell membrane		
References	[64–67]	[68–72]	[72–77]

particles that are coated with DNA into the cells(Dunaevsky, 2013). This method is applicable to tissues, cells and organelles, and can be used both *in vitro* and *in vivo*. In general, three major steps are needed to inject the DNA into cells/tissues: (i) coating the particle with DNA, (ii) transferring the coated particles into a cartridge, and (iii) firing the DNA-coated microcarriers into cells/tissues using a pulse of helium gas (O'Brien and Lummis, 2006).The transfection efficiency in brain slices using a gene gun can reach around 30%(O'Brien and Lummis, 2006). Up to now, only a few reports are available where successful biolistic gene has been transferred into neurons or neuronal tissues(Wirth and Wahle, 2003; Wellmann et al., 1999). Biolistic transfection can overcome physical barriers such as the stratum corneum of the epidermis. It also allows multiple transfection with more than one type of DNA within the same sample(O'Brien et al., 2001; Jiao et al., 1993). However, the major drawbacks of biolistic transfection method are high cell death and high cost of equipment, although the consumable costs thereafter are relatively low(O'Brien and Lummis, 2006).

Taken together, lipofection is the most commonly used method for the transfection of neuronal cells due to its high transfection efficiency and low cytotoxicity. As compared to electrical and physical transfection methods, lipofection is more applicable for transfecting a large number of neurons in one go. On the other hand, for single cell studies, single-cell electroporation and microinjection are more appealing due to their transfection accuracy. However, these methods are recommended for the transfection of robust neurons (i.e. PC12 and invertebrate neurons) as the electrical and mechanical stimuli could jeopardize cell viability. Altogether, several factors such as neuronal cell types and their survival rates should be taken into consideration before deciding on the transfection approach.

Although a plethora of transfection methods have been established, efficient transfection of post-mitotic cells, such as mammalian neurons, remains a challenging task. While numerous studies are exploring efficient platforms for transfecting neurons, most of these studies focused on the evaluation of the transfection efficiencies. Hence, the biological

Table 2
An overview of different transfection methods on neurons *in vitro*.

Delivery methods	Nucleic acids	Vectors	Target neurons	Max. transfection efficiency achieved	Amount of nucleic acids used	Reference
Calcium-phosphate/DNA co-precipitation	Plasmid encoding EGFP	Calcium phosphate	Hippocampal neurons	50%	1-4 μg of plasmid DNA	(Sun et al., 2013)
	Plasmid encoding Bcl-xL	Calcium phosphate	Hippocampal neurons	1-5%	Not mentioned	(Alavian et al., 2011)
	Plasmid encoding GFP	Calcium phosphate	Hippocampal neurons	13.4%	3-5 μg of plasmid DNA	(Köhrmann et al., 1999)
Lipofection	Plasmid encoding GFP	Lipofectamine 2000	Cortical neurons	8%	400 ng of plasmid DNA	(Halterman et al., 2009)
	Plasmid encoding NGF	Lipofectamine	PC12 cell	3-4%	1 μg plasmid encoding NGF gene	(Zou et al., 1999)
	Plasmid encoding β-gal	DOTAP	Hippocampal neurons	3%	1.5 μg of plasmid DNA	(Kaech et al., 1996)
	Plasmid encoding β-gal	FuGene	Primary Mesencephalic Neurons	12.7%	255 ng of plasmid DNA	(Wiesenhofer and Humpel, 2000)
	pCMV-EGFP	M9-assisted lipofectamine	Embryonic rat hippocampal neurons	25%	1 μg of plasmid DNA	(Ma et al., 2002)
	mRNA	Lipofectamine 2000	Neurosphere	40-50%	50-100 ng	(McLenachan et al., 2013)
	mRNA	Lipofectamine 2000	DRG neurons	25%	50-1000 ng of mRNA	(Williams et al., 2010)
	siRNA	Cyclodextrins	Hypothalamic neurons	45%	50 nM of siRNA	(O'Mahony et al., 2012)
	siRNA	Lipofectamine, Stearyl-R8 and AVPs	Hippocampal neurons	83%,73% and 75%, respectively	10 pmol of siRNA	(Tonges et al., 2006)
	siRNA	Octaarginine-PEG-lipid	Hypothalamic neurons	20%	50-100 nM of siRNA	(O'Mahony et al., 2013)
	siRNA	PEG-PEI	Neural stem cells	86.05 ± 5.22%	80 pmol of siRNA	(Liang et al., 2012)
	miR-21	Lipofectamine RNAiMAX	Cortical neurons	38.70%	600-700 ng of miR-21	(Han et al., 2015)
	Plasmid encoding GFP	Electroporation	Mouse cerebellar granule neurons	26.8% ± 8.6%	1-5 μg of plasmid cDNA	**(Buchser et al., 2006)**
	Plasmid encoding GFP	Electroporation	Hippocampus neurons	17.3% ± 3.2%	1-5 μg of plasmid cDNA	**(Buchser et al., 2006)**
	plasmid encoding EGFP	Electroporation	Brain slices	30%	0.25-2 μg of plasmid DNA	(Haas et al., 2001)
Electroporation	Plasmid encoding EGFP	Electroporation	DRG neurons	15-20%	1-2 μg of plasmid DNA	(Dib-Hajj et al., 2009)
	mRNA	Electroporation	Neurosphere	60-70%	50-100 ng	**(McLenachan et al., 2013)**
	Dextran-fluorescein/ siRNA	Electroporation	DRG neurons	59 ± 1.2%	2.4-2.8 μg of siRNA	(Boudes et al., 2008b)
	miR-124	Electroporation	Neural stem cells	50-60%	5 g miR mimic	(Jiang et al., 2016)
Biolistic	Plasmid encoding EGFP	Gene gun	Brain slices	30%	Not mentioned	(O'Brien and Lummis, 2006)
	Plasmid encoding BDNF/ NT-4	Gene gun	Rat visual cortex	0.6–0.8%	30 μg of plasmid DNA	(Wirth and Wahle, 2003)
	DNA/gold particles	Gene gun	Cerebellar granule cells/ Hippocampal neurons	10%	1 μg DNA/mg of gold particles	(Wellmann et al., 1999)

Table 3
Non-viral delivery of nucleic acids for nerve system repair.

