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Technology digest: non-viral vectors

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Introduction

The advent of gene therapies has offered hope to address previously untreatable genetic disorders, such as protein deficiency disorders or monogenetic deficiencies. Using various techniques to correct or simply replace disease-causing genetic fragments, inherited and acquired diseases have been treated, although currently with varying success. Identifying the most suitable vector to deliver genetic materials in the production of an investigational therapeutic product is key to reducing manufacturing risk, ensure consistency and reproducibility, and realize the revolutionary potential of genome engineering [1.]

Freya Leask
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Genetic delivery vectors

In gene therapies, vectors, such as adeno-associated viruses (AAVs) or lentiviruses, are used to transfer genetic material into human cells to alter their function [2]. The nature of this genetic delivery system has a great impact on the efficacy of the final treatment as well as any adverse events experienced by the patient. Viral vectors, including AAVs, were some of the first applied to cell and gene therapy clinical studies; they can be highly efficient in transferring genetic information but are often not specific in their insertion locations, leading to random, or close-to-random, insertions throughout the genome. They sometimes fail to fully integrate the genetic material into their target cells and are limited in how much DNA they can carry. Viral vectors can have cytotoxic effects and transduced cells produced via viral vectors are also prone to mutagenesis and immunogenicity. This makes them challenging components to incorporate into clinical studies and current medical practice [2].

By contrast, non-viral vectors, such as liposomes, naked DNA, oligonucleotides and transposons, are simpler systems which can both insert, edit and delete desired genetic sequences using technologies such as CRISPR-Cas9 and zinc finger nucleases. They are also easier to manufacture at scale and induce low host immunogenicity. As such, despite their novelty, clinical trials involving use of non-viral vectors are likely to increase, especially after the high-profile successes in the Pfizer/BioNTech and Moderna COVID-19 vaccines, which utilize mRNA.

Transposons

Transposons are discrete fragments of nucleic acid, consisting in their natural form of a single gene encoding the transposase enzyme flanked by terminal inverted repeats (ITRs). Transposons can move between loci within the chromosome and cause mutations or rearrangements within the genome. They also have a much higher genome integration efficiency; this makes transposon-based gene therapies attractive development targets as this integration efficiency enables more persistent expression of therapeutic genes [2].

Transposases can also act on almost any DNA sequence flanked by the ITR, enabling novel methods of genetic engineering in a range of scenarios including animal models, human cells and clinicals trials. Two commonly utilized transposons are

piggyBac (PB) and Sleeping Beauty (SB). PB, isolated from insects, offers efficient genomic integration, robust gene expression and seamless excision, and can also carry very large DNA fragments; for this reason, PB has shown promise in generating iPSCs which are genetically 'clean' of exogenous elements. However, use of PB runs the risk of transposase integration; other potential challenges are that the delivery of PB plasmid vectors cannot take place without additional transfection reagents, which could impact the cost and subsequent scalability of this technique, and there is little control over insertion sites [2].

SB has been molecularly reconstructed from the genomes of marine relics, eliminating inactivating mutations to produce a tool capable of precise 'cut-and-paste' transposition [3]. As SB is non-human, there is a reduced risk of mobilizing endogenous transposon elements. SB integration is also non-random, showing statistical target preferences towards AT-rich palindromes, and to a lesser extent transcriptional units and their upstream regulatory sequences. Unfortunately, transposition efficiencies of current variations of SB remain low, at approximately 20% [4].

TcBuster™ [5] (Bio-Techne, MN, USA) is a relatively new transposon-based hyperactive transposon system consisting of two components: a plasmid containing the cargo flanked by inverted tandem repeats (ITRs) and mRNA encoding the TcBuster transposase. The TcBuster transposon originated from the red flour beetle and is considered "a rising star for gene transfer" [6]; preliminary results have shown it to be versatile, cost-effective and clinically scalable, especially for cells such as engineered NK and T cells, which shows great potential [7].

Unlike gene delivery via viral vectors, where genetic material must be packaged prior to transduction, transposons such as TcBuster can be quickly generated and electroporated into target cells. Viral vectors are typically capable of integrating much smaller cargo sizes, around 4-5 kilobases (kb) of DNA compared to more than 10kb for TcBuster. Transposon-based vectors maintain a higher gene transfer efficiency – at least 40% [8] – and greater stability, allowing for more rapid generation of transgenic mammalian cells with limited secondary effects. TcBuster has also been shown to have an integration pattern comparable to PB and SB, demonstrating a reduced risk of insertional mutagenesis compared with viral vectors [9].

Speaking to RegMedNet, Nathan Allen, Director of Product Management, Cell and Gene Therapy at Bio-Techne, explained, "TcBuster is a tool that provides researchers and developers with an alternative that overcomes the common challenges our industry faces today." Quicker and more efficient gene delivery ultimately means lower cost of genomic modification, making cell- or gene-based therapeutic agents that incorporate elements of genetic modification more cost-effective.

Non-viral vectors in clinical study

Non-viral vectors show immense potential as alternatives to viral vectors in terms of safety and practicality for clinical applications. However, future improvement can be made to ensure optimal gene transfer efficiency and minimal host-protein interaction. Previously, a challenge with production and clinical relevance was the time-consuming cell culture and expansion required, which could compromise therapeutic effectiveness, making an unattractive intervention. Pre-clinical and early-stage

clinical trials rely on the stable transposition of the genes of interest within the target cell genome; transposon-based genetic editing has now been validated for both ex vivo and in vivo therapy for several indications and clinical targets [9]. However, when deciding on a method of gene editing, consideration should be given to various confounding variables, including disease target, cell type and transfection method [10].

As gene therapy continues to see success in clinical study, further attention will be given to the durability of therapeutic effect as we move beyond quality-of-life improvements and towards curative treatments. Whilst vector optimization is certainly one important factor in therapy efficacy, improvements in disease modelling, drug testing and therapeutic delivery will also be needed to fully realize the potential benefits of gene therapy. In particular, the location of gene therapy delivery can significantly improve or lessen the effectiveness of a genetic therapy; intravenous delivery is the most studied, but the administered genetic material is subject to enzymatic degradation from the point of entry [11]. Direct administration to the disordered organ reduces this impact but physical barriers, such as the blood brain barrier and the blood retinal barrier, have developed over thousands of years to make this challenging.

As therapy developers and vendors, such as Bio-Techne, strive towards the same goal, knowledge sharing and learning from pioneers who have come before will be crucial to help the cell and gene therapy industry to maturity as quickly as possible.

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No writing assistance was utilized in the production of this manuscript.

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Review

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Regenerative Medicine

Current reprogramming systems in regenerative medicine: from somatic cells to induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) paved the way for research fields including cell therapy, drug screening, disease modeling and the mechanism of embryonic development. Although iPSC technology has been improved by various delivery systems, direct transduction and small molecule regulation, low reprogramming efficiency and genomic modification steps still inhibit its clinical use. Improvements in current vectors and the exploration of novel vectors are required to balance efficiency and genomic modification for reprogramming. Herein, we set out a comprehensive analysis of current reprogramming systems for the generation of iPSCs from somatic cells. By clarifying advantages and disadvantages of the current reprogramming systems, we are striding toward an effective route to generate clinical grade iPSCs.

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First draft submitted: 6 April 2015; **Accepted for publication:** 4 September 2015; **Published online:** 18 December 2015

Keywords: efficiency • genomic modification • induced pluripotent stem cells • reprogramming system • vector

Induced pluripotent stem cells (iPSCs) paved the way for research fields including cell therapy, drug screening, disease modeling and the mechanism of embryonic development. Previously, nuclear transfer or the fusion with embryonic stem cells (ESCs) in somatic cells was fraught with technical, ethical, immune and logistical barriers [1]. Cell extracts from embryonic carcinoma cells or ESCs, which mediated nuclear reprogramming, constituted an attractive alternative to cell fusion or nuclear transfer. Notably, they upregulated ESC genes and downregulated somatic cell markers and epigenetically modified histones [2]. Thus far, these extracts have not successfully reprogrammed somatic cells into iPSCs with full differentiation potential.

Excluding embryonic materials has been deemed as the obligatory approach to obtaining available iPSCs. By the retroviral transduction of 24 candidate genes and subsequent narrowing down to four transcription factors (TFs), namely Oct4, Sox2, Klf4c, and c-Myc (OSKM), Takahashi and Yamanaka [3] made a breakthrough in 2006, converting mouse fibroblasts to iPSCs. These reprogrammed cells complied with the major aspects of pluripotency (Figure 1), including morphology, proliferation, pluripotent marker expression, self-renewal, multilineage potency and germ-line transmissibility, which were similar to ESCs [4]. Although the viral transduction of OSKM remains the most common strategy to provide a fast way to produce iPSCs with numerous therapeutic implications, poor reprogramming efficiency is still a key concern [5].

To improve the reprogramming efficacy, alternative factors and newer methods should be rigorously tested to ensure quality of the resultant iPSCs. For successful generation of iPSCs from mouse fibroblasts, Sox1 and Sox3 are perfect substitutes for Sox2; furthermore, L-Myc and N-Myc can replace c-Myc [4,6]. In addition, somatic cell reprogramming was originally achieved by gene delivery systems

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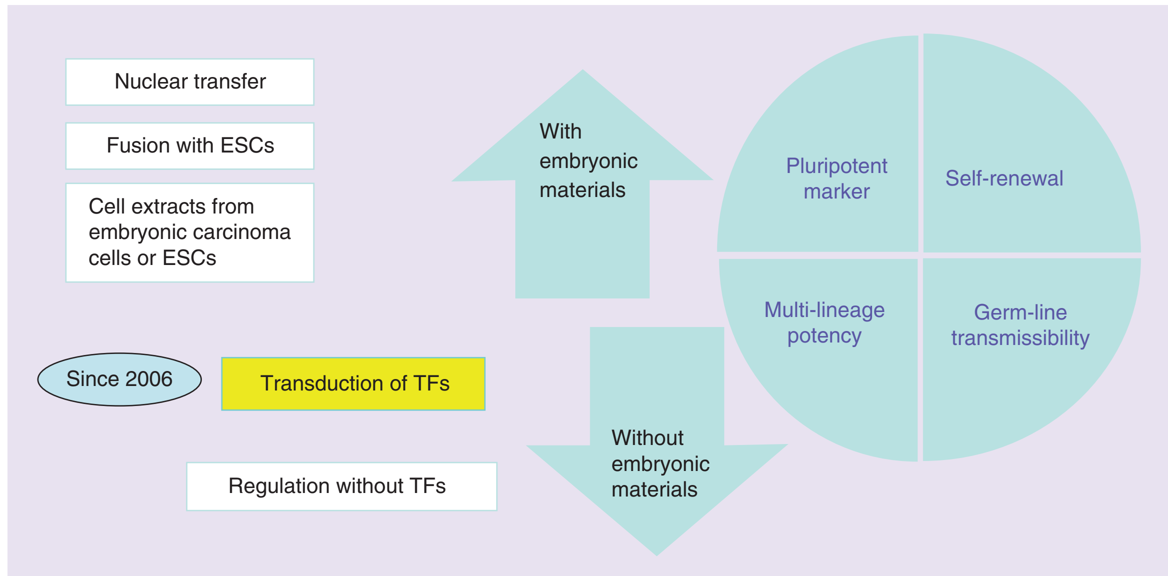

Figure 1. Reprogramming with or without embryonic materials and the characteristics of the resulting cells are demonstrated.
ESC: Embryonic stem cell; TF: Transcription factor.

via integrating viruses, but this resulted in integration into the host genome and caused random mutations within target cells [7]. Since the complete elimination of transgene integrations has been the major goal for delivery systems, several nonviral delivery systems for introducing TFs to somatic cells have been developed, with the aim of enhancing reprogramming efficacy and reducing abnormal chromosomes. In this review article, we set out a comprehensive analysis of reprogramming systems for the generation of iPSCs from somatic cells as a guide to the application of current generation systems **(Figure 2)**. By analyzing advantages and disadvantages of the current reprogramming systems, we are striding toward an effective route to generate clinical grade iPSCs **(Table 1)**.

The reprogramming kinetics & efficiencies

To better evaluate the reprogramming systems, investigators are seeking for various methods and markers to determine the efficiency in the reprogramming process. Epithelial characteristics and activation of some ESC markers are acquired in somatic cells after initiation of reprogramming through MET transition, which is deemed as a critical but nonessential step for reprogramming. Later, pluripotency-related genes are activated, and markers of AP, SSEA1, NANOG and the surface marker TRA-1-60 gradually turn to be expressed in the different reprogramming stages [8–11]. Cell surface markers of CD44 and ICAM1 can be used to indicate the gradual reprogramming process including mesenchymal state, epidermal state, early pluripotent state and late pluripotent state [12]. The ratio between the number of original cells receiving the set of TFs and the number of genuine iPSC colonies and the kinetics of reprogramming are important for the successful reprogramming, while they are hard to be measured. Besides, the donor cell type and defined culture conditions will undoubtedly influence the reprogramming efficiencies and kinetics. Compared with fibroblasts, human primary keratinocytes can be reprogrammed 100-times more efficiently than MEFs [13]. And the intrinsic epigenetic states in specific donor cells contribute to the higher efficiency, fewer TFs and the quality of the resulting iPSCs [14]. For example, neural stem cells with endogenous expression of Sox2 can be reprogrammed in the absence of Sox2 or with Oct4 alone [15,16]. Concurrently, an increase in proliferation rate and a decrease in cell size are molecularly accompanied with the sequential transition [17]. Telomerase reverse transcriptase and the SV40 large T antigen, which have positive effects on proliferation, can also increase the quantity of resulting iPSCs [18]. Small molecules and miRNA which are able to regulate the cell cycle may take effect to increase the number of fully reprogrammed colonies [19,20]. Intriguingly, hypoxic conditions [21], growth factors secreted by feeder cells [22] and additions in culture medium [23] can absolutely improve the reprogramming efficiency. The kinetics are regulated by multiple factors, consequently there is no golden standard for accurate evaluation about the reprogramming for various reprogramming conditions.

Viral vector approaches for reprogramming

During the reprogramming process, induction silencing occurs gradually but viral genes are expressed constitutively. Despite the possibility of making safe

iPSCs, nonintegrating viruses display a rather low gene transfer capacity and thus repeated infections are often required for many cell types. Consequently, retroviruses [24] and lentiviruses [25] are still the widely applicable delivery systems.

Retrovirus

As the most common choice in studies, retroviruses from replication-defective vectors can infect their target cells and deliver their viral payload but avoid cell lysis and death by inhibiting the lytic pathway. The infectivity of retroviruses is limited to dividing cells, thus the cell type for reprogramming is under restrictions. Retrovirus-mediated iPSCs stained positive for alkaline phosphatase, showed renewed expression of pluripotency genes, exhibited ultrastructural features including massive glycogen granules in the cytoplasm [26] and formed teratomas *in vivo* [27]. Recently a polycistronic cassette encoding four TFs separated by 2A peptides was tested in a retrovirus under an LTR or EF1α promoter, and the efficiency was much higher (up to 0.6%) than any other vectors [28]. In brief, the insertional mutagenesis, residual expression and reactivation of TFs, as well as titer loss during viral concentration and storage inhibited the infection of species and cell types resulting in reprogramming limitations.

Lentivirus

HIV1-based VSV-G-pseudotyped lentiviruses, as a subclass of retroviruses, are efficient and easy to transduce nondividing cells. However, the unpredictable integration will disturb the internal genes and bring about the activation of oncogenes. In the pluripotent state, their poor silence will make their constitutive versions less suitable for reprogramming attempts [29]. Adult mouse fibroblasts can be efficiently converted to iPSCs by using the Stem Cell Cassette (STEMCCA) polycistronic lentiviral vector [30]. Similarly, a single polycistronic Dox-inducible lentiviral vector was developed and successfully reprogrammed somatic cells with relative higher efficiency [31]. What is more, only a single proviral copy with high fidelity was required in the reprogramming process [30,31]. Cells reprogrammed with the stemgent human TF lentivirus set [32] began to show iPSCs morphology four days posttransduction. As it uses a type of retrovirus, this technique is limited by the same fundamental drawbacks. Moreover, its inefficient packaging cell lines also contribute to VSV-G toxicity.

Adenovirus

Adeno-associated virus (AAV) delivery systems lack pathogenicity, are capable of infecting dividing and

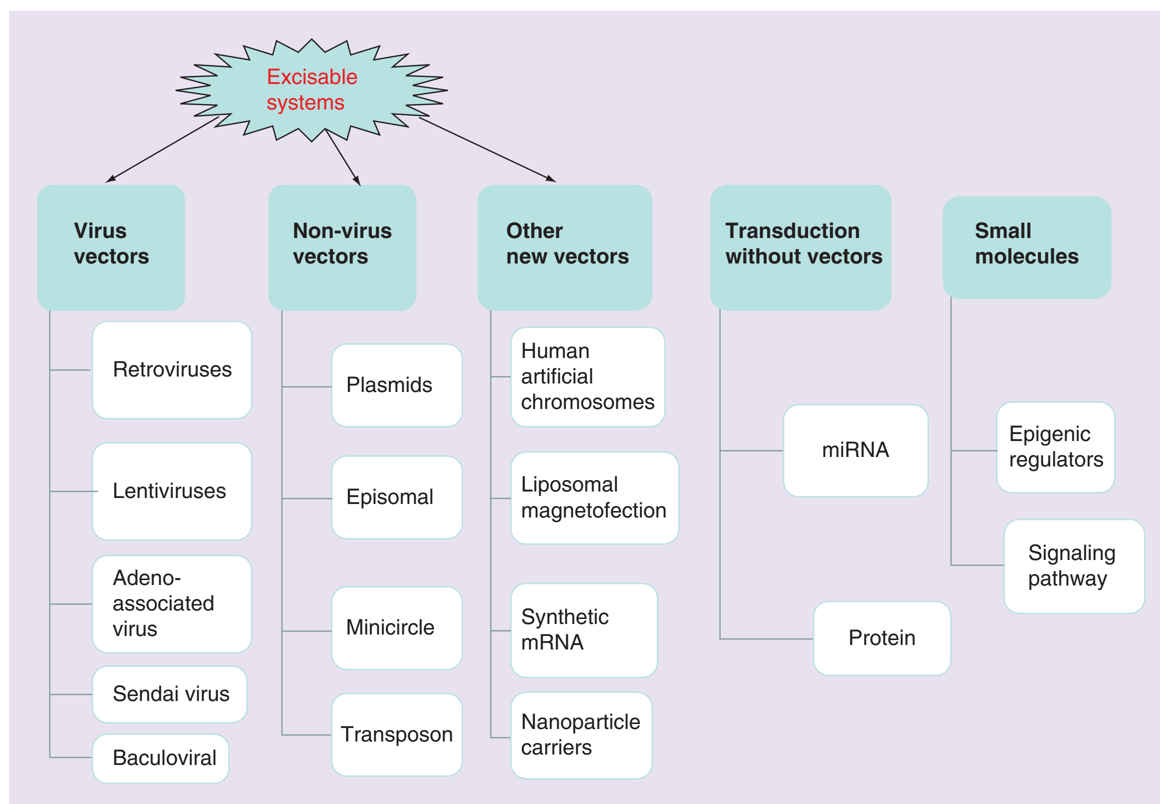

Figure 2. A comprehensive survey of reprogramming systems for the generation of induced pluripotent stem cells from somatic cells as a guide to the application of current generation systems.

Table 1. Advantages and disadvantages of the current reprogramming systems.

Classification	Vectors	Advantages	Disadvantages
Virus	Retrovirus	High efficiency, avoid cell lysis and death	Limited cell type, insertional mutagenesis, residual expression of TFs, titer loss during viral concentration
	Lentivirus	High efficiency, broadened tropism, ease of handling, availability of inducible systems	Unpredictable integration site, poor silence, insertional mutagenesis, residual expression of TFs, titer loss during viral concentration, inefficient packaging cell lines
	Adenovirus	Lack pathogenicity, broadened tropism, site-specific integration	Low efficiency
	Sendai virus	High efficiency, without integration into the host genome, broadened tropism	Sustained cytoplasmic replication of viral vectors
	Baculoviral	High efficiency, broadened tropism, site-specific integration, without appreciable cytotoxicity, flexibility in transgene exchange, low genome toxicity	Perturb the transcription of 12 genes involved in the Toll-like receptor signaling pathway
Nonviral approaches	Standard plasmids	Without integration into the host genome, without chromosomal abnormalities	Low efficiency, additional TFs requirement, result in cell death, transiently express transgenes, low efficiency, insufficient excision of integrated vectors
	Episomal plasmids	Simplest disintegration approach, reasonable efficiency, broadened tropism	Low efficiency, repetitive induction, insufficient excision of integrated vectors
	Minicircle plasmids	Nonintegrating, inexpensive, low immunogenicity	Low efficiency, occasional integration, additional TFs requirement, multiple transfections, insufficient excision of integrated vectors
	Transposons	Higher efficiency than plasmids, broadened tropism	Mutations in the genome, low efficiency, repetitive induction, insufficient excision of integrated vectors
Other burgeoning delivery systems	miRNA	Small, without genome integration, readily synthesized, function longer than the coding RNAs,	Low efficiency, transient action, multiple transfections
	Artificial chromosome system	Episomal transmission, transfer of multiple large transgenes long-term stable maintenance of single copy episomes, without integration into the host chromosomes, can be transferred from one cell to another	Low efficiency, limited by technical difficulties of gene loading into the HAC, ill-defined structures
	Synthetic messenger RNA	High efficiency, without transgene integration, rapid kinetics, obviation of a clean-up phase to purge the vector.	Relatively laborious
	Liposomal magnetofection	How efficiency, stable, integration-free, under the least toxic conditions	The distribution of aggregate complexes over the cell surface may be ununiform
Direct protein transduction		Without the genetic modification, simpler and faster	Low efficiency, require repetitive induction, and/or produce insufficient excision of integrated vectors
Small molecules		High efficiency, improve the efficiency of TF-mediated reprogramming, reduce the transcriptional factors	Unable to recapitulate the series of TFs and generate iPSCs with full pluripotency and differentiation potency

HAC: Human artificial chromosome; iPSC: Induced pluripotent stem cell; TF: Trancription factor.

nondividing cells and can stably integrate into the host cell genome at a specific site, which distinguishes them from lentivirus-based approaches. Adenoviruses can infect all cell types with the exception of some lymphoid cells, and their gene expression is not consistently and sufficiently long enough within this system. Transgene-free human iPSCs can be generated through the site-specific integration and excision of transgenes combined with the LoxP/Cre system. AAV serotypes 2 and 6 were superior to other serotypes in their transduction efficiency, and this is correlated with the abundance of their respective receptors [33]. Even so, the reprogramming efficiency of these two serotypes is very low both in mouse and human cells [34,35]. As an alternative for standard adenovirus, introducing artificial DNA double-strand breaks is unnecessary in the reprogramming process by helperdependent adenoviral vector (HDAdV), and 7–81% of colonies were gene-targeted for complete iPSCs generation [36,37]. In consequence, considerable works are obligatory for optimized transgene expression and higher efficiencies in the reprogramming process.

Sendai virus

The Sendai virus vector, a negative-strand RNA virus in the paramyxovirdae family, is nonpathogenic to humans. It will replicate in the cytoplasm of target cells but does not go through a DNA phase [38]. It is gradually depleted from the iPSCs cytoplasm after several passages, efficiently generating transgene-free iPSCs starting with different cell types as well as in feeder-free conditions [39,40]. During the division of iPSCs, although viral vectors were slowly diluted, the sustained replication of viral vectors had to be cleared [41]. In a cost-effective manner, this vector efficiently demonstrates constant reprogramming results [42]. Then temperature-sensitive mutations, which can accelerate future clinical application of iPSCs by less invasive methods, were introduced for the complete removal of viral constructs at nonpermissive temperatures [43].

Baculoviral

In addition to mentioned viral vectors, baculoviral (BV) can transduce various mammalian cells without considerable cytotoxicity [44,45]. This virus delivers genes with high efficiency in human ESCs and delivers genes in almost all medaka ESCs [46]. After three successive transductions of mouse embryonic fibroblasts (MEFs) with BacMam particles, iPSCs colonies were generated and the efficacy was shown to be increased to 64–98% [47]. Although BV may trigger innate responses in mammalian cells [33,34], the transduction of MSCs activates only slight and transient responses in the Toll-like receptor 3 pathway [48], and no well-known cytokines and sensors or their downstream signaling mediators were altered by this way [49]. Recently, BV transduction successfully reprogrammed human fibroblasts by site-specific integration into the AAVS1 locus [50]. Attributing to the high integration efficiency, flexible transgene exchange and low genome toxicity, BV-transcription activator-like effector nuclease system may offer great potential for precise genetic manipulation in iPSCs generation [51,52]. To promote the technology far away, BV as transgenic vector of radionuclide reporter gene imaging technology bring up to monitor stem cell transplantation therapy [53].

Nonviral approaches to reprogramming

To avoid interference with the host genome during the reprogramming process, safer methods must be developed. Several nonviral vectors including plasmids [54], episomal vectors [55], minicircles [56] and transposons [57] have been described for iPSC reprogramming. However, these nonviral approaches are inefficient, require repetitive integration, and produce deficient excision of vectors.

Standard plasmids

Plasmids are nonvirus vectors that do not integrate into the genome of iPSCs and produce chromosomal abnormalities [58] but are characterized by low reprogramming efficiency [59]. Their occasional genomic integration requires additional TFs and results in cell death when nucleofection occurs. Most regular plasmid vectors lack the ability to replicate themselves in mammalian cells, leading to gradual cell division, and even then, they only transiently express transgenes. Established iPSCs are morphologically similar to ESCs, and express pluripotent markers of ESCs at comparable levels [60]. To ensure efficient and controlled generation, reprogramming plasmids have been equipped with a particular bacteriophage site and a specific expression vector to enhance integration into the genome [61].

Episomal plasmids

The Epstein–Barr nuclear antigen-1-based episomal system, a simplest disintegration approach, indicates appreciable efficiency while only requires one transfection with Maxiprep DNA. The vectors have been extensively used to generate footprint-free iPSCs, replicate themselves autonomously as extrachromosomal elements in both dividing and nondividing cells, persist throughout reprogramming and subsequently diminish in iPSCs [62]. As current protocols of generating integration-free human iPSCs from keratinocytes are generally inefficient, the simple transfection of episomal vectors was able to achieve a reprogramming efficiency of approximately 0.14% on average [63]. The delivery of episomal vectors into cells

may be a problem for primary somatic cells, which may be solved by using the adenovirus episomal vector hybrid system [64], a system utilizing Cre-mediated site-specific recombination to excise an episomal vector from a target recombinant adenovirus genome. In summary, episomal vectors are superior to conventional plasmid vectors because of the increased duration of reprogramming factor expression in target cells.

Minicircle plasmids

Minicircle plasmids are nonintegrating and inexpensive delivery vectors of low immunogenicity, but protocols for their use are inefficient, result in occasional integration, and require additional factors and multiple transfections. Minicircles are special episomal DNA vectors devoid of any bacterial plasmid backbone [65] and are significantly smaller than standard plasmids. The repeated transfection of minicircle DNA vectors into somatic cells and abundant cell sources are amenable to efficient reprogramming into transgene-free iPSCs [65]. In comparison with standard plasmids, minicircle DNA benefits from higher efficiency and longer ectopic expression but accompanied with lower activation of exogenous silencing [56], which enhances its transfection efficiency and the survival rate of target cells. Although the minicircle theoretically should not integrate into the target cells, there is still a relatively small chance of integration. Intriguingly, the minicircle-based generation of iPSCs is compatible with the production of chicken chimeras [66].

Transposons

Transposons are able to move from one locus to another within the chromosome and cause mutations or genomic rearrangements within the genome. First discovered by Barbara McClintock in 1950 [67], they can be grouped into two classes: class I copy themselves after being firstly transcribed to RNA, then being reversely transcribed to DNA, finally they are inserted at another position into the genome; class II move directly from one locus to another but excise themselves from the original location and insert themselves into a new locus. Transposases are normally located at each end of the transposon, can act on almost any DNA sequence which is flanked by the terminal repeat sequences [68] and mediate a higher genome integration efficiency than plasmids [69]. Genetic screens conducted on this transposase have since resulted in a hyperactive variant capable of efficient transposition in vertebrates and mammalian cells [69], enabling novel methods of genetic engineering in animal models, a variety of human cell types and gene therapy trials [70]. Furthermore, following stable genomic integration, the reexpression of the transposase can result in transposon excision [71]. The piggyBac (PB) transposon belongs to the class II mobile genetic elements and requires only the inverted terminal repeats (ITR) and active transposase to catalyze insertion and/or excision [69,70]. The unique characteristics of PB transposons including efficient genomic integration, unlimited cargo capacity, robust gene expression, and even seamless excision [72] make this system one of the best choices for generating 'genetically clean' iPSCs. The use of PB in a plasmid containing both a transposase and transposon greatly increased the probability of transposase integration, but using a transposon and transposase from separate vectors circumvented this. In addition, the delivery of PB plasmid vectors into cells is dependent on transfection reagents, and the insertion sites in each cell are uncontrolled. The Sleeping Beauty (SB) transposon system was reconstructed from fragments belonging to the Tc1/mariner superfamily and resembles an ancestral transposon [73]. The SB transposon does not exhibit an integration bias towards particular genetic elements, thereby reducing the risk of insertional mutagenesis. Furthermore, unlike the alternative transposon PB, SB has no SB-like elements within the human genome, which minimizes the possibility of mobilizing endogenous transposon elements [57]. The SB transposon-reprogrammed iPSCs showed long-term proliferation *in vitro* over 40 passages and expressed typical surface markers of ESCs [74]. Together with its simple and inexpensive production, SB-mediated gene transfer can be used to generate mouse iPSCs from different genetic backgrounds [75].