Injuries	Delivery methods	Therapeutic nucleic acids	Delivery vectors	Therapeutic outcomes	Amount of nucleic acids used	Reference
CNS	Injection	Plasmid DNA	Polyethylenimine (PEI)	Increase transgene expression	2-40 μg of plasmid DNA	(Shi et al., 2003)
	Injection	Plasmid DNA	PEI PEGlyation	Decrease apoptosis	4 μg of plasmid DNA	(Shi et al., 2003)
	Nose instillation	Plasmid encoding RFP	Chitosan and PEI-coated magnetic micelles	Vectors enter the brain parenchyma	10 μg of plasmid DNA	(Das et al., 2014)
	Injection	Plasmid encoding Bcl-2	Lipofectamine	Reduce atrophy and loss of neurons	3~75 μg/25 μg of plasmid DNA	(Takahashi et al., 1999)(Shibata et al., 2000)
	Injection	Plasmid encoding GDNF	DC-Chol-liposomes	CST regeneration and function recovery	Not mentioned	(Lu et al., 2002)
	Scaffold implantation	Plasmid DNA	Lipofectamine	Significant transgene expression	800 μg of plasmid DNA	(De Laporte et al., 2009b)
	Scaffold implantation	Plasmid encoding NT-3	PEGylated DMAEMA	Promote robust axonal regeneration	Not mentioned	(Yao et al., 2012)
	Scaffold implantation	Plasmid DNA	Transfast	Transgene expression lasted for 3 weeks	3 μg of plasmid DNA	(De Laporte et al., 2009a)
	Scaffold implantation	Plasmids encoding NT-3	PEI PEGylation	Improved axonal regeneration and functional recovery	1 μg of plasmid DNA	(Yao et al., 2013)
	Injection	mRNA	Polyplex nanomicelle	Decrease immune responses	2 μg of mRNA	(Uchida et al., 2013)
	Injection	siRhoA	PgP	Promote axon regeneration and decrease apoptosis	20 μg of siRNA	(Gwak et al., 2017)
	Injection	siRNA	Hiperfect	Promote axon regeneration	0.5 μg of siRNA	(Singh et al., 2014)
	Scaffold implantation	miR-222	TKO	Promote nerve regeneration	48 μg of microRNA	(Nguyen et al., 2017b)
	Cell transplantation	NT-3 overexpressed OEGs	liposomes	Promote nerve regeneration and hindlimb functional recovery	Not mentioned	(Wu et al., 2008)
	Cell transplantation	E-cadherin overexpressed NSCs	SuperFect	Induce differentiation of NSCs into neurons	5 μg	(Zhang et al., 2014)
	Cell transplantation	BDNF overexpressed MSCs	PAsp(DET)	Promote the recovery of motor function	12 μg	(Uchida et al., 2016)
	Cell transplantation	NRG1 overexpressed SCs	FuGene6	Promote neuroprotective and anti-apoptotic effects	3 μg	(Zhang et al., 2010)
PNS	Injection	Plasmid encoding VEGF	Up vector plus electroporation	Promote DRG neurons survival and nerve regeneration	50 μg of plasmid DNA	(Pereira Lopes et al., 2011)
	Injection	Plasmid encoding VEGF and G-CSF	Up vector plus electroporation	Promote motor function, nerve regeneration and blood vessel reformation	50 μg of plasmid DNA	(Pereira Lopes et al., 2013)
	Scaffold implantation	Plasmid encoding FGF-2	Metafectene™	The recovery of sensory and motor function	Not mentioned	(Haastert et al., 2006)
	Scaffold implantation	Plasmid encoding VEGF	Lipofectamine 2000	Promote nerve regeneration and blood vessel reformation	Not mentioned	(Kempton et al., 2009)
	Cell transplantation	Gene modified SCs	pLVTHM	Potential in stimulating nerve regeneration	Not mentioned	(Shakhbazau et al., 2014)

outcomes that may be induced by functional nucleic acids remain to be evaluated. Therefore, future evaluations of the functionalities of the transfected neurons are required.

2.5. In vivo studies

Although several *in vitro* transfection methods have been explored and optimised for neuronal transfection, not all approaches are applicable for *in vivo* utilization. Among the transfection methods discussed above, chemical transfection is the most commonly used approach for *in vivo* studies due to their high transfection efficiency, ease of modification and low cytotoxicity. Electrical and physical transfection methods, on the other hand, are much less used due to the risk of inducing secondary injuries. Studies related to the non-viral delivery of nucleic acids for *in vivo* nervous system repair, such as the vectors used, the delivery methods and the therapeutic outcomes were summarized in Table 3.