Excisable systems

Cre–loxP system

Cre-deletable systems have made it possible for the removal of the integrated transgenes from the genome [64]. During the normal viral reverse transcription cycle before integration, the loxP sequence is duplicated into the 5′ LTR region to create a loxP-flanked version, and then integrates into the targeted genome. The deletion of the loxP-flanked transgene cassette requires the introduction of Cre recombinase activity, which has been accomplished with Cre-encoding plasmids [76], lentiviral Cre constructs [64] and adenoviral Cre constructs [77]. By contrast, the delivery of Cre mRNA [78] to obtain transgene-free iPSCs involves the daily transfection of mRNA for a week to perform excision, so this mRNA-mediated progress is more inefficient, laborious and less appealing. Then, transgene-free iPSCs can be obtained by treatment with Cre recombinase and selection of excised iPSC clones. Both excised and non-excised iPSCs expressed pluripotency markers and were able to differentiate *in vitro*, and non-excised cells can form germ-line competent chimeras *in vivo* [64]. More recently, by a single application

of TAT–Cre recombinant protein for 5 h, the process of obtaining transgene-free iPSCs with minimal technical complexity was accelerated [79]. Cre recombinase resulted in multiple transgene excisions, potentially leading to genome rearrangement and genomic instability. The efficient and reliable induction of Cre recombinase activity in loxP-modified iPSCs and subsequent selection of cleaned clones represents a roadblock for the widespread use of Cre-deletable systems [79].

Exercisable site-specific integration

Although the nonintegrating methods are rapidly becoming a standard approach, methods based on the site-specific integration of reprogramming factor genes hold the potential for the efficient generation of genetically amenable iPSCs suitable for future gene therapy applications. As a class of artificial restriction enzymes, transcription activator-like effector nucleases (TALENs), can be efficiently delivered by the type III secretion system [80], and significantly promote homologous recombination over 1000-fold. A recent study using plasmid transfection of human primary cells has demonstrated the generation of iPSCs by zinc finger nuclease (ZFN)-mediated targeted insertion of TF genes into the CCR5 locus, though a relatively low reprogramming efficiency was reported [81]. It appears that the low cotransfection efficiency of ZFN and a large donor DNA carrying TF genes represents a major obstacle for the reprogramming of human primary cells by ZFN technology. The expression of TFs can be efficiently accomplished in almost every transduced cell when combining with a single polycistronic vector by inserting a 'self-cleaving' 2A peptide or an internal ribosome entry site sequence between two consecutive open reading frames [31].

Other burgeoning delivery systems

miRNA transduction

miRNAs, which are very small, can be readily synthesized and delivered into cells. After that, they remain stable for several days and function longer than the coding RNAs but without risk of genome integration. Specific miRNA mimics or miRNA inhibitors promote the reprogramming of somatic cells into iPSCs [82]. The overexpression of miR-302a, miR-302b and miR-200c can improve the reprogramming efficiency but reduce the handling time and tumorigenicity efficiently [83]. In an episomal system, ESC-specific miRNAs (miR-302/367 cluster) increased the iPSC colony-forming efficiency in fibroblasts and epithelial cells [84]. Another ESC-specific miRNA (miR-294) can replace exogenous c-Myc in the reprogramming of MEFs towards iPSCs and improve the reprogramming efficiency without c-Myc. miR-302b, which shares the same seed sequence as miR-294, can also improve the reprogramming efficiency [20]. Based on the regulation of the miRNA processing, Lin28 can replace Klf4 and c-Myc and improve the reprogramming efficiency in combination with Nanog [85]. Miyoshi *et al.* generated mouse and human iPSCs by direct delivery of ESC-specific miRNAs without any vector-based gene transfer [86]. However, attributing to the transient action, multiple transfections are required for complete reprogramming with miRNAs.

Artificial chromosome system

Human artificial chromosomes (HACs), which can be transferred from one cell to another, are used for episomal transmission and the transfer of multiple large transgenes. The functional centromere of HACs enables long-term stable maintenance of single copy episomes but without integration into the targeted genome. Despite these obvious advantages over viral vectors, the use of HACs for reprogramming was limited by technical difficulties of transgene insertion and the undefined structures [87,88]. Recently, by the use of 21HAC vector, MEFs was successfully reprogrammed to iPSCs [89]. Global gene expression patterns demonstrated that the HAC-based iPSCs are relatively uniform at a level comparable to retrovirus-based iPSCs [89]. Next, the cells which spontaneously lost the HAC were isolated and, consequently, HAC-free iPSCs were established [89]. HAC1 carried four TF partially reprogrammed MEFs, but HAC2 carried four TFs and a p53-knockdown cassette efficiently reprogrammed MEFs [89]. Satellite-DNA-based artificial chromosomes (SATACs) have already passed the obstacles, including large-scale purification, transfer into various cells and embryos, germ-line transmission and generation of transgenic animals [90]. The reprogramming of MEFs was efficiently induced by HACs with engineered OSKM factors carrying an N-terminal flag-tag and a C-terminal polyarginine tail [91].

Synthetic messenger RNA

The use of synthetic mRNA to generate iPSCs is extremely attractive for regenerative medicine, which benefits from the avoidance of common drawbacks in DNA-mediated or virus-mediated reprogramming strategies. Exogenous DNA must be delivered into the cytoplasm and placed into the chromosome for successful reprogramming. In contrast, exogenous mRNA only needs to be transmitted through the cell cytoplasm and leaves the integration out undoubtedly. In contrast to retrovirus-derived iPSCs, synthetic mRNA-derived iPSCs do not differ significantly from the parental fibroblasts. Thus synthetic mRNA gradually became an important alternative to DNA-based integration for cell reprogramming. Furthermore, hepatic differentiation studies indicated that mRNA-based iPSCs can

differentiate into hepatoblasts efficiently [92]. Synthetic mRNA-based integration-free techniques successfully generated iPSCs from adipose tissues of a patient under feeder-free conditions and put forward iPSCs as a potential personalized regenerative medicine [93]. This system can reprogram enormous cell types to pluripotency with high efficiency and direct the resulted iPSCs into terminally differentiated cells [94]. Notable advantages of the mRNA approach include high efficiency, rapid kinetics, and obviation of a clean-up phase to purge the vector. Still, this method is relatively laborious, but when reprogramming without feeders, there is reduced labor and material costs [95].

Liposomal magnetofection

Liposomal magnetofection (LMF) is based on the use of superparamagnetic particles and cationic lipids and shows better transfection efficiency than other nonviral delivery systems; however, the ununiform distribution of aggregate complexes on the cell surface should be eliminated. Under a dynamic gradient magnetic field, the transfection was less cytotoxic and the efficiency was greater by almost 21 and 42% in comparison with LMF and lipofection, respectively [89]. LMF based-iPSCs are able to present similar characteristics to ESCs, including cellular morphology, surface marker expression, embryoid body formation, teratoma formation, direct differentiation into terminal cells, and chimeric mouse production [96]. Park *et al.* produced a stable and integration-free iPSC line by a single LMF procedure and a half-dose of plasmid, while the *in vitro* and *in vivo* pluripotency were similar to other cell lines. Thus, LMF may represent an outstanding technique for the generation of virus-free iPSC lines and could lead to enhanced stem cell therapy [96].

Synthetic carriers

There is growing applications in nanoparticle and synthetic carriers as reprogramming systems for generation of iPSCs. For instance, after retinoic acid (RA) was efficiently incorporated into poly(N-isopropylacrylamide)-co-acrylamide nanoparticles, this nanoparticle could be a potentially powerful carrier for effective RA delivery to direct human iPSC fate to the neuronal lineage [97]. Tavernier *et al.* generated mouse iPSCs from MEFs using a different cationic lipid carrier fused with OSKM mRNAs [98].

Direct protein transduction

Protein delivery, without genetic modification, provides a substantially simpler and faster approach than the currently progressive genetic reprogramming systems, but the efficiency is too low to be practical for research and clinical applications. Protein transduction of TFs tagged with polyarginine has generated mouse iPSCs in the presence of valproic acid (VPA) [99] and generated human iPSCs without VPA [100]. By fusing in frame to a glutathione-S-transferase tag and to the transactivator transcription-nuclear localization signal polypeptide, recombinant OKSM proteins successfully generated stable iPSCs [101]. After optimizing cationic bolaamphiphile-protein complex ratio to 7:1 and incubating for 3 hours, the reprogrammed human fibroblasts were shown to exhibit the characteristics of ESCs, including the expression of pluripotent genes, teratoma formation, and differentiation into various terminal cells [102].

Although protein transduction is able to convert the immature fetal cells, adult somatic cells are difficult to be reprogrammed [18]. More recently, Human umbilical cord blood neural stem cells have been successfully reprogrammed with HEK293cell extracts containing three TFs recombinant proteins in combination with additional small molecules under low oxygen condition [103]. What's more, after the cell penetrating TAT domain from HIV1 to be conjugated with cationic liposomes or combination with VPA, the transduction efficiency was increased [104,105]. In the absence of any chemical treatment, the system may allow the translation of iPSC technology into the clinical applications [94,100]. However, to successfully reprogram somatic cells to pluripotent state, purification of sufficient desired proteins is necessary.

Small molecules

There is growing evidence indicating that small molecules may revolutionize the iPSC field by replacing current delivery systems and extremely enhancing reprogramming efficiency. They are particularly useful for partially reprogrammed cells and cells resistant to reprogramming. A majority of these chemicals are inhibitors of epigenetic regulators and inhibitors of signaling pathways.

Epigenetic regulators

To potentially reprogram somatic cells by sole chemical supplements, high-throughput screening technologies can be used to identify detailed small molecules for modulating the expression and regulating pluripotency. Huangfu *et al.* [106] demonstrated that the treatment of MEFs with a histone deacetylase inhibitor (VPA) could improve the reprogramming efficiency in OSKM- and OSK-infected MEFs by 100-fold and 50-fold, respectively. Although overexpression of Mbd3/NuRD does not have any positive or negative effect on iPSC induction efficiency, combined with Nanog overexpression improves both reprogramming kinetics and efficiency [107]. However, another recent study reported that Mbd3/NuRD is required for effi-

cient iPSC generation from neural stem cells, pre-iPSCs and epiblast-derived stem cells [108]. In the context of iPSC reprogramming, combined overexpression of the histone variants TH2A and TH2B, the efficiency of iPSC generation was improved and further enhanced by additional overexpression of the phosphorylation-mimic form of nucleoplasmin through the induction of an open chromatin structure [109]. More recently, Li *et al.* [110] demonstrated that under the transduction with lentiviral vectors expressing only Oct4, treatment with small molecules is sufficient to generate functional iPSCs. Intriguingly, the combination of VPA, CHIR99021, 616452, tranylcypromine, forskolin and dznep can reprogram MEF into iPSCs with 2i media [111]. What is more, under the condition without any transgene, mouse iPSCs were efficiently generated only with a combination of seven small molecule compounds [9].

Signal pathways

In the same manner, specific signaling modulators are also sufficient to generate functional fibroblast-derived iPSCs [112]. The combination of TGF-β, WNT and FGF pathways resulted in regulating pluripotency in different species [113]. With the combination of TGF-β receptor inhibitor, MEK inhibitor and thiazovivin, the reprogramming efficiency was improved for more than 200-fold [114]. Recent studies demonstrated that the Ink4/Arf and p53–p21 pathways serve as a barrier to iPSC generation [115–117]. Thus, it will be worthwhile to test whether the combination of transient p53 inhibition and delivery of reprogramming factors via nonintegrating vectors could generate genetic-modification-free human iPSCs with a higher reprogramming efficiency. Furthermore, the signaling pathway regulators can also eliminate the requirement for transduction with certain reprogramming factors. In the absence of exogenous c-Myc, Wnt3a-conditioned medium can also help to reprogram somatic cells with high efficiency [23]. In addition, the MEK and TGF-β pathways without delivery of exogenous transcription factors efficiently generated iPSCs [118]. Above all, small molecules are the most promising resources for successful reprogramming of high-quality clinical-grade iPSCs with a minimum of genomic operation. However, it is currently unknown whether small molecules alone can recapitulate the series of TFs and generate iPSCs with full pluripotency and differentiation potency.

Epigenetic barriers & reprogramming process

The epigenetic status may be altered during reprogramming process [119], chromatin remodeling complexes and certain histone variants play important roles in the acquisition and subsequent maintenance of the permissive pluripotent chromatin state [120]. Reconfiguration of chromatin structure including DNA methylation, histone modifications and nucleosome remodeling come out after initiation of reprogramming. Repres-

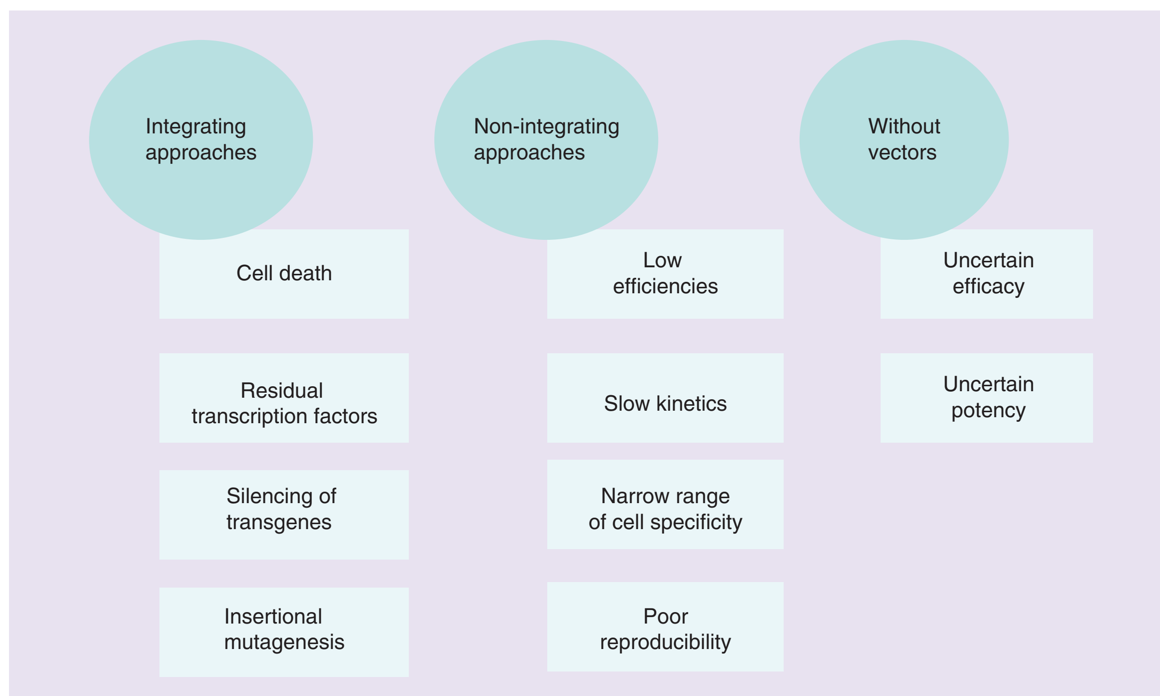

Figure 3. The obstacles exist in systems with or without vectors for induced pluripotent stem cells generation.

sive chromatin comprises a major mechanistic barrier in reprogramming process [121,122], and global DNA demethylation is a conserved and required feature of reprogramming [123]. Most histone variants incorporated into chromatin in a replication-independent manner and may contribute to the epigenetic barrier during reprogramming [124]. Additionally, both repressive H3K9me2/3 histone methylation and the presence of 5mC may act as a barrier to the reprogramming process [125,126]. In the initial phase of reprogramming, rapid genome-wide changes of H3K4me2 distribution are one of the earliest events [17], then dramatic changes at promoter and enhancer regions of more than a thousand genes were observed. On the other hand, NANOG overexpression and inhibition of DNA methylation synergistically enhance the final phase of reprogramming [127]. Clearly, reprogramming process was accompanied by silencing of somatic cell genes, resetting of pluripotency, and altered epigenetic status. In the contrast, evidence appeared to show that some epigenetic alteration is not obligatory for successful reprogramming. Although somatic methylome is altered after initiation of reprogramming, *de novo* deposition of methylation is not a requirement [128]. Depletion of Tet1 and Tet2 may result in significantly reduced efficiency of iPSC colony formation [129], while they are clarified to be only necessary for somatic cells to undergo the MET during iPSC reprogramming [129].

To clarify the detailed transition during the reprogramming process, comparative analysis of genetically matched mouse ESCs and iPSCs was performed and revealed identical transcriptional and methylation profiles [130], while in other cases, iPSCs are not identical but with transcriptional, epigenetic and phenotypical heterogeneous lines when compared with ESCs [131]. The differences between ESCs and iPSCs may be due to the preexist mutations in original cells or long time culture or the reprogramming technology [132]. With integrative vectors, the reprogrammed cells tend to be heterogeneous by transgene insertions [133]. By a whole exome sequencing of human foreskin fibroblasts and their derived iPSCs, the aberrations can be attributed to *in vitro* passaging for 7%, preexist mutations in the parental fibroblasts for 19%, and the remaining 74% of the mutations were acquired during cellular reprogramming. Another report suggested that the mutation intensity during reprogramming is nine fold higher than the background mutation rate in culture [134]. What is more, genomic copy number variation rates were negatively associated with the dosages of TFs, and high-performance engineered factors may result in less genomic copy number variation rates than the classic TFs at the same dosage [135]. The genomic integrity of the partially purified reprogramming protein-based mouse iPSCs was compared with mouse iPSCs developed from viral-based strategies, and they were able to maintain genomic integrity better than current viral reprogramming methods [136]. While some researchers demonstrated that most of the genetic variation in iPSC clones is not caused by reprogramming *per se*, but is rather a consequence of the mutational history derived from individual cells [137]. In consist with this, one case demonstrated that genome stability can persist throughout reprogramming, and it is possible to generate iPSCs without gene mutations with current reprogramming methods [138]. Taken together, it is obligatory to cross epigenetic barriers in somatic cells for successful reprogramming, but various delivery systems will lead to epigenetic alterations in resulting pluripotent cells.

Conclusion & future perspective

Somatic cell reprogramming was originally achieved by gene-delivery systems via integrating viruses. To apply these systems to clinical usage, obstacles including resulting cell death, remnant expression of transgenes, immunogenicity and insertional mutagenesis should be stepped over for generation of virus-free and transgene-free iPSCs. One major goal of reprogramming research is to eliminate or reduce transgene integrations since the advent of iPSC technology. There is no gold standard for an iPSC reprogramming strategy because these nonintegrating approaches exhibit limitations such as low reprogramming efficiencies, slow reprogramming kinetics, a narrow range of cell specificity, and poor reproducibility [79]. Thus, gene-delivery reprogramming approaches remain major strategies for generation of iPSCs for basic research. Excisable vectors are applicable for most virus-based systems; once the efficiency of disintegration vectors, miRNA mimics, direct protein transduction and small molecules is enhanced, the alternative routes may be the most promising approaches to avoid genome alterations. The obstacles to overcome in all of the systems for iPSC generation are summarized in Figure 3. In addition to the mentioned delivery systems, these values are also subject to the donors' age, the different combination of reprogramming factors, the somatic cell types and the passage number of target cells. Meanwhile, reprogramming systems absolutely overcome the epigenetic barriers and may lead to epigenetic abbreviations. Considering the mentioned factors for successful and available reprogramming, the further optimization of the reprogramming protocols, accompanied with a thorough analysis of the generated iPSCs, will facilitate the clinical applications of the iPSC technology and produce desired terminal cells for regenerative medicine.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assitance was used in the production of this manuscript.

Executive summary

Various reprogramming systems & induced pluripotent stem cell generation

- Induced pluripotent stem cell (iPSC) technology has been improved by various reprogramming systems.
- Low reprogramming efficiency and genomic modification steps still inhibit clinical use of iPSCs.
- One major goal of reprogramming research is to eliminate or reduce transgene integrations since the advent of iPSC technology.

Epigenetic status & iPSC generation

- It is obligatory to cross epigenetic barriers in somatic cells for successful reprogramming.
- The epigenetic status may be altered during reprogramming process.

Conclusion & future perspective

- Advantages and disadvantages of the current reprogramming systems may help scientists to generate clinical grade iPSCs.
- Improvements in current vectors and the exploration of novel vectors are required to be investigated thoroughly.

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Sleeping Beauty transposon system – future trend in T-cell-based gene therapies?

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Evaluation of: Huang X, Wilber AC, Bao L *et al.*: Stable gene transfer and expression in human primary T cells by the Sleeping Beauty transposon system. *Blood* 107, 483–491 (2006). The Sleeping Beauty (SB) transposon system can mediate stable gene transfer and expression in primary human T cells. Optimal *in vitro* conditions for maximum gene transfer efficiencies have been developed with regard to further application of the SB transposon system in T cell based gene therapies. This raises the question of whether or not the SB transposon system is a convincing alternative for virus-mediated gene transfer based on the currently available data. Here, we will discuss controversial safety and efficacy issues in transposon and viral gene transfer technology.

Transposons are discrete nucleic acid fragments that are able to move and replicate in the genome by a cut-and-paste mechanism. In their natural form, DNA transposons consist of a single gene encoding the transposase enzyme that is flanked by terminal inverted repeats (IRs). The transposase gene can be artificially separated from the IR-containing binding sites and replaced by a gene of interest. Under this condition, the transposase gene can mobilize transposons *in trans* as long as they retain the IRs. In flies and worms, endogenous DNA transposons have been utilized for germline transgenesis and insertional mutagenesis. However, thus far, no functional DNA transposons have been found in vertebrate genomes. By eliminating the inactivating mutations in vertebrate transposon relics found in the fish genome, the Sleeping Beauty (SB) transposon has been molecularly reconstructed [1]. The mediation of precise cut-and-paste SB-transposition in fish, as well as in mouse and human cells, has been demonstrated, providing the basis for a potential novel gene therapeutic approach in vertebrates. Since then, the SB transposon has been the subject of numerous studies. It has been shown to mediate transposition and long-term expression in cultured mammalian cells [1–3]. *In vivo* experiments were performed in mice with transposition directed to liver and lung tissues [4,5]. Chromosomal transposition of SB has been accomplished in mouse embryonic stem cells [6]. Furthermore, mouse germline-transmission and expression from transformed elements [7] and insertional mutagenesis was demonstrated [8].

With the perspective of future nonviral T-cell-based gene therapies, Huang and colleagues raised the question of whether the SB transposon system can mediate stable, long-term gene expression in primary human T cells *in vitro* [9].

Nucleofection of primary blood lymphocytes (PBLs) has been performed with SB plasmid vectors that do (pT2/DsRed//-*SB10*), or do not (pT2/DsRed), contain the *SB10* transposase gene in addition to a red fluorescent protein (DsRed) coded reporter gene. Dose response experiments with different amounts of SB transposon (20, 10, 5, 2.5, 1.2 and 0.6 μg/5×10^6 PBLs) exhibited more DsRed$^+$ cells at day 9 after transfection with pT2/DsRed than with pT2/DsRed//-*SB10*. However, DsRed expression after pT2/DsRed transfection declined and became undetectable, whereas reporter gene expression was maintained in cells after pT2/DsRed//-*SB10* transfection on day 50 in culture. The highest level of DsRed$^+$ cells under these conditions has been accomplished with 10 μg of SB10-containing transposon.

Huang and colleagues analyzed the effect of *trans*-delivery on stable gene expression in human PBLs by conucleofection of 5 μg SB transposon (pT2/DsRed) and varying amounts of vector expressing SB10 (20, 10, 5, 2.5 and 0 μg/5×10^6 PBLs) or the improved SB11 transposase [2]. By contrast with nucleofection of pT2/DsRed alone, by which the DsRed expression was lost, conucleofection with the transposase encoding plasmid maintained the transgene expression. The most effective transfection in primary T cells was achieved using 10 μg of SB10-transposase-encoding plasmid with *trans*-delivery (11%) versus *cis*-delivery (3%) of SB-transposase after 21 days.

To study the conditions by which both CD4$^+$ and CD8$^+$ T cells express the reporter gene, Huang and colleagues nucleofected PBLs with

Keywords: cancer, gene therapy, gene transfer, integration, retrovirus, safety, Sleeping Beauty, T cell receptor, transduction, transposon

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pT2/DsRed only or with pT2/DsRed plus the SP10-containing vector, and performed immunophenotyping by flow cytometry. Both CD4+ and CD8+ T cells expressed DsRed on day 1, independently of the nucleofection conditions used. On day 29 however, CD4+ and CD8+ T cells expressed DsRed only after conucleofection with a combination of both pT2/DsRed plus SB10-containing vector.

For a molecular analysis of transposition, T cell clones were generated after conucleofection with pT2/DsRed and SB10-containing plasmid. A total of six DsRed+ clones were identified and maintained in culture for up to 4 months with stable reporter gene expression. Southern blot analysis revealed 4–19 copy numbers of integrated transgene in all of the six clones. Splinkerette polymerase chain reaction (PCR) recovered the up- and downstream flanking regions of the transposon inserts. Typical TA-dinucleotide insertion sites and parts of human chromosomes were identified, precluding the presence of ectopic plasmids as the cause of reporter gene expression.

Owing to the usual requirement for coexpression of several genes in gene therapy approaches, Huang and colleagues developed SB transposon vectors expressing multiple genes. Neither the commonly used internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV), nor the 18 amino acid 2A self-cleaving oligopeptide from the foot-and-mouth disease virus proved to be suitable for the expression of multiple genes in the context of the SB transposon. However, the integration of a synthetic bidirectional promoter used in lentiviral vector systems can mediate the stable expression of two gene products in human primary T cells after nucleofection. For that purpose, PBLs were cotransfected with an SB10-containing vector and a bidirectional SB vector containing the gene of the enhanced green fluorescent protein (EGFP) and the nerve growth factor receptor (NGFR). It was found that 3 weeks after transfection, 2–4.7% of PBLs were positive for EGFP and NGFR.

A bidirectional SB transposon containing the *luciferase* gene, a fusion gene of the NGFR and the cytosine deaminase (NGCD) [10], were generated to demonstrate the expression of a therapeutically relevant gene product via transposition. Conucleofection of this SB transposon with the SB10-containing vector resulted in 6.3 and 8.5% NGFR expressing T cells on day 7. In a cytotoxicity assay, single cell-sorted NGFR+ T cells were killed by 5-fluorocytosine (5-FC) in a dose-dependent manner. However, luciferase activity in the NGFR+ T cells was low, indicating either a low susceptibility for luciferase expression, or the inefficiency of this bidirectional SB transposon.

Gene therapeutic technologies are required to meet widespread safety and efficiency concerns. Although the demands for current approaches are high, gene transfer via transposition as well as by retro- or lenti-viruses bear as yet unsolved problems.

Due to the safety concerns in gene transfer approaches, research focussed on the insertion sites of the SB transposon. Recently, a large-scale, genome-wide analysis of SB transposon integration in mammalian cells was accomplished [3]. DNA from more than 1300 independent, SB-mediated integrations in human and mouse cells was isolated and mapped to their respective genomes. SB integration was found not to be random, showing statistical target site preferences. The SB integration is significantly biased towards AT-rich palindromes, which are particularly susceptible to local melting and have been shown to adopt a bendable DNA structure. Furthermore, SB integration shows a small statistical bias towards transcriptional units and their upstream regulatory sequences. However, 96% of the insertions that occur in genes are located in introns. This can be explained by base composition and length that make introns more attractive targets for the transposons than exons or promoters. With respect to intergenic regions, there is a strong bias towards microsatellite repeats. There is no indication that SB transposition would be accompanied with recombination or deletion events at the integration sites. Owing to the observed integration preference for bent DNA, it can be assumed that physical properties may be the major factor determining SB target site selection [3,11].

Considering these results, transposons seem to be safer vehicles for therapeutic gene transfer compared with retro- or lentivirus vector systems. Obviously, the safety of virus-mediated gene delivery regarding the integration sites and their cell-biological consequences is still under discussion.