2.5.1. Non-viral delivery of nucleic acids for central nervous system (CNS) regeneration

Injuries to the CNS often lead to long-term disability, mortality and psychological symptoms(Shoichet et al., 2008). Primary injuries often result in contusions and bleeding while secondary injuries can occur months after the initial damage and include axonal damage, demyelination and vascular injuries(Mckee and Daneshvar, 2015). Generally, the injured axon may regenerate if the microenvironment is favourable for regrowth(Yao et al., 2012). Hence, nucleic acid-based therapy has emerged as a promising strategy for treating different nervous system injuries by either up-regulating the growth promoting molecules or down-regulating the growth inhibitory factors(Blits and Bunge, 2006).

Comparatively, although proteins can also be administered to an injury site, one advantage of using nucleic acids is that multiple therapeutic genes can be delivered from the same delivery systems(Blits and Bunge, 2006). In addition, the administration of proteins to the injured site via local injection is often transient due to the labile nature of proteins, especially under the injured microenvironment(Storer et al., 2003). Hence, by modifying the genome of the transfected cells, protein expression can be prolonged(Lu et al., 2002). Besides that, when transfecting cells that can proliferate, protein expression may be passed on(Haastert et al., 2006), thereby enabling long term therapeutic effects. More importantly, protein treatment works through the recognition by protein receptors. For proteins which receptors are lacking on the neurons, protein treatment is not applicable(Cameron et al., 2006). Nucleic acid delivery could also overexpress transcription factors(Nie et al., 2015), which cannot be achieved by protein delivery.

2.5.2. Bolus delivery of polymer-based carriers

Polymers play critical roles in non-viral gene delivery by providing controlled release of therapeutic nucleic acids over long durations. Due to the transient nature of nucleic acids, such as their fast degradation rate, repeated administrations are typically needed to achieve the long-term expression of therapeutic genes in the treatment of nervous system injuries. As such, sustained delivery of nucleic acids by polymer-based carriers is of great necessity.

Polymer-based carriers have been used to deliver both large plasmid DNAs and small oligonucleotides. Among the non-viral vectors used *in vivo*, the polycationic polymer, polyethyleneimine (PEI, 50-kDa), exhibits one of the highest transfection efficiency(Boussif et al., 1995). Shi *et al.* studied the effects of PEI/DNA on the injured rat spinal cord by intrathecal administration(Shi et al., 2003). In particular, the naked DNA that encoded luciferase or PEI/DNA complexes were injected into the spinal cord lumbar levels. Thereafter, the transgene expression was improved significantly in the presence of PEI. In particular, the expression level induced by the PEI complexes containing 4 μg of DNA was 40-fold higher than that induced by the same amount of naked plasmid DNA. The data showed that the luciferase activity at the lumbar and thoracic levels accounted for 50% of the total activity in the whole spinal cord. Positively stained cells were also observed to display typical morphologies of astrocytes and neurons. In addition, long-term gene expression was achieved with repeated injections of PEI/DNA complexes. However, when repeated dosages were administered, a 70% attenuation of gene expression was observed following reinjection at a 2-week interval due to apoptotic cell death(Shi et al., 2003).

To circumvent the problem of cytotoxicity, other studies demonstrated that the modification of PEI by polyethylene glycol (i.e. PEGylation) could improve biocompatibility(Ogris et al., 1999; Nguyen et al., 2000; Kichler et al., 2002). Specifically, as compared to PEI alone, PEGylated PEI was significantly less toxic to neuronal precursor cell lines such as PC12 and NT2/D1 cells(Shi et al., 2003). Furthermore, by using PEG-grafted PEI for DNA complexation, the attenuation of gene expression (which was observed due to the cytotoxicity of PEI) was not detected after repeated intrathecal injections(Shi et al., 2003). Hence, PEGylated PEI could significantly reduce the cell death which was caused by using PEI alone.

Overcoming mRNA instability is vital for effective mRNA delivery (Heil et al., 2004; Karikó et al., 2004). Correspondingly, polymer-based carriers have shown their potential in solving these issues(Itaka and Kataoka, 2009). A polyplex nanomicelle system using the polycation, poly [N9-[N-(2-aminoethyl)-2-aminoethyl] aspartamide] ([PAsp (DET)]), was reported recently(Uchida et al., 2013). Due to its core-shell architecture based on the self-assembly of block copolymers (which consisted of PEG and polyamino acids), this polyplex nanomicelle served as an effective mRNA carrier. As the mRNAs were entrapped within the micelle, both stability and immunogenicity issues could be simultaneously resolved(Itaka and Kataoka, 2009). Besides that, this system could enhance endosomal escape due to pH-responsive membrane destabilisation by [PAsp(DET)](Miyata et al., 2008). Furthermore, it also rapidly degraded into non-toxic forms under physiological conditions, which further facilitated endosomal escape and minimized cell damage and toxicity after administration(Masago et al., 2007; Itaka et al., 2010).

Since PEI induces strong cytotoxicity, chitosan (CS), a natural linear cationic polysaccharide, was explored as an alternative for *in vivo* gene delivery. Chitosan has been widely used as drug carriers, wound dressings, and scaffolds for tissue engineering due to its biocompatibility, biodegradability and low toxicity(Lu et al., 2013; Malmo et al., 2013). As such, chitosan or chitosan-functionalized nanoparticles (CNPs) have been widely investigated for non-viral gene delivery(Nice, 2004). However, the low transfection efficiency of chitosan has hindered its applications. Recently, modifications, such as grafting PEI onto chitosan, or creating a chitosan-PEI composite have been developed for gene delivery *in vivo*(*Lou* et al., *2009*). Das *et al.* tested chitosan and PEI-coated magnetic micelles (CPmag micelles or CPMMs) as gene delivery carriers and the efficacy of this carrier was evaluated in a mild traumatic brain injury (mTBI) model. CPMM-tomato plasmid (ptd) conjugates expressing a red fluorescent protein (RFP) were instilled into the nose of sham-operated rats or rats subjected to mTBI. CPMM-ptd conjugates were shown to successfully enter the brain, and the red fluorescent protein (RFP) expression was identified in the cortex and hippocampus at 48 hours after mTBI without evoking any inflammatory response. These observations indicated the possibility of using intranasally administered CPmag as a theragnostic vehicle for mTBI(Das et al., 2014).

2.5.3. Lipid-based carriers

Besides serving as effective gene carriers for *in vitro* studies, lipid-based carriers are also widely used in animal works due to their biocompatibility, biodegradability and low toxicity(Helm and Fricker, 2015; Immordino et al., 2006). Takahashi *et al.* used lipofectamine-plasmid complexes (Plasmids: Lipofectamine = 2:1) to regulate the expression of B-cell lymphoma-2 (Bcl-2), which is a protein that has been shown to prevent apoptosis(Adams and Cory, 1998; Beattie et al.,

1998). Therefore, targeting the expression of Bcl-2 could potentially prevent neuronal cell death after CNS injuries. Hence, lipofectamine-complexed plasmids encoding pα22β-galα4bcl-2 gene were injected into the right side of the T8 segment after spinal cord hemi-incision. The transgene expression was then confirmed by observing the expression of the reporter gene, LacZ, three days after administration. Colocalization of LacZ expression and Clarke's Nucleus (CN) neurons were detected at the spinal cord L1 level. Correspondingly, this treatment significantly reduced atrophy and the loss of axotomized Clarke's Nucleus (CN) neurons(Takahashi et al., 1999). Besides that, the axotomized red nucleus (RN) neurons were also protected by this treatment. Results showed that 87% of RN neurons survived two months after C3/C4 subtotal hemi-incision, suggesting that intraspinal administration of Bcl-2 gene could prevent retrograde cell loss and reduce atrophy of damaged RN(Shibata et al., 2000).

Glial-derived neurotrophic factor (GDNF) supports the survival of motor neurons and promotes axonal regeneration after axotomy (Henderson et al., 1994; Watabe et al., 2000). Lu *et al.* showed that the administration of complexes of liposome plasmids that encoded GDNF promoted axon regeneration after spinal cord injury(Lu et al., 2002). The liposome plasmid complexes were injected directly into the grey matter of the rat spinal cord (T7-T8 level). Thereafter, the expression of GDNF mRNA was detected one week after injection. Moreover, the expression of EGFP-GDNF was observed in the cells around the injection site 4 weeks after injection, indicating that these plasmids lasted for at least one month. Furthermore, anterograde tracing confirmed the regeneration of corticospinal tracts four weeks after treatment. Behaviour tests, such as the inclined plane test and Basso, Beattie, and Bresnahan (BBB) scores exhibited improved functional recovery of the rats' hindlimbs. These observations suggested that the delivery of plasmids encoding GDNF could promote nerve repair after SCI. However, the transfection efficiencies and the cell damage after lipoplexed plasmids injection were not assessed. Also, the exact cell types that were transfected by lipoplexes remains unknown.

Thus far, intraspinal injection is one of the most common administration routes of nucleic acids for treating traumatic injuries in the CNS. Usually, the injected nucleic acid therapeutics can last for 1 month and the time-course of observing its expression/effects is around 3-7 days (Lu et al., 2002; Das et al., 2014; Takahashi et al., 1999; Shibata et al., 2000). However, prolonged expression of the transgenes (eg. several months) is often needed to achieve more prominent functional recovery (Nguyen et al., 2017a). While most studies have focused on evaluating the functional outcomes that are induced by the administration of nucleic acids, it is also crucial to understand the possible side effects of gene delivery, the extent of cellular uptake of transgenes, clearance durations as well as the transfection efficiencies.

2.5.4. Scaffold-mediated non-viral nuclei acids delivery for SCI treatment

Scaffolds serve a crucial role in tissue regeneration by providing a means to control the local extracellular environment. These substrates may present biochemical(Diao et al., 2015), topographical(Jiang et al., 2012) and mechanical(Jiang et al., 2014) cues to cells. Beyond that, scaffolds may also be employed as controlled release vehicles for biomolecules and therapeutic drugs(Magnani, 1998; Salvay and Shea, 2006). Specifically, drug encapsulation within scaffolds can help to protect nucleic acids from biodegradation by shielding them from immune attacks and retain nucleic acids locally, thereby preventing systemic clearance (Roy et al., 2003). Importantly, the sustained nucleic acid delivery via scaffolds also increases the opportunity for cellular internalisation and the likelihood of successful transfection due to local availability of drugs(Cao et al., 2010). Consequently, scaffolds and nucleic acid-incorporated substrates are employed to guide neuronal cell growth, direct neuronal differentiation(Cao et al., 2009; Mahairaki et al., 2011) and promote functional recovery for the treatment of traumatic nerve injuries(De Laporte et al., 2010; Houchin-Ray et al., 2009).

In one study, lipoplexed plasmid DNA was encapsulated in multichannel poly[lactide-co-glycolide] (PLG) neural conduits(De Laporte et al., 2009a). Before implanting into the animals, different extracellular matrix (ECM) components (fibronectin, collagen I, laminin I) were coated onto PLG to immobilise DNA. *In vitro* studies revealed that fibronectin produced the highest immobilisation efficiencies as compared with the other two coatings. Thereafter, luciferase assay indicated that 25 or 50 μg of fibronectin coating elicited the highest levels of transgene expression. The plasmid DNA-encapsulated PLG conduits were subsequently implanted into spinal cord hemi-sectioned rats (T9-T10 level)(De Laporte et al., 2009a). Three weeks after implantation, the transgene expression level was 2-fold higher than that of naked plasmids. Additionally, the transgene expression persisted for three weeks and axon regeneration was observed inside the channels. However, the regenerated axons did not exit the conduits and the functional recovery after treatment remains unknown.