The potential risk of insertional oncogenesis was realized in a human gene therapy trial for X-linked severe combined immunodeficiency (SCID). Patients were treated with autologous hematopoietic stem cells transduced with a recombinant retrovirus containing interleukin

receptor common γ chain (γc). Most patients showed almost fully restored immune systems, but a few of them developed leukemia 3 years after the treatment. Leukemic clones from two of these patients showed retrovirus vector integration in proximity to the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2. It is still under discussion whether deregulation of LMO2 alone was responsible for oncogenic transformation. Factors specific to the constitutive expression of the γc transgene and a predisposition specific to an expanded abnormal T-cell progenitor population in X-SCID might also have contributed to the transformation process [12].

To analyze the risk of insertional oncogenesis in general, a long-term preclinical trial was performed with 65 nonhuman primates and 17 dogs that were administered progenitor cells transduced with retroviral vectors expressing marker or drug-resistance genes, receptors or enzymes. None of the animals developed abnormal hematopoiesis or leukemia, although cell doses, vector backbones and transduction conditions utilized were very similar to those employed in human clinical trials, including the SCID trials [13].

However, large-scale mapping studies of retroviral integration sites in human cells revealed that retroviral integration is not random, but favors sites of transcriptionally active gene expression, in particular those next to promoters, bearing the risk of their uncontrolled activation. Lentiviral vectors integrate across the entire transcribed region of genes with no preference to be close to the transcriptional start, due to their ability to infect nondividing cells. Nevertheless, it is known that viral vector integration depends on the infected cell type and on the vector system used [14].

Recently, the effects of retroviral transduction has been examined in T cells from leukemic patients treated with donor lymphocytes that had been genetically modified with a suicide gene (*herpes simplex virus-thymidine kinase*). Molecular analysis confirmed previous results, demonstrating that retroviral vectors integrate preferentially within, or near to, transcribed regions of the genome and close to promoters in particular. Quantitative transcript analysis showed that one fifth of the integrations affect the expression of nearby genes. However, transduced T-cell populations maintain stable gene expression profiles, phenotypic and biological functions *in vivo*, with no indication of clonal selection up to 9 years after administration. Analysis of integrated proviruses in transduced cells before and after transplantation suggests that integrations interfering with normal T cell function are more likely to lead to clonal ablation than to expansion *in vivo* [15].

This discussion about retro- and lenti-viral gene delivery demonstrates that the safety profile of any gene transfer strategy needs to be addressed individually for each disease and in relation to its pathophysiology, the functions of the transgene products and the recipient cell type. The safety of transposition should thus be evaluated *in vivo* for several different transgenes in order to achieve a more comprehensive estimation of the risk for mutational insertion.

In terms of efficiency, the transposition technique certainly requires improvement and further development. Whereas transfer efficiencies are high after viral administration of a broad spectrum of transgenes, the transposition-mediated gene delivery efficiencies are as yet low and have so far been demonstrated with a few different transgenes only. Huang and colleagues have achieved maximum gene transfer efficiencies of 11% after conucleofection of SB transposon and SB-transposase encoding plasmids *in trans*. As reported, these transposition efficiencies are sufficient to induce 4–19 gene copy numbers per clone. However, a proportion of the 11% of transgene-positive cells after transposition is likely to be insufficient for clinical application. For any therapeutic use, it is certainly an advantage to scale the proportion of effectively transduced cells up or down.

By contrast, retroviral gene transfer efficiencies are usually high. To correlate gene transfer efficiency with retroviral vector copy numbers, primary hematopoietic progenitor and cultured K562 cells were transduced with the EGFP-expressing vector at different multiplicities of infection. The gene expression and copy numbers after transduction were analyzed in mass cultures and subsequent cell clones. The results indicate an exponential increase of integration numbers correlated to gene transfer rates, and a linear increase of expression levels with insertion frequency averages of: 1 vector insertion per transduced cell with a gene transfer efficiency of less than 30%, 3 insertions with 60% and 9 insertions with an almost maximum 90% efficiency in K562. For therapeutic gene transfer, however, it is suggested that limiting retroviral gene transfer efficiency to approximately 30% could avoid the generation of cell clones containing multiple insertions, thereby minimizing the risk for mutational insertion [16].

Future perspective

Huang and colleagues optimized the *in vitro* conditions for stable gene transfer in primary human PBLs utilizing the SB transposon system. However, several questions concerning T-cell-directed gene transfer in particular, and the SB transposon system in general, need to be addressed before this technology will be ready for early clinical gene therapy trials.

Similarly, it is essential to perform *in vivo* experiments with the SB transposon system to test the survival and expression kinetics of the transfected T cells and to evaluate the therapeutic value of different transgenes.

In general, the gene transfer efficiency of the SB transposon system requires significant improvement. Indeed, there are approaches in generating hyperactive versions of the transposase, which obtain higher activities when used with improved transposon vectors [2], but transduction efficiencies remain far below those obtained with viral gene transfer. Repeated rounds of SB transposon administration offer the possibility to increase overall transgene expression levels. Nevertheless, the resulting cytological and immunological consequences have not yet been determined. Another approach is to generate transposase mutants of lower sensitivity to overproduction inhibition in order to circumvent the inhibitory effect of supra-optimal transposase concentration. To finally increase the efficiency of gene transfer, it would be helpful to understand more about the interaction between transposon and the host cell. The SB system has in fact been demonstrated to be able to interact with various host proteins, some of which limit transposon activity [17].

Safety concerns should also be addressed, because transposons have the capacity to integrate into transcribed regions of the genome. Moreover, transposon-based gene transfer has to compete with other promising nonviral gene-transfer systems which are based on the site-specific integration of the transgene, such as integrases, bacteriophage ΦC31 [18] or zinc-finger-nucleases [19]. A promising future direction could be the establishment of targeted SB transposition by adopting molecular strategies of Ty retrotransposons in yeast [20] or in certain bacteriophages [18].

Executive summary

Objective

- Can the Sleeping Beauty (SB) transposon system mediate stable, long-term gene expression in primary human T cells *in vitro*?

Approach

- The SB10 transposase was predominantly used.
- Plasmid vectors contain the SB transposase on the same molecule (*cis*) or on a molecule separate from the SB transposon (*trans*).
- Nucleofection of primary peripheral blood lymphocytes (PBLs) with SB plasmid vectors carrying a *DsRed* reporter gene encoding a red fluorescent protein or the therapeutically relevant gene product of the nerve growth factor receptor (NGFR) and cytosine deaminase fusion gene (NGCD).
- For dual gene expression, SB plasmid vectors contain an internal ribosome entry site (IRES) for bicistronic expression, a self-cleaving 2A peptide for bi- and tri-cistronic expression or a synthetic bidirectional promoter.

Results

- *Cis*- and *trans*-delivery vectors can mediate stable gene transfer and expression of the *DsRed* reporter gene in primary human $CD4^+$ and $CD8^+$ T cells.
- Transposition efficiency: conucleofection of *trans*-delivered plasmids and SB10-transposase-encoding plasmids is more efficient than nucleofection of *cis*-delivered vectors (maximum of 11 vs 3% with 10 µg plasmid/5×10^6 PBLs on day 21).
- Long-term gene expression *in vitro*: after nucleofection with *trans*-SB-transposon, $DsRed^+$ T cells were cloned. The $DsRed^+$ clones were cultured for up to 4 months without losing visible transgene expression, compared with $DsRed^-$ T-cell clones.
- Integration: the analyzed 6 $DsRed^+$ T-cell clones had 4–19 copies of integrated transgene at TA-dinucleotide sites in the genome.
- Expression of multiple genes: a synthetic bidirectional promoter cloned into the SB transposon vector, but not an IRES or the self-cleaving 2A peptide, was suitable for stable expression of two gene products in primary human T cells.
- Application: 6.3–8.5% of T cells express the NGFR after nucleofection of PBLs with the *trans*-delivered SB vector containing NGCD encoding a therapeutically relevant protein. Cell sorted $NGCD^+$-T cells are killed by 5-fluorocytosine (5-FC) in a dose-dependent manner, $NGCD^-$-T cells are not.

Conclusion

- In recent years, SB transposons have been shown to mediate gene transfer and long-term expression in a wide range of cultured mammalian cells, such as mouse liver and embryonic stem cells. This new study demonstrates for the first time that the SB transposon system can mediate stable gene transfer in primary human T cells, and is trendsetting for its use in T-cell-based gene therapy applications.

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Review

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Regenerative Medicine

Biomaterials and stem cells as drug/gene-delivery vehicles for Parkinson's treatment: an update

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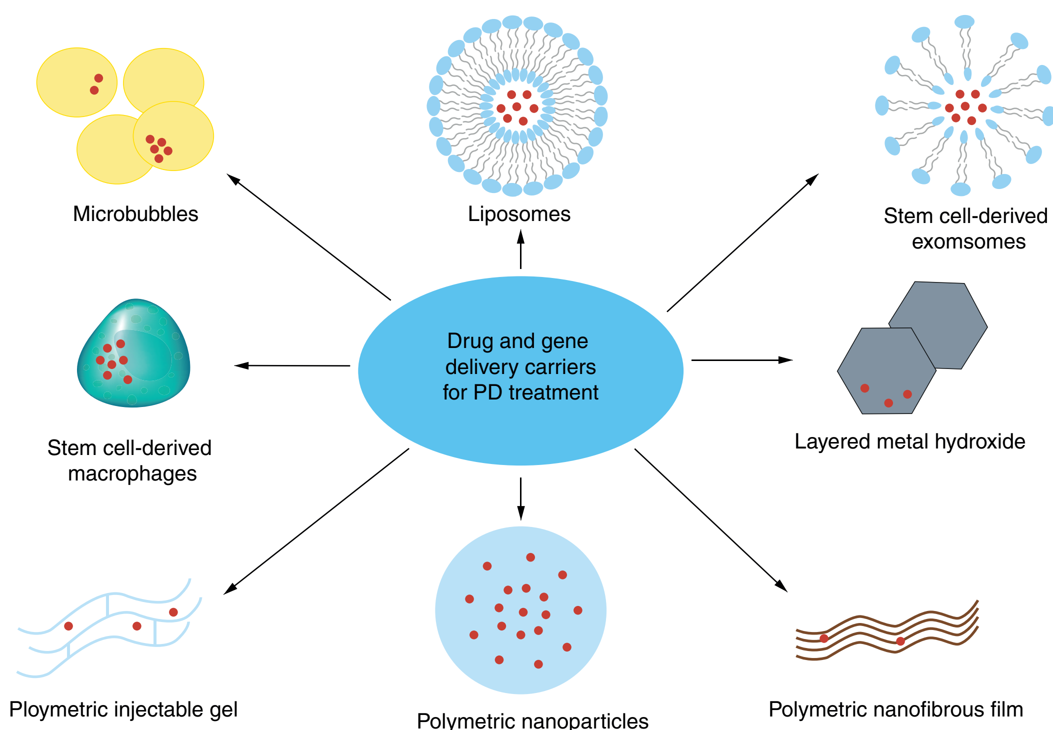
By introducing biomaterials and stem cells into Parkinson's disease (PD), therapeutic approaches have led to promising results due to facilitating brain targeting and blood–brain barrier permeation of the drugs and genes. Here, after reviewing the most recent drug- and gene-delivery vehicles including liposomes, exosomes, natural/synthetic polymeric particles/fibers, metallic/ceramic nanoparticles and microbubbles, used for Parkinson's disease treatment, the effect of stem cells as a reservoir of neurotrophic factors and exosomes is provided.

Graphical abstract:



First draft submitted: 1 April 2021; Accepted for publication: 26 October 2021; Published online: 6 December 2021

Keywords: biomaterials • drug-delivery systems • nonviral gene delivery • Parkinson's disease • stems cells

Future Medicine

Treatment, preventing the progression and alleviating the symptoms of Parkinson's disease (PD), a common degenerative disorder of the CNS has been the aim of many researchers' attempts for several decades. Deep brain stimulation surgeries, gene therapy and stem-cell therapy have been introduced as approaches for PD management while drug therapy is still the main PD treatment option [1].

Different drugs including L-dopa, dopamine agonists, promotors of dopamine levels such as inhibitors of MAOB and COMT enzymes are prescribed to PD patients [2]. However, conventional drug therapies for PD treatment are encountered by two problems: the existence of the blood–brain barrier (BBB) which restricts the drugs from reaching the brain and the need for lifetime consumption of drugs which is inconvenient especially for PD patients who develop movement and swallowing problems. Researches have shown that drug-delivery systems are effective tools for solving these two problems.

To solve the first problem, different nano- and microcarriers are designed and fabricated from biomaterials to facilitate PD drug transport to the brain. Drug carriers exploit the same mechanisms as brain cells use to pull vital molecules inside the brain to successfully traverse the BBB and release their cargo into the damaged brain. Expression of transporters, specific receptors and efflux pumps in the endothelial cells of BBB are mechanisms for the transport of small hydrophilic molecules, large hydrophilic molecules and small lipophilic molecules, respectively [3,4]. However, in designing drug-delivery systems for PD treatment, alterations to the the BBB compared with the healthy individuals should be considered. Disruption and increased permeability of the BBB take place following neurodegeneration in PD [5]. Alteration in the expression of P-gp transporters in the BBB lead to BBB disfunction, which is followed by altered α-syn transport to the brain and worsening PD progression as a vicious cycle [6]. Drug carriers could also be made from mucoadhesive biomaterials to improve the nose-to-brain delivery of drugs. There is a shortcut to the brain without the need to cross the BBB, which starts from the nose epithelium, passes the trigeminal nerve path and ends to the olfactory region of the brain. This route has several benefits including patient compliance, large absorption area and preventing drugs from the first-pass metabolism. However, there is still a lack of data regarding whether drug-loaded carriers are transported across the neural path to the brain, where they release their cargo, or the released drug is being absorbed by nasal epithelium [7,8].

To solve the second problem, drug carriers with sustained-release were made from biomaterials. Since PD patients develop muscle rigidity, dyskinesia and swallowing problems, sustained-release systems eliminate the need for daily oral intake and frequent self-administration which means improving the quality of life for PD patients. Otherwise, sustained-release systems not only improve patient compliance but also preserve the loaded therapeutics from plasma degradation and fast clearance by the immune system. This enables highly sensitive molecules, such as genes that are rapidly degraded by deoxyribonucleases, to be loaded into carriers and released intact into the cells.

Considering the beneficial role of drug carriers in PD treatment, in this review, a collection of biomaterials that are used in recent studies as drug-delivery vehicles or nonviral gene delivery vectors for PD treatment are gathered. Not only biomaterials but also stem cells as systems to deliver therapeutics to the PD-affected brains are discussed here.