A follow-up study by the same group then applied the multichannel PLG conduits to deliver DNA plasmids to support and direct cellular processes and promote gene transfer following spinal cord hemisection at T9-T10(De Laporte et al., 2009b). The expression of the transgene was shown to last for 44 days *in vivo*. Furthermore, the implantation of multichannel conduits supported cell infiltration and axon growth. Immunohistochemistry confirmed that the transfected cells at the implant site were mainly Schwann cells, fibroblasts, and macrophages. These observations suggested that the synergistic effects of functional gene expression and topographical cues could significantly improve nerve regeneration(De Laporte et al., 2009b). However, the transgene expression was mainly detected in glial or immune-related cells. Therefore, future studies are needed to analyse how these transfected cells affect nerve regeneration.

Although multiple groups have explored the delivery of large nucleic acids, the delivery of small oligonucleotides and gene silencing is less explored. Nonetheless, a study done by our group introduced a three-dimensional (3D) nanofiber hybrid scaffold that directed and enhanced axonal regeneration after SCI(Nguyen et al., 2017b). The fabrication of this 3D hybrid scaffold involved the combination of electrospun aligned fibres and collagen matrix. Mir-222, an inhibitor of the PI3K pathway that is important to central axon growth(Zhou et al., 2012), was then chosen as the additional biochemical signal to enhance nerve regeneration after SCI. As a biofunctionalized platform, the 3D aligned nanofiber-hydrogel scaffold provided sustained non-viral delivery of proteins (NT-3) and miR-222, along with synergistic contact guidance for nerve repair. Correspondingly, aligned axon regeneration was observed as early as one-week post-injury. Furthermore, no excessive inflammatory response and scar tissue formation was triggered after scaffold implantation.

Taken together, studies thus far have indicated that functionalized scaffolds serve as promising nucleic acid delivery platforms for SCI treatment. As compared to intraspinal injection, scaffold-mediated nucleic acid delivery can last for several months (eg. 3 months)(Nguyen et al., 2017a) and the transgene expression was observed up to 3-4 weeks after treatment(De Laporte et al., 2009a; De Laporte et al., 2009b). However, in contrast to *in vitro* neuronal cell transfection, the transfection efficiency and the side effects of gene delivery after CNS injuries have not been clearly discussed in the above studies. One possible reason might be due to the lack of robust experimental methods to evaluate cellular uptake, transgene expression and gene silencing effects under the injured microenvironment. In general, as compared to protein therapeutics such as NT-3, BDNF and GDNF(Elliott Donaghue et al., 2016; Ikeda et al., 2002; Zhang et al., 2009), *in vivo* nucleic acids transfection is not commonly used to treat CNS injuries. However, given the promising outcomes of these studies and the lack of robust regeneration using conventional protein-based methods, it may be highly worthwhile to continue to establish more robust non-viral nucleic acid transfection methods for CNS injury treatment.

2.6. Non-viral delivery of nucleic acids for peripheral nervous system (PNS) regeneration

The PNS has some regenerative capacity. However, its ability to grow remains limited when crossing large lesion gaps. Hence, in patients with PNS injuries, nerve reconnection is often incomplete over large lesions due to the low rate of nerve regeneration (i.e. 1 mm/ day (Sulaiman and Gordon, 2013)) and misrouting of the regenerated axons (Allodi et al., 2012). Hence, locomotor recovery remains limited(Guo et al., 2014) and more effective therapeutic strategies are needed (Hoyng et al., 2015). Gene therapy-based strategies aim to provide target-specific neurotrophic support to enhance the survival and regeneration of both sensory and motor axons and finally, the recovery of function(Zacchigna and Giacca, 2009; Lim et al., 2010). To achieve this, artificial nerve guidance conduits are commonly utilized to bridge large nerve defect gaps. The synergistic effects of nucleic acids and topographical cues provided by the nerve conduits could further direct and enhance nerve regeneration and functional recovery after PNS injuries.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor which stimulates the function of new blood vessels and enhances vascular permeability(Leung et al., 1989). Lope *et al.* reported the use of plasmid vectors, which expressed human VEGF165 gene(Pereira Lopes et al., 2011) for sciatic nerve injury treatment. Plasmid vectors carrying the VEGF gene were then injected into the thigh musculature below the sciatic nerve followed by electroporation. Ten minutes later, the sciatic nerve was transected and a 6 mm collagen nerve guide conduit was implanted to bridge the injury gap. Consequently, the number of regenerated myelinated axons and blood vessels were notably larger in VEGF-encoding plasmid (VEGF plasmid)-treated animals as compared to the control group (vectors alone). Moreover, the functional sciatic index and gastrocnemius muscle weight significantly increased in VEGF plasmid-treated animals versus vectors alone. While the results indicated that VEGF plasmid administration supported and enhanced nerve regeneration, this method of gene transfection before an injury may not be clinically relevant.