Drug-delivery systems for PD treatment

The majority of drug-delivery systems encapsulate L-dopa, which turn into dopamine after carboxyl removal by aromatic L-amino acid decarboxylase enzyme within the body. Tyrosine hydroxylase (TH) responsible for translating tyrosine into aromatic L-amino acid decarboxylase is an essential molecule in this biosynthetic pathway. The reason behind using L-dopa instead of free dopamine is the risk of producing 6-hydroxydopamine (6-OHDA) and neurotoxins following free domain release [9]. Alternatively, other dopamine derivatives and antioxidants have been incorporated into carriers for PD treatment. Here, recent drug carriers for PD improvement are gathered.

Liposomes

Liposomes are an important class of delivery systems due to their capacity to entrap hydrophilic and hydrophobic drugs simultaneously between multiple phospholipid bilayers of their structure. Other attributes of liposomes including biocompatibility, lack of toxicity, lack of immune stimulation, having US FDA approval and the ability to cross the BBB owing to their lipophilic shell have made liposomes a good option for carrying anti-PD drugs [10]. Unilamellar liposomes composed of dimyristoyl phosphatidylcholine and cholesterol were used for sustain releasing L-dopa dimeric prodrugs in the dialysate rat striatum, which produced a 2.5-fold higher local drug concentration rather than free prodrugs [11]. Intraperitoneally (ip.) injected unilamellar liposomes with sustained

release of fumaric-diamides of (O,O-diacetyl)-L-dopa-methyl ester increased L-dopa pro-drug bioavailability in the rat's striatum [12,13]. In addition to L-dopa, selegiline hydrochloride was loaded into liposomes with high entrapment efficiency and its administration from nose-to-brain restored behavioral parameters in PD animal models [14]. A dopamine receptor agonist, apomorphine, was also incorporated into liposomes and successfully delivered in a prolonged manner to the damaged parts of the brain [15]. Loading antioxidants into liposomes to reduce dopaminergic neurons loss following increased oxidative stress is another PD management option. Delivery of glutathione enzyme or resveratrol, both as reactive oxygen species (ROS) scavengers, from liposomes, improve symptoms in PD rat models following increased bioavailability of antioxidants [16].

Several targeting ligands have been explored to enhance the brain-targeting potential of drug-carrying liposomes. In one study, glutamate-conjugated multilamellar liposomes carrying dopamine hydrochloride efficiently delivered the drug to the brain [17]. In another case, polyethylene glycol (PEG)-attached liposomes as a dopamine carrier were conjugated to an antibody against OX26 to cross the BBB via the transferrin receptor (TfR). Such targeted liposomes showed a low clearance rate and high blood residence time in PD rats [18]. Dopamine-loaded liposomes functionalized with transferrin also showed a high permeability across human cerebral microvascular endothelial cells as an *in vitro* model of the BBB [19]. Chlorotoxin modification of liposome surface is also suggested and improved the BBB permeation was evidenced by increased uptake of drug-loaded liposomes by brain-microvascular endothelial cells *in vitro*. After ip. injection of L-dopa-loaded chlorotoxin-modified liposomes to the mitochondrial permeability transition pore (MPTP)-induced mouse, increased dopamine distribution in *substantia nigra* (SN) was observed *in vivo* [20]. Decorating the liposome surface with a brain-targeting peptide named RVG-29 increases BBB permeation, following interaction of RVG-29 and the acetylcholine receptors on the brain endothelial cells. Intravenous (iv.) injection of RVG-29-modified liposomes loaded with a dopamine derivative, N-3,4-bis(pivaloyloxy)-dopamine, increased drug concentration in striatum and SN of a unilateral 6-OHDA-lesioned mouse model [21]. Applying an external force could further assist BBB leakage. Embedding Fe_3O_4 nanoparticles into delivery vehicles and then applying a magnetic force has been used frequently for targeted drug release applications [22]. Either incorporating Fe_3O_4 nanoparticles into liposomes or decorating the liposome surface with magnetic constitutes facilitate BBB permeation of liposome particles under the magnetic field, so that a 2.5-fold higher concentration of liposomes was achieved in the brain of PD rats after attachment to Fe_3O_4 nanoparticles and being exposed to MRI [23]. Liposomes embodying Fe_3O_4 nanoparticles and loaded with resveratrol showed greater BBB penetration under a magnetic field, as well as sustained delivery of the neuroprotective drug in a PD rat model [24].

The ratio of loaded drugs to the lipids in liposome structure affects the recovery of behavioral deficits and suppression of PD symptoms [2]. To increase the dopamine/lipids ratio of the dopamine-loaded liposomes, producing a transmembrane ammonium sulfate gradient has been offered. Enhancing dopamine-loading efficiency of liposomes resulted in alleviation of the symptoms in PD-suffering C57BL/6 mice [25].

Controlling drug release from anti-PD drug-loaded liposomes is also pointed out by modifying the liposome surface with hollow gold nanoshells (HGNs). Noticing the ability of HGNs to absorb near-infrared (NIR), a NIR laser with highly precise femtosecond stimulation could produce programmed drug release from liposomes in a way mimicking the pattern of neurotransmitter release in the brain, as evidenced by cyclic voltammetry results shown in Figure 1. HGNs modification of liposomes also makes them responsive to the ultrasound wave, so that liposome contraction and drug release take place over prolonged periods of ultrasound exposure [26].

Exosomes

Exosomes are nanometric bodies (30–120 nm) released from mesenchymal stem cells, neurons, microglia, astrocytes, lymphocytes and monocytes. These cell-derived vesicles are a suitable carrier for anti-PD drugs due to their intrinsic ability to pass the BBB, as well as their natural lipid bilayers enriched with adhesive proteins suitable for nose-to-brain delivery [27,28].

Loading small hydrophilic drugs into exosomes is practicable through a simple incubation with drug solution at room temperature. For example, Qu *et al.* loaded dopamine into macrophage-derived exosomes through 24 h incubation and then removed unloaded dopamine molecules by ultracentrifugation [29]. Sonication and extrusion are suitable loading strategies for large proteins [30].

Selective dopamine delivery to the brain via blood exosomes has resulted in dopaminergic neurogenesis in a mouse PD model. Blood exosomes, which are released into the bloodstream during maturation of reticulocytes to erythrocytes, bind to the brain cells after the formation of transferrin dimer which links TfR on the exosome to the TfR on the target cells. As evidenced by computer modeling, the combination of transferrin-exosome internalizes

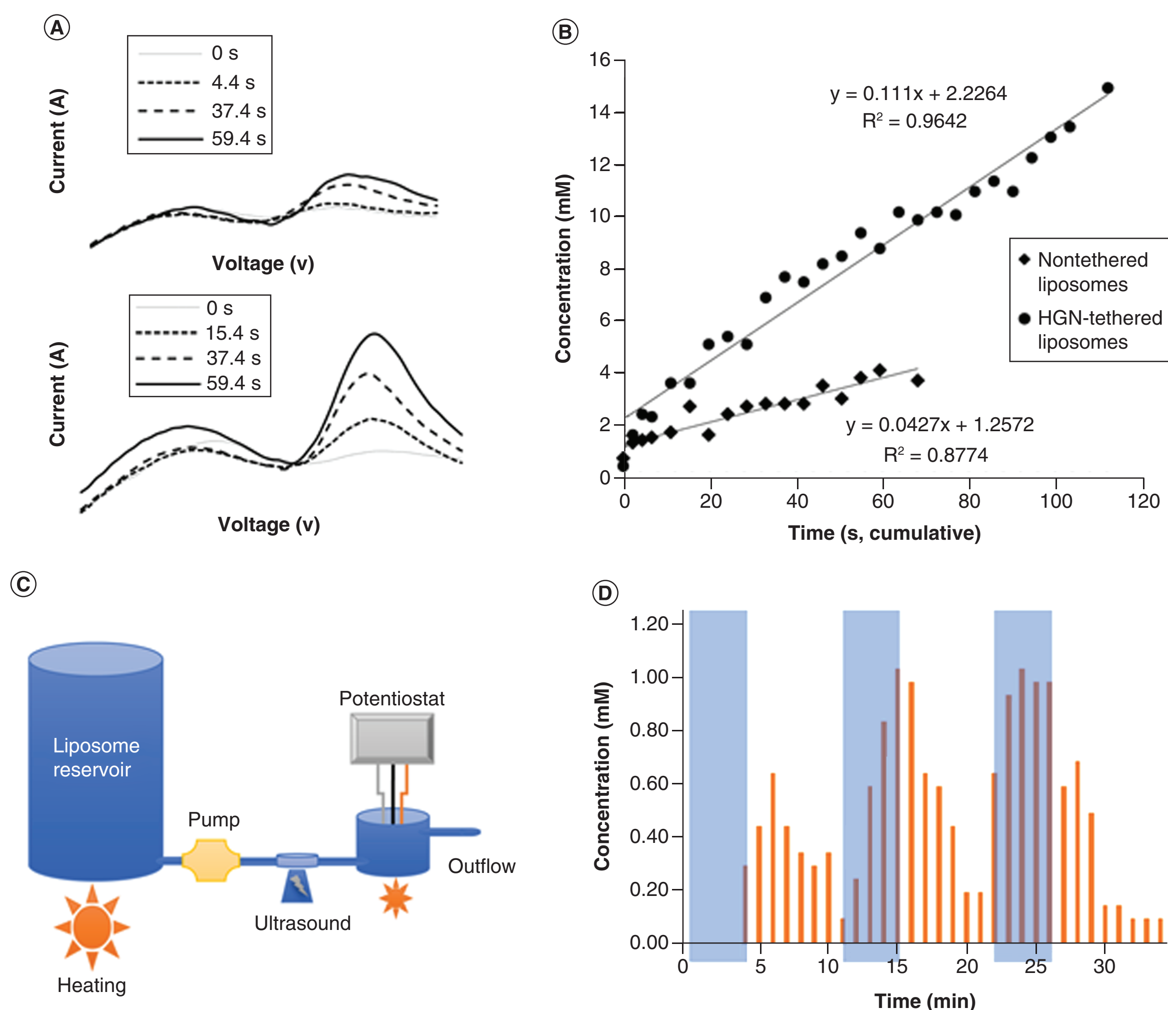


Figure 1. Detection of dopamine by cyclic voltammetry in response to ultrasound. (A) Representative cyclic voltammograms used to verify the presence of dopamine released from liposomes. Top: Nontethered liposomes. Bottom: HGN-tethered liposomes. **(B)** Representative example of linear release of dopamine from HGN-tethered and nontethered liposomes over successive ultrasound applications at 20°C (room temperature). Note: Each data point represents 2–5 s of 2.4 W cm^{-2} ultrasound exposure applied every 3 min. The total experiment run time is approximately 50 min for control nontethered liposomes, and approximately 80 min for HGN-tethered liposomes. **(C)** Schematic illustration of the circulating flow system. **(D)** Representative example of phasic dopamine release at 35°C in a circulating flow system. Blue bars indicate a 9 s continuous ultrasound application at 2.4 W cm^{-2} (0.64 MPa) every 20 s.

HGN: Hollow gold nanoshell.

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into the cells where iron releases from transferrin. Afterward, dissociation of iron-deficient transferrin with TfR takes place which is followed by returning receptor to the cell surface for further exosome attachment [29].

Catalase, effective for reducing ROS secreted in the brain following microglia activation during PD progression, has been loaded into exosomes. Exosomes loaded with this enzyme successfully passed the BBB, accumulated in microglial cells and inhibited ROS-generated neurodegeneration [31]. Reducing oxidative stress in the inflamed brain tissues via intranasal administration of catalase-loaded exosomes to the PD mice's brain and successful uptake by PC12 cells has been performed previously [32]. To achieve catalase-loaded exosomes, either monocytes and macrophages were transfected with catalase DNA or naive exosomes were loaded with catalase. Sustained release of the enzyme from exosomes and protecting the loaded enzyme from proteases degradation resulted in sufficient catalase concentration in the target cells [33].

Exosomes are not only a potential carrier for anti-PD drugs but also they could provide a high sensitivity biomarkers for PD, so that CNS-derived exosomes, which could be simply isolated from patients' plasma or cerebrospinal fluid (CSF) samples, induce α-syn oligomerization, a highly sensitive PD recognition event [34]. However, the beneficial outcomes of using exosomes as drug-delivery vehicles or biomarkers are hindered by its safety issues which come from the complex structure of exosomes and the differences between exosomes from various cell sources [35].

Natural polymer-based systems

Chitosan is the most important natural polymer for anti-PD drugs delivery with attention to its mucoadhesive properties, as well as its ability to overcome the BBB. Mucoadhesive chitosan reduces mucociliary clearance that means higher nasal mucus retention for efficient nose-to-brain drug delivery. As well, the positive charge on the chitosan molecules opens the tight junctions transiently for BBB transport trough transcytosis [36]. It has been shown that glycol form of chitosan suppresses the activity of multidrug resistance protein 1, an efflux pump P-gp, to encourage BBB transport [16].

There are several examples of loading anti-PD drugs into chitosan nanoparticles for improving brain uptake of the drug after nose-to-brain administration. Loading rivastigmine, an anti-PD drug, into chitosan nanoparticles improved the drug bioavailability in the brain following intranasal delivery of the carrier [37]. Nose-to-brain delivery of bromocriptine-loaded chitosan nanoparticles was also effective for the treatment of haloperidol-induced Parkinsonism [38]. Additionally, chitosan loaded with rotigotine and administered intranasally improved the results of catalepsy, swimming and akinesia tests, reduced lactate dehydrogenase and increased catalase activity compared with the intranasal delivery of rotigotine solution [39].

It is shown that injected dopamine-loaded chitosan nanoparticles traverse the BBB, increase dopamine levels in the rat striatum and reduce ROS production [16]. Dopamine loading into chitosan nanoparticles is achieved via the chitosan interaction with dopamine, while the stability of the drug structure is preserved [16]. Preparing dopamine-loaded injectable chitosan gel is also feasible with regard to the coupling between chitosan and oxidized dopamine molecules. Ren *et al.* used an oxidizing agent, $NaIO_4$, to produce polydopamine from dopamine which could cross-link the gel composed of quaternized chitosan and gelatin *in situ* and the entrapped dopamine could be released in a long period [40].

Collagen and hyaluronic acid are among naturally occurring polymers with the potential for PD drug-delivery applications. Tunesi *et al.* prepared a semi-interpenetrating network by stimulating collagen fibrillogenesis in the presence of hyaluronic acid and gelatin, and then loaded transactivator of transcription (Tat) conjugated Hsp70 into the resulting injectable hydrogel. While Hsp70 acts on α-syn oligomers through colocalization with Lewy bodies and reduce α-syn toxicity, its short half-life and low cell membrane permeability necessitate loading into a carrier such as the gel system introduced in this study [41]. Releasing Tat-Hsp70 from the hydrogel into the striatum protected dopaminergic neurons in a 6-OHDA-induced model of PD and improved motor functions. An important achievement of Tunesi *et al.* was generating a refillable gel for reducing invasive surgeries [42].

Another natural polymer that has been used for Levodopa encapsulation is Zein, an amphiphilic amorphous polymer taken from corn endosperm. Zein was produced by electrospinning in the form of nanofibrous films with sustained release of Levodopa [43].

Synthetic polymer-based systems

The virtue of synthetic polymers to be used as a carrier for anti-PD drugs is the control over biodegradation and drug release via controlling their synthesis process. Encapsulating dopamine into biodegradable polymeric particles increases dopamine bioavailability and prevents its metabolism. Poly(lactic-*co*-glycolic acid) (PLGA) are the frequently used synthetic polymers for drug delivery to PD models. Sustained release of Levodopa/benserazide from PLGA microspheres, as presented in Figure 2, reduces L-dopa-induced dyskinesia in 6-OHDA rats through the mechanism of lowering β-arrestin2 [44]. PLGA microspheres were also used for the delivery of growth factors including GDNF and VEGF to the 6-OHDA rat. The results of the amphetamine rotation behavior test showed a decreased number of rotations and TH + immune histochemical analysis confirmed increased neurons into the striatum and external SN [45]. Co-polymerization of PLGA with other polymers produces a wide range of carriers for drug loading. An example is the FDA-approved co-polymer of PEG–PLGA modified with borneol and lactoferrin receptors to release dopamine via nose-to-brain rout. Improved uptake by SH-SY5Y and 16HBE cells in the case of lactoferrin-attached polymeric particles was observed *in vitro*, as well as increased striatum absorption in 6-OHDA-

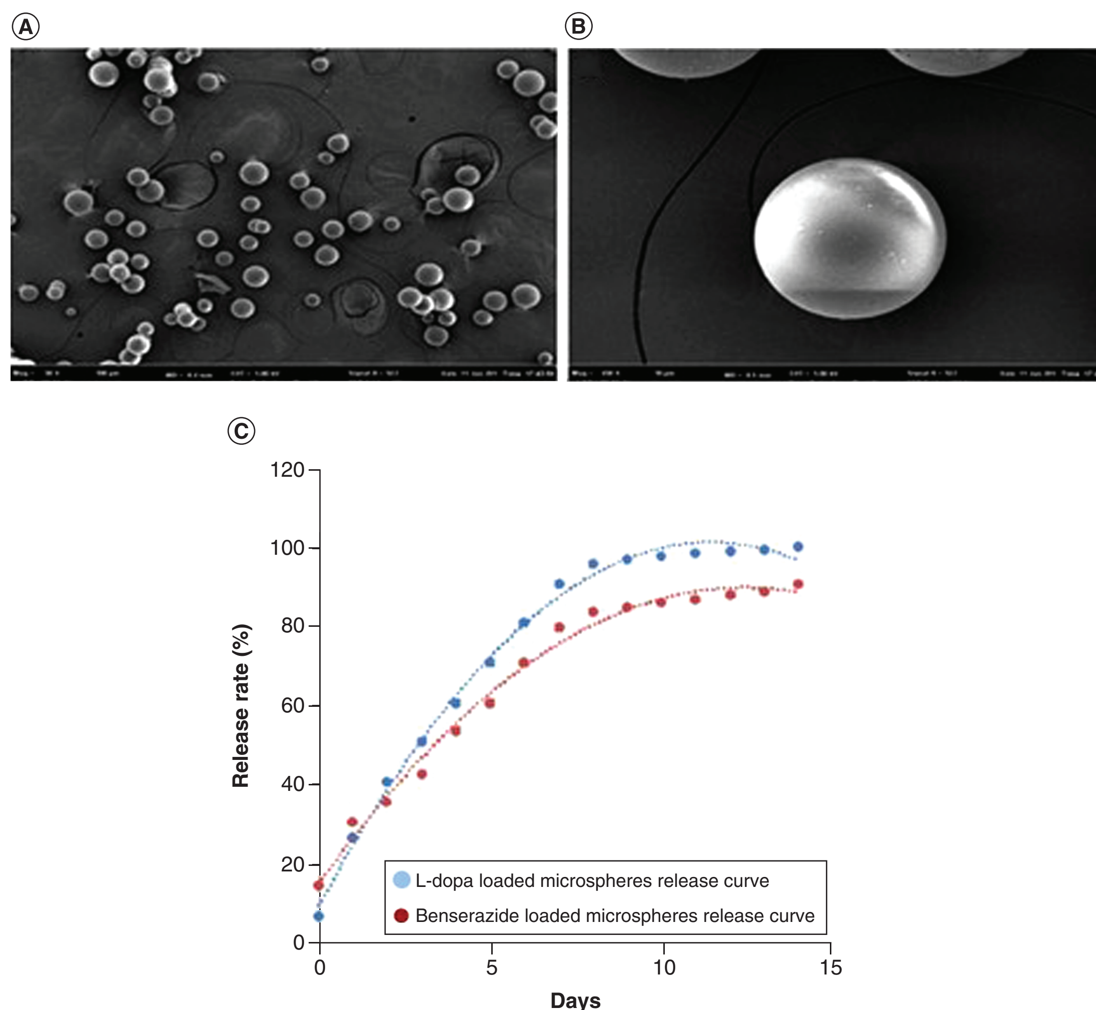

Figure 2. Sustained release of levodopa/benserazide from poly(lactic-*co*-glycolic acid) microspheres. (A & B) Scanning electron microscopy of the levodopa/benserazide poly(lactic-*co*-glycolic acid) microsphere nanoparticles. **(C)** Release profile of levodopa/benserazide from composite levodopa/benserazidepPoly(lactic-*co*-glycolic acid) microsphere *in vitro* n = 4.
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induced PD rats *in vivo*. Due to the expression of lactoferrin receptors on the nasal olfactory epithelium, dopamine nanocarriers modified with lactoferrin and administered intranasally facilitated BBB transport [46]. Modification of PEG–PLGA nanoparticles with lactoferrin has been also reported previously for refinement of mucoadhesive system and better nose-to-brain delivery of rotigotine in a PD animal model [47]. Additionally, conjugation of Odorranalectin, a 17 amino acid peptide, to PEG–PLGA improved the BBB passage following the nasal delivery of particles [48].

The undesirable generation of acidic by-products following *in vivo* PLGA degradation shifted the focus of researchers to other synthetic polymers. As a case, a sustained drug release system was developed based on polycaprolactone-incorporated Rasagiline mesylate, a confirmed monotherapy in early PD, and adjunctive therapy in moderate-to-advanced PD cases. Incorporation of Rasagiline mesylate into polymer not only improved Rasagiline mesylate half-life and the need for daily oral administration but is more compatible with PD patients who suffer from dysphagia, the difficulty in swallowing. A single subcutaneous injection of polymeric microspheres fulfilled drug release for a month [49].

PF127 is an *in situ* gel-forming polymer with the ability to cross-link in the body temperature [50]. Incorporating Ropinirole, a dopamine agonist for PD treatment, into PF127 has been accompanied by satisfactory results, so that the brain bioavailability showed a fivefold increase after nasal administration of PF127 to the sheep with the lowest

deteriorative effects on the mucosa [51]. Mixing PF127 with other polymers such as chitosan improved mucoadhesive feature of the thermo-reversible gel and its ability to protect the loaded levodopa from rapid degradation [52].

Polyethylenimine (PEI)-dextran sulfate nanoparticles loaded with retinoic acid were released into the MPTP-induced mouse model of PD. The potential of retinoic acid to increase the expression of Nurr1 and Pitx3, transcription factors important for dopaminergic neurons protection, led to preserving dopaminergic neurons in the SN following administration of polymeric nanoparticles [53].

A polymeric blend system composed of methacrylate co-polymer/methacrylate co-polymer blend named Poly-x-Lipo Nano-enabled Tablets (PXLNET) was developed to release L-dopa at a constant rate in the gastric environment. The PXLNET containing L-dopa and the decarboxylase inhibitor, benserazide, was delivered via the catheter to the pig and increased dopamine concentration in the CSF and urine samples. Adding benserazide to the tablet prevented dopamine-to-L-dopa conversion and kept the dopamine level constant in the formulations [54,55].

Metal/ceramic-based systems

Drug-delivery systems made from metal or ceramic nanoparticles are of special interest for PD treatment due to the neuroprotective effects of these particles. Gao *et al.* demonstrated the inhibitory effect of gold nanoclusters on α-syn aggregation *in vitro* and Lewy body formation in MPTP-induced mouse [56]. Furthermore, Umarao *et al.* confirmed the neuroprotective effect of superparamagnetic iron oxide nanoparticles (SPIONs) implanted into the striatum of a 6-OHDA rat and this beneficial effect was attributed to the antioxidant feature of SPIONs due to the presence of Fe ions on SPIONs surface [57]. The results of Umarao *et al.* showed increased dopaminergic neurons survival, decreased lesioned area of the striatum and elevated cytochrome c levels. Based on these studies, a group of researchers used layered zinc hydroxide as a carrier with a controlled and pH-dependent release of carbidopa. The layered structure of metal hydroxide and subsequent incorporation of the whole system into a chitosan matrix provided desirable carbidopa loading and release [58]. In a recent study, hollow Na+-titanate nanospheres (TiNS) were synthesized and loaded with Cerebrolysin®, a composition of neurotrophic factors and peptide fragments taken from porcine brain proteins. Delivery of Cerebrolysin was linked to the amelioration of the level of dopamine and its metabolites, such as 3,4-dihydroxyphenylacetic acid and homovanillic acid, as well as increasing TH-producing cells in PD mice. Additionally reduced α-syn, oxidative stress and brain edema formation was observed [59].

Nonviral gene delivery systems for PD treatment

Gene therapy has been an intriguing field of research in the recent decade. Application of gene delivery approach for PD treatment is mainly based on targeting whether α-syn-related genes or GDNF, a neurotrophic factor responsible for survival, differentiation and synapsis of DAergic neurons. α-syn is a protein which its overproduction and aggregation of its phosphorylated state is a cause of neurodegeneration in PD. α-syn phosphorylation at serine 129 is performed by PLK2 enzyme and causes oligomer formation [60]. Targeting *SNCA*, the gene related to α-syn protein, with the promise of reducing the gene expression or mRNA degradation is used for addressing PD.

So far, different adeno-associated viruses were utilized to carry shRNA and siRNA aiming at targeting and silencing SNCA [61]; however, using biomaterials as nonviral gene delivery systems in comparison to viral methods have the superiority of lower immune responses stimulation, preferable brain transduction and additional capacity of gene packaging [62,63]. Here, different nonviral gene delivery vectors for incorporating α-syn-related or GDNF genes are discussed.

Liposomes

The use of liposomes as nonviral gene delivery agents has been considered in recent years owing to their lipid outer layer, which facilitates cell fusion and gene transfection, as well as the cationic nature of some phospholipids in liposome structure which are important for the incorporation of negatively charged genes [64]. Liposomes as a vector for the *GDNF* gene have been studies before; however, few studies have suggested the role of liposome as a gene delivery vehicle for PD treatment.

Incorporating the *GDNF* gene into cationic liposomes has been effective for the *in vivo* model of spinal cord injuries [65,66]. For PD treatment, Trojan horse liposomes functionalized with a monoclonal antibody to the TfR and loaded with GDNF improved gene expression in SN of a 6-OHDA rat [67]. Such gene therapy method resulted in reduced apomorphine-induced contralateral and amphetamine-induced ipsilateral rotations, reduced whisker-induced forelimb placement abnormalities and increased TH enzyme activity [68]. To improve DNA encapsulation

into the liposomes, the ethanol-mediated DNA condensation method was undertaken by Zhou *et al.*, which resulted in better therapeutic effects in PD rats [69].

Exosomes

mRNA or miRNA targeting α-syn-related genes could be loaded into exosomes via electroporation [30]. Tail vein injection of exosomes loaded with siRNA-targeting SNCA reduced SNCA mRNA up to 50% in the midbrain and striatum of the mouse. Exosome fusion with RVG resulted in better recognition by acetylcholine receptors [70]. Suppression of miRNA-155, an important mediator of the inflammatory response of microglia to α-syn, is also effective in PD treatment. Genetic engineering of exosomes to express LAMP2 and RVG lead to the sufficient delivery of siRNAs targeting miRNA-155 to the microglia [71].

Polymer-based systems

Polymers, especially cationic ones, are good candidates to be used as nonviral gene delivery vectors. PEI is among cationic polymers with wide applications in gene delivery which, however, show cytotoxicity [72]. For PD treatment, this polymer successfully incorporated siRNA-targeting SNCA and produced a 65% reduction of mRNA expression and α-syn protein expression in the striatum [70]. Polyamidoamine (PAMAM), another cationic polymer with dendrimer structures, has the advantage of being less cytotoxic and having higher transfection efficiency than PEI [73]. In PD gene therapy, PAMAM was used for the delivery of the human *GDNF* gene. The surface of PAMAM nanoparticles was modified with lactoferrin-targeting ligand to improve BBB traverse of the particles after transvascular administration. The second modification of carriers with PEG also improved their blood circulation time. Finally, using this gene delivery system in a rotenone-induced mouse model improved locomotor activity, decreased dopaminergic neuronal expression and increased dopamine levels [48]. PEG not only decrease blood clearance of nanoparticles but is itself a good gene carrier with the ability to bypass the BBB. In a study by Harmon *et al.*, DNA-encoding human GDNF were attached to a 10 kDa PEG-containing polymer (named as CK30PEG10k, a polymer in which 30-mer lysine is substituted with PEG), and successfully were transported to the rat brain via intranasal administration [74].

Another platform for PD gene therapy was an N-isopropylacrylamide (NIPAm)-based vector, a pH sensitive and thermally responsive material, combined with acrylic acid and functionalized with superparamagnetic nerve growth factors, for loading shRNA-targeting SNCA. These magnetic particles reduced α-syn expression and improved PD *in vitro* [75].

Using PLGA as a gene vector is also offered when it is combined to a cationic moiety, such as cationic peptide protamine sulfate, to incorporate negatively charged miR-124. Since the vector preserves the miR-124 stability, uptake of miR-124 by stem cells activated Notch-1 signaling and led to stem cell differentiation by targeting Sox9, Dlx2, PTBT1 and CTDSP1. Then, stem cells differentiated into neuronal cells and replaced lost dopaminergic neurons in the 6-OHDA animal model of PD [76].