The granulocyte colony-stimulating factor (G-CSF), a cytokine that induces survival, proliferation and differentiation of hematopoietic lineage cells(Metcalf, 2008), was introduced and evaluated by the same group. The synergistic effects of G-CSF and VEGF were further investigated using the same delivery vectors in the treatment of sciatic nerve injuries(Pereira Lopes et al., 2013). In particular, plasmids encoding VEGF and/or G-CSF genes were injected locally (below the sciatic nerve in adult mice) and transfected via electroporation. The sciatic nerves were then transected followed by the implantation of a polycaprolactone (PCL) nerve guide. The administration of G-CSF alone and G-CSF-VEGF cocktail improved nerve regeneration, and the improvement was even more significant in the cocktail treated groups. G-CSF-VEGF cocktail-treated animals showed remarkably improved motor function recovery as compared with the control groups (vectors alone). In addition, the myelinated axons, blood vessels and gastrocnemius muscle weight were also significantly increased with VEGF and G-CSF treatment. These works suggest that the combined treatment acted synergistically in improving regeneration after sciatic nerve transection lesions(Pereira Lopes et al., 2013).

Generally, electroporation was used in the above studies for the transfection of foreign genes *in vivo*, but these studies mainly focused on the effects of the transgenes on nerve regeneration outcomes with minimal attention spent on evaluating secondary damages due to such physical transfection methods. Importantly, the introduction of these nucleic acid therapeutics before an injury has low clinical relevance for unpredictable traumatic nerve injuries. Furthermore, the studies did not evaluate the transfection efficiencies and the expression of transgenes, which makes it difficult to make comparisons with *in vitro* electroporation outcomes.

Although non-viral gene delivery approaches have been used for the treatment of PNS injuries, existing studies regarding scaffold-mediated gene delivery via non-viral methods remain limited. As axotomized nerve terminals are usually far from their cell bodies, therapeutic nucleic acids that modulate gene expression in the cell soma (i.e. at DRGs) might not exert their effects efficiently due to the long distance. Hence, exploring therapeutic nucleic acids that target the injured axons, such as nucleic acids that facilitate local protein synthesis or new growth cone formation, might serve as an alternative for the treatment of PNS injuries.

2.7. Gene therapy-based cell transplantation for nervous system injury treatment

To precisely monitor the transfection process and the transfection efficiencies of target cells, the implantation of gene-modified cells to the injured nervous system has also been explored as an alternative to enhance nerve regeneration. This method has been used in both CNS and PNS injury treatments.

Primary olfactory-ensheathing glial (OEG) were transfected with cationic liposome-complexed recombinant plasmids that encoded NT-3. *In vitro* transgene expression analysis demonstrated that higher amount of NT-3 was released from NT-3-transfected OEG as compared to cells that were transfected with transfection reagent only. Subsequently, the transfected cells were implanted into the rat spinal cord directly after a thoracic spinal cord (T9) contusive lesion. Seven days after transplantation, spinal cord tissues that were injected with transfected OEG expressed high levels of NT-3 mRNA. More importantly, robust nerve regeneration and hindlimb functional recovery were observed three months after implantation(Wu et al., 2008). Table 3 summarises various studies on gene-modified cell transplantation for promoting nerve regeneration after CNS injuries. The study mentioned above was highlighted due to their comprehensive work *in vitro* and *in vivo*. Besides that, their results strongly suggested that gene-modified cell transplantation is effective after SCI. However, the use of scaffolds for delivering non-viral gene-modified cells for CNS injury treatment, to our knowledge, has not been attempted. We speculated that this might be due to many impeding factors that could affect the regeneration outcomes. Some examples include the viability of cells encapsulated within the scaffold, the level of transgene expression, the migration rate of the transplanted cells along with the mass of functional molecules that are ultimately released from the scaffolds.

The implantation of gene-modified cells is also applicable for PNS injuries. Schwann cells (SCs) are regarded as the therapeutic targets of PNS due to their role in promoting tissue regeneration by secreting growth-promoting molecules, guiding regenerating axons towards a target region and myelinating regenerated axons(Jessen and Mirsky, 1991; Lobsiger et al., 2002). Therefore, restoring the function of SCs is crucial for PNS regeneration. Specifically, the entrapment of the low molecular weight (18-kDa) isoform of fibroblast growth factor-2 (FGF-2) in artificial nerve guidance conduits significantly enhanced the growth of myelinated and unmyelinated axons across large lesion gaps (Aebischer et al., 1989). SCs transfection was carried by Haastert *et al.* (*Haastert* et al., *2006*)using complexed Metafectene™ and plasmids that encoded 18-kDa-FGF-2 isoform and 21/23-kDa-FGF-2 isoform. Thereafter, the transfected SCs were seeded into silicone tubes and the tubes were implanted into the rat sciatic nerve to bridge a 15-mm rat ischiatic nerve defect. Consequently, functional assessment indicated a more robust regeneration of sensory function by grafted SCs that over-expressed different FGF-2 isoforms as compared to normal untreated SCs. Furthermore, the over-expression of the high molecular weight 21/23-kDa-FGF-2 isoforms by grafted Schwann cells resulted in earlier signs of sensory recovery as compared to the over-expression of 18-kDa-FGF-2. In contrast, motor recovery was detected after the over-expression of 18-kDa-FGF-2, as revealed by the recording of compound muscle action potentials (CMAP) (Haastert et al., 2006).

Kempton *et al.* bridged a 10-mm gap using a collagen nerve guidance conduit with gene-modified mesenchymal stem cells (MSCs) that

overexpressed VEGF(Kempton et al., 2009). These nerve guidance conduits were filled with saline, Matrigel with mesenchymal stem cells (MSCs) or Matrigel with gene-modified MSCs (transfected with complexed Lipofectamine 2000 and plasmids that encoded VEGF165 gene). The treatment with VEGF-transfected MSCs significantly promoted nerve regeneration and facilitated blood vessel formation three weeks after implantation. However, the differentiation and function of MSCs after VEGF transfection were not evaluated. Hence, it remains unknown if the MSCs participated directly in tissue function or provided biochemical support through paracrine signalling.

Modifying genes of cells before transfection allows better control over the transfection efficiency of target cells. However, there are also some concerns, such as the low survival rate of the transplanted cells and their ability to function in the injured microenvironment.

Gene therapy has shown its potential in enhancing nerve regeneration after injuries by overexpressing growth-promoting factors and preventing neurons from cell death. The incorporation of scaffolds provides additional topographical cues, which further facilitate and modulate nerve regeneration. However, some inevitable challenges should be taken into consideration, such as low transfection efficiency, uncertain cellular uptake and unpredictable side effects in the process of non-viral gene transfection *in vivo*.

3. Design considerations for better control over the delivery and uptake of nucleic acids by injured neurons to enhance nerve regeneration

From the non-viral delivery systems reviewed thus far, bulk of these systems depend on physical transfection methods, polymer-based as well as lipid-based carriers. While physical transfection methods such as electroporation have achieved significant levels of successful nucleic acid transfection *in vitro*, the possibility of causing secondary nerve tissue damage has limited their *in vivo* applications. On the other hand, the systemic delivery of nucleic acids via non-viral carriers faces a variety of problems. Although improvements have been made to enhance transfection efficacy of these non-viral carriers, it is undeniable that these delivery systems are still exposed to systemic clearance and serum nucleases – both of which lead to transient modulation of gene expression, which is often insufficient in achieving desired therapeutic effects. Most importantly, these delivery systems cannot provide topographical cues which are essential for guiding neurite extension across an injury site.

Scaffolds serve as supporting structures and provide physical signals that may direct cell fate, aid cell infiltration, attachment, growth (Salvay and Shea, 2006; Langer, 1998; Langer, 2001) and modulate gene transfection (Kong et al., 2005). Additionally, the delivery of nucleic acids via scaffolds provides protection against nucleases and allows localised and sustained delivery of nucleic acids at the injury site. While scaffold-mediated non-viral delivery of nucleic acids appears promising based on available works, numerous areas still require improvements for better functional outcomes. The following sections will look at some design alternatives to improve non-viral nucleic acids delivery platforms for nerve regeneration. Specifically, these improvements aim to enhance the efficiency of delivery, transfection and uptake of nucleic acids by neurons at the site of injury.

3.1. Enhancing stability of nucleic acids

Nucleic acids are susceptible to bio-degradation and clearance from the body due to the presence of extracellular nucleases and the immune system (Roy et al., 2003). The chronic inflammatory environment and the presence of reactive oxidative species secreted by activated inflammatory cells render nucleic acids vulnerable to degradation at the trauma site within the nervous system (Li et al., 1999; Kao, 1999; Krieg et al., 1995; DiProspero et al., 1997; Brewer et al., 1999; Shah et al., 1992).

Bio-responsive delivery systems can be considered to minimise such undesirable nucleic acid degradation. These systems change their properties in response to a biological trigger such as changes in pH, temperature, light or presence of biomolecules such as enzymes (You et al., 2010). Their abilities to adapt to the environment provide novel modes of release, e.g. release of nucleic acids only in a well-defined disease or injury state. Along these lines, cell-matrix interactions represent an interesting trigger for releasing nucleic acids from scaffolds. For example, matrix metalloproteinases (MMPs) are enzymes that degrade both matrix and non-matrix proteins. They have great importance in remodelling the extracellular environment of cells (Nagase et al., 2006). Correspondingly, MMP-degradable hydrogels supported cell growth and modulated cell migration (Raeber et al., 2005; Lee et al., 2007; Lutolf et al., 2003). Since MMP expression increases during spinal cord injury (Xu et al., 2001; Noble et al., 2002; Birkedal-Hansen et al., 1993; Davies and Silver, 1998; Mautes et al., 2000), such MMP-degradable scaffolds could serve as an additional protective measure for nucleic acids that have been encapsulated within the matrix. As cells penetrate the matrix, MMPs are released locally, and nucleic acids that have been encapsulated within the matrix may then be released. Consequently, this limits the exposure of the encapsulated nucleic acids only to cells that are migrating into and residing within the matrix.

3.2. Modulating mechanical properties of scaffolds to increase cellular uptake of nucleic acids

The mechanical properties of scaffolds and matrices that cells adhere to are increasingly being recognised for their roles in regulating cellular response (Discher et al., 2005; Xu et al., 2006). Similarly, the efficiency of gene uptake and cellular transfection have also been investigated as a function of matrix compliance(Kong et al., 2005). In particular, increasing substrate stiffness led to an increase in polyplex uptake, de-condensation and eventual delivery to the nucleus (Kong et al., 2005). Besides that, stiffer substrates were identified to promote higher nucleic acid transfection efficiency due to the enhanced rate of cell proliferation (Kong et al., 2005; Escriou et al., 2001; Tseng et al., 1999) (although it should be noted that in the context of neurons, these cells do not undergo proliferation (Herrup and Yang, 2007)). Interestingly, when neurons were cultured on soft substrates, they formed significantly more branches than on stiffer matrices (Flanagan et al., 2002; Georges et al., 2006). Hence, coupling the delivery of nucleic acids that enhance axonal growth cone advancement with softer substrates may potentially promote nerve regeneration (Singh et al., 2014; Sengottuvel et al., 2011; Hellal et al., 2011; Iyer et al., 2014).