Microbubbles

One approach to facilitate BBB cross of drug carriers is applying focused ultrasound (FUS) to produce a localized delivery of energy and disrupt the BBB temporarily while preserving BBB integrity. This technology has provided reassuring therapeutic results for PD treatment as reviewed by others [77–80]. In FUS technology, iv. administered microbubbles are required for BBB cavitation. Microbubbles are microspheres filled with perfluorocarbon with a lipidic coating which gives them low solubility. They absorb ultrasound radiation force to collapse and release their conjugated gene carrying moiety at the site of FUS exposure. What matters for the safety of this technology is the concentration and size distribution of microbubbles [77–80].

Microbubbles could be used as a carrier for anti-PD drugs such as apomorphine to prevent the problem of short half-life and first-pass metabolism of drugs; as Hwang *et al.* achieved a sustained release of apomorphine after sonication at 1 MHz [81]. Though, most cases of microbubbles use are devoted to the delivery of genes rather than drugs. Fan *et al.* developed cationic microbubbles containing the *GDNF* gene and after injection of microbubbles, exposed the brain of PD rats to the FUS. Successful BBB permeation followed by expression of GDNF-produced neuroprotection [82]. In a later study, FUS improved the delivery of BDNF from the nasal cavity to the brain of an MPTP mouse model and improved dopaminergic neurons function [83]. Using microbubbles for local GDNF delivery resulted in behavioral motor deficits restoration and dopamine amelioration in the PD models of rats [84].

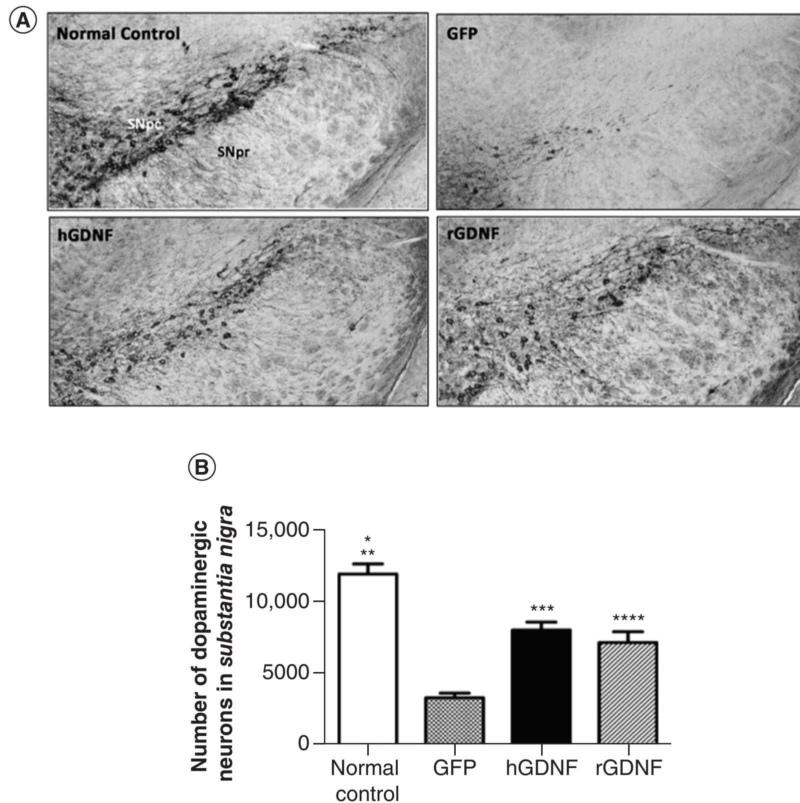

Figure 3. GDNF therapy protected loss of dopamine neurons in the *substantia nigra* of MitoPark mice. (A) The 30-μm thick coronal midbrain sections were from randomly selected brain samples of hGDNF-, rGDNF-, GFP-transplanted MitoPark mice and GFP-transplanted wild-type normal control mice at 9 weeks post-transplantation. The sections were stained with TH immunohistochemistry. Red-stained TH-immunoreactive neurons are dopamine neurons distributed in *substantia nigra* at 100× magnification. **(B)** The number of dopaminergic neurons in *substantia nigra* of hGDNF-, rGDNF- and GFP-transplanted MitoPark mice and GFP-transplanted wild-type normal control mice. The total number of Nissl+/TH-immunooreactive neurons were estimated by quantitative stereological analysis using the Zeiss AxioImager A1 microscope with Stereo Investigator software. Each bar represents mean ± standard error of mean from six animals per treatment group (n = 6). *$p < 0.001$ versus GFP-transplanted-MitoPark mice; **$p < 0.01$ versus hGDNF- and rGDNF-transplanted MitoPark mice; ***$p < 0.001$; ****$p < 0.01$.
SNpc: *Substantia nigra* pars compacta; SNpr: *Substantia nigra* pars reticulate; TH: Tyrosine hydroxylase.
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Long *et al.* used microbubbles in combination with MRI-guided FUS to deliver Nrf2 to the 6-OHDA rats and reported reduced ROS generation and dopaminergic neurons protection following *Nrf2* gene transfection [85]. In another attempt, the administration of microbubbles coupled with PEG-liposomes was done in the 6-OHDA rats. FUS increased accumulation of microbubble-liposome complexes into the brain and local release of GDNF and nuclear receptor-related factor1 from liposomes led to dopaminergic neurons loss reduction [86]. Applying MRI-guided FUS to microbubbles-coupled pegylated-liposomes increased GDNF expression, reduced amphetamine-induced rotations and increased TH activity [87].

Stem cells as drug- & gene-delivery vehicles for PD treatment

Replacing the lost neurons using human pluripotent stem cells has been a prospect for PD treatment and other neurodegenerative disorders [88]. Different scaffold materials and designs have been suggested to trigger stem-cell differentiation into neuronal lineages [89–91]. Another interesting aspect of using stem cells, that is also a focus of this review, is the possibility of using stem cells as drug-delivery vehicles. Stem cells are a good reservoir for neurotrophic factors which could block PD progression.

GDNF and neurturin are potent neurotrophic factors and their direct injection to the brain has restored damaged dopaminergic neurons in the SN and improved motor deficits in animal models of PD. In a recent randomized

Table 1. Comparing different drug-delivery systems for Parkinson's disease treatment.

Drug-delivery system type	Benefits	Limitations	Ref.
Liposomes (such as DMPC/CHOL liposomes)	– Possibility of simultaneous hydrophilic and hydrophobic drug loading – Biocompatibility – Lack of toxicity – Lack of immune stimulation – Having FDA approval – Ability to cross the BBB – Potential of being functionalized with targeting ligands	– Rapid systemic elimination – Sensitivity of phospholipids to metabolic degradation – Storage concerns – Difficult control over sustained release of drugs – Poor reproducibility – Low drug encapsulation efficiency	[10,14,15,17–19,96,97]
Exosomes	– Ability to pass BBB – Its natural lipid bilayers for dual drug loading – Having adhesive proteins suitable for nose-to-brain delivery – Being a biomarkers for PD	– Safety concerns – Variations between exosomes from different cell sources	[27,28,34,35]
Natural polymer-based carriers (chitosan, zein, collagen, hyaluronic acid)	– Mucoadhesive properties – BBB permeation	– Material batch variation – Limited modulation of pore size and degradation – Poor processability	[16,43,98]
Synthetic polymer-based carriers (PLGA, co-polymer of PEG–PLGA, PF127, polycaprolactone, PEI/dextran sulfate, methacrylate co-polymers)	– Control over biodegradation and drug release via controlling the synthesis process – Tunability and forming into nanoparticles, and injectable gels	– Higher risk of immunogenicity	[44,46]
Metal/ceramic-based carriers (gold nanoclusters, SPIONs, layered zinc hydroxide, TiNS)	– Neuroprotective effects	– Risk of toxicity – Low entrapment efficiency of drug	[56,57,99]

BBB: Blood–brain barrier; CHOL: Cholesterol; DMPC: Dimyristoyl phosphatidylcholine; PD: Parkinson's disease; PEG: Polyethylene glycol; PEI: Polyethylenimine; PLGA: Poly(lactic-co-glycolic acid); SPION: Superparamagnetic iron oxide nanoparticle; TiNS: Titanate nanosphere.

Table 2. Comparing different gene delivery systems for Parkinson's disease treatment.

Gene delivery system type	Benefits	Limitations	Ref.
Liposomes (THLs)	– Good cell fusion and gene transfection due to lipid outer layer – Facilitated incorporation of negatively charged genes due to the cationic nature of some phospholipids	– Rapid systemic elimination – Sensitivity of phospholipids to metabolic degradation – Storage concerns – Poor reproducibility	[96,97]
Exosomes	– Good cell fusion and gene transfection due to lipid outer layer – Being an inherent gene carrier	– Safety concerns – Variations between exosomes from different cell sources	[35,70]
Cationic polymers (PEI, PAMAM, NIPAm, PLGA/protamine sulfate)	– Facilitated incorporation of negatively charged genes – Potential of being conjugated to targeting ligand and PEG – Simplicity of preparation – pH and thermal sensitivity	– Risk of toxicity due to tissue accumulation	[72,76,100]
Microbubbles	– Site-specific and controlled gene delivery – Blood–brain barrier permeation	– Need for an external force – Control of safety	[77–80]

PEG: Polyethylene glycol; PEI: Polyethylenimine; PAMAM: Polyamidoamine; NIPAm: N-isopropylacrylamide; THL: Trojan horse liposome.

placebo-controlled study, convection-enhanced delivery of GDNF across the putamen using a skull-mounted transcutaneous port containing four microcatheters resulted in improved motor function [92]. Nonetheless, the direct injection of neurotrophic factors to the brain is not patient compliant. Thus, to prevent direct injection and achieve BBB transport, stem cell-derived macrophages are used as a carrier for GDNF. Such macrophages could be taken from a transplanted hematopoietic stem cells. Released GDNF from macrophages resulted in stopping dopaminergic neurodegeneration in MitoPark mouse as a genetically engineered model of PD with a deficiency in transcription factor A, mitochondrial. Reduction of dopaminergic neurons loss following release of GDNF from transplanted cells, which was assessed by quantitative stereological analysis of TH+ neurons in the SN, is presented in Figure 3 [93].

Stem cells' encapsulation is a straightforward method to keep the cells alive, prevent immunological reactions and fulfill diffusion of secreted molecules from the stem cells into the target implanted site. Therefore, Emerich

et al. fabricated semipermeable hollow fiber membranes from commercially available polyethersulfone membrane filled with filaments of polyethylene terephthalate yarn to encapsulate stem cells. Then, they implanted stem cell-containing capsules into the rodent striatum. Released GDNF from cells was distributed into the brain and exerted neurotrophic effects [94].

In addition to neurotrophic factors, the release of antioxidants such as catalase to the brain of PD rats is performed by bone-marrow-derived macrophages. To prevent enzyme degradation and preserve its catalytic activity, PEI–PEG co-polymer and catalase were mixed and self-assembled into particles. Then, the particles were taken up rapidly by bone-marrow-derived macrophages. Slowed release of the enzyme from macrophages after exposure to PD mice activated either α-syn or TNF-α and reduced oxidative stress in the SN [95].

Stem cells might be used to deliver genes, instead of neurotrophic factors or antioxidant enzymes, to the brain. Implanting exosomes-releasing engineered cells into the animal models of PD is an example of mRNA delivery to the brain [31]. Macrophages, which are transfected with plasmid DNA encoding catalase, release exosomes that contain catalase mRNA, active catalase and NF-κB transcription factor. Delivery of these genetic materials to the neurons by exosomes resulted in the synthesis of catalase protein [35] (Tables 1 & 2).

Conclusion & future perspective

In this review, it was shown that loading drugs into biomaterial and cell-derived compartments increase the transport of anti-PD drugs across the BBB and improve neurons' survival. Also, biomaterials and exosomes effectively incorporate genes and improve the transfection of the desirable brain site. Furthermore, stem cell usage as sources of GDNF and neurturin, as well as macrophages-derived exosomes has opened a new horizon to the PD treatment.

Reviewing the recent drug and gene delivery systems for PD treatment suggests that using microbubbles and FUS is of great importance with the potential for clinical PD gene therapy. Liposome-based gene delivery systems for PD have become nearly extinct despite the previous beneficial results and recent studies have focused on using liposome-based gene vectors in combination with microbubbles and FUS technology. Liposomes for drug-delivery applications also could be replaced with niosomes, nanosized nonionic surfactant vesicles, owing to the lower price and complexity of niosome structure compared with liposomes, as Gunay *et al.* showed facilitated BBB penetration following the release of pramipexole from PEGylated niosomes into 6-OHDA rats brain [101]. Cerasome nanoparticles are another choice to be used instead of liposomes as Zhang *et al.* made polysorbate 80-modified cerasomes loaded with curcumin and conjugated with microbubbles for the BBB opening and PD treatment [102]. With regard to antioxidant and cell-protective mechanisms of curcumin, it has the potential to be used for PD treatment in the future after being loaded into drug-delivery vehicles to pass BBB [103,104].

Executive summary

- Liposomes are suitable carriers for the targeted and sustained release of drugs and antioxidants into the brain of patients suffering from Parkinson's disease (PD). The blood–brain barrier traverse and drug release of liposomes could be potentiated by incorporating specific metallic components and and applying an external magnetic force or near infrared laser.
- Cell-derived and macrophage-derived exosomes can also be used not only for dopamine and antioxidant delivery to the brain but also as a highly sensitive biomarker for PD recognition.
- For effective delivery of anti-PD drugs to the brain, nanocarriers in different forms of nanoparticles, gels and nanofibers are made from naturally occurring polymers such as chitosan, collagen, hyaluronic acid and Zein.
- Use of synthetic polymers such as poly(lactic-*co*-glycolic acid) (PLGA), co-polymer of polyethylene glycol–PLGA, PF127, polycaprolactone, polyethylenimine (PEI)/dextran sulfate and methacrylate co-polymers for delivery of anti-PD drugs to the brain is also commonplace.
- Besides polymers, neuroprotective and antioxidant effects of some metal or ceramic nanoparticles have made them interesting carriers for anti-PD drugs.
- Targeting SNCA, the gene related to α-syn protein, delivery of *GDNF* gene and suppressing miRNA-155 are other therapeutic approaches for PD treatment that have been done using vehicles made of cationic liposomes, exosomes and cationic polymers such as PEI, polyamidoamine, N-isopropylacrylamide and PLGA/protamine sulfate.
- Using focused ultrasound technology for temporary disruption of blood–brain barrier by microbubbles is another way of drug- and gene-delivery to the brain of PD cases. Microbubbles could be combined with other carrier systems, such as liposomes, to improve their therapeutic efficiency.

Overall, in recent years, many signs of progress in PD treatment have been made through designing cells and biomaterials in the form of drug and gene delivery systems. However, clinical translation of these systems requires more attempts and in-depth analysis of stem cell-derived macrophages and exosomes as platforms for the delivery of therapeutics.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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