On the contrary, glial cells seem to prefer stiffer substrates(Pogoda and Janmey, 2018; Moshayedi et al., 2014a). The mechanical mismatch between nerve tissues and implanted electrodes led to glial cell activation (Moshayedi et al., 2014b). While glial scar formation is undesirable for nerve regeneration across spinal cord injuries, increasing evidence indicates that the components of glial scar are important in triggering the proliferation of neural progenitors and stem cells. Astrocytes, in particular, have been identified as one of the key regulators of these processes (Walton, 2006; Laywell et al., 2007). Therefore, while bio-functionalised scaffolds should be mechanically soft to prevent mismatch in stiffness at the host-implant interface, these scaffolds should also have the appropriate stiffness to allow glial cells to infiltrate and attract the migration of stem cells into spinal cord injury sites.

The hybrid fibre-hydrogel system from our group(Nguyen et al., 2017b) could serve as a potential platform to amalgamate various matrices of different mechanical stiffness. In particular, the hydrogel matrix provides a mechanically soft interface between the scaffold and the injured tissue. Hence, this hybrid scaffold may help to reduce glial scar formation while promoting neurite branching and ingrowth into the scaffold. Furthermore, the encapsulation of nucleic acids into this hydrogel matrix allows nucleic acid transfection to occur at the growth cones of neurite extensions. Concurrently, the fibres within this hybrid

scaffold serve as the relatively stiffer matrix. Hence, these fibres may promote glial cell infiltration since glial cells prefer stiffer substrates. As glial cells reside within the hybrid scaffold, their presence could bring about the eventual attraction of neuronal stem cells, which play important roles during nerve regeneration especially across large lesion sites.

3.3. Increasing specificity of cellular binding and uptake

The direct administration of cationic polyplexes to the site of injury often result in non-specific cell association through interactions with anionic membrane proteins such as proteoglycans(Mislick and Baldeschwieler, 1996). Despite these drawbacks, the delivery of nucleic acids via these cationic polyplexes is still being employed for transfecting neurons *in vitro* and *in vivo* due to ease of application (Boussif et al., 1995; Abdallah et al., 1996). In addition, understanding and predicting the efficiency of neuronal nucleic acid uptake is complicated by the fact that neurons are highly polarised cells with soma, axonal and dendritic domains that possess distinct membrane compositions (Cameron et al., 1991; Dotti et al., 1991). Furthermore, the physicochemical characteristics of nucleic acid carriers, including the size, charge and surface composition, may strongly influence the nature of their interaction with the neuronal plasma membrane. Hence, the design of non-viral nucleic acid delivery platforms is crucial to ensure that cells within the nervous system can specifically bind and uptake these therapeutic nucleic acids.

Bio-functionalised scaffolds could be designed to attract neurite extensions within the injured nervous system such that nucleic acids can reach these targeted group of cells/ regions of the neuron. The covalent coupling of bioactive laminin epitope, IKVAV, along with the incorporation of a full-length laminin chain provided a permissive environment that attracted neurite outgrowth (Frick et al., 2017). This approach could be considered as a way to attract the extension of neurites from injured neurons into scaffolds that are loaded with nucleic acids, such that growth cones at the ends of these neurite extensions can preferentially uptake these molecules.

3.4. Improving temporal control

Tissue development and regeneration generally occur in sequential phases (Shea et al., 2000). Therefore, regeneration strategies should incorporate precise spatial and temporal controls to engineer mature and functional tissues (Nishimura et al., 2003). Since the release profile of nucleic acid from scaffolds does not directly correlate with transgenic expression, obtaining temporal control remains challenging (Jang et al., 2005). In this respect, modifications in biomaterial designs can allow sequential delivery to be established. In particular, one type of nucleic acid may be encapsulated in a rapidly degrading polymer while the other type of nucleic acid may be held within a slow degrading polymer to achieve sequential delivery of multiple factors (Jang and Shea, 2003). Alternatively, the layer-by-layer strategy may be engaged, where nucleic acids are loaded in between two materials. Correspondingly, the degradation of one material layer will be accompanied by the release of nucleic acids at the closest proximity to the degraded material layer (Zhang et al., 2004). MMP-degradable scaffolds can also be adapted for such differential delivery of nucleic acids (You et al., 2010).

4. Conclusions

The injured nervous system holds limited regenerative capability, especially within the CNS. Although the PNS has some regenerative capacity, its ability to grow remains limited when crossing large lesion gaps. Although the delivery of nucleic acids via viral vehicles into the injured nervous system holds great potential in enhancing nerve regeneration *in vivo*, the lack of safe and efficient delivery systems has limited the application of these molecules in patients who suffer from traumatic nerve injuries. Therefore, the exploration of non-viral delivery approaches is of great necessity. Along this line, this review has highlighted the important nucleic acid candidates, which participate in cellular activities within the nervous system, and their mechanisms of gene modulation. Following that, we focused on the transfection of neuronal cells and summarised the non-viral vectors that have been used *in vitro* as well as their corresponding transfection efficiencies. More importantly, we reviewed recent animal studies on non-viral delivery of therapeutic nucleic acids for nerve regeneration. Specifically, the combinatorial approach of nucleic acid therapeutics with scaffolds provides a synergistic and promising treatment option for nerve regrowth. Besides that, scaffold-mediated non-viral nucleic acid delivery allows controlled modulation of gene expression while providing topographical cues to guide axonal regeneration.

Although scaffold-mediated non-viral nucleic acid delivery approaches have been applied to nerve injury repair, many challenges remain in the development of these bio-functionalized scaffolds. These challenges include maintaining or enhancing the stability of nucleic acids against biodegradation, improving cellular uptake efficiencies as well as the temporal control of the expression of targeted genes. As such, these aspects should be taken into consideration when designing scaffolds for more effective therapeutic treatment for nerve repair.

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