Gene therapy aims to introduce genetic information into a cell-type of interest to replace, correct, silence, or modify defective genes. Gene therapy in its broadest sense can theoretically prevent, halt, or cure any condition that affects mankind. In addition to that, the introduction and/or manipulation of genes is one of the major research areas in biological sciences, aimed to deepen our knowledge on how biological systems work. Scientific advances have made it possible to induce changes ranging from manipulations of large stretches of the genome to the change of single nucleotides. The gold-standard vehicles to bring the genetic information into the target cells are viral vectors, amongst which the adeno-associated virus (AAV) is the most commonly used. AAV-vectors are small single stranded DNA viruses that naturally infect cells in humans and other primate species, thereby making them a perfect candidate for gene therapy. In contrast to retroviruses, such as lentiviral vectors, AAVs are replication deficient and do not integrate into the host genome thus reducing the risk of insertional mutagenesis. Furthermore, it is currently not known to cause any diseases in humans. The AAVs have an excellent biosafety profile and approximately 80–90% of the human population is already carrying the virus. There are currently about 13 naturally occurring variants known (serotypes), each with a different tropism profile to each cell/tissue type (Vance et al., 2015). In the clinic, AAV-vectors have been shown to achieve stable and long-term transgene expression. Currently over 215 clinical trials are either ongoing or completed using the AAV-vector platform (www.clinicaltrials.gov), targeted to a wide range of disorders such as haemophilia A and B, Duchenne muscular dystrophy, spinal muscular atrophy, Leber congenital amaurosis, but also neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease (Ginn et al., 2018). Two AAV-based drugs have already achieved FDA approval, Luxturna in 2017 for rare inherited retinal dystrophy and Zolgensma in 2019 for spinal muscular atrophy. For preclinical research, the AAV-vector is the workhorse to achieve transgene expression in different tissue types in a multitude of biological systems. By use of cell-type specific enhancers/promoters (Blankvoort et al., 2020), tissue selective capsid variants (Davidsson et al., 2019; Weinmann et al., 2020), or conditional expression systems (e.g., Cre recombinase or tetracycline) (Chen et al., 2013), disease modelling (e.g., protein overexpression) (Kirk et al., 2002), and gene editing (e.g., CRISPR/Cas) (Jinek et al., 2012), Future work on AAV development is largely focussed on achieving higher selectively in cell type specificity (tropism), transduction efficiency (transgene expression), avoiding host immune responses, and increasing the packaging capacity of the viral capsid. Additionally, increasing the production yield is a challenge for the future as the demand has increased in recent years as very large volumes of high genome copy (gc) titer vector are used in clinical applications and preclinical systemic injection studies. To achieve cell specific tropism, increased and cell specific transgene expression, larger cargo capacity, as well as increased production yield, several lines of research are ongoing focussing on i) capsid development, ii) expression cassette, and iii) production methods (Figure 1, top). New natural and synthetic AAV-capsid variants are constantly being identified and developed. Development of new capsids is based on rational design, directed evolution (e.g. CREATE) (Deverman et al., 2016), barcoding (e.g. BRAVE) (Davidsson et al., 2019), or computer guided generation for new peptide/capsid configurations (Ogden et al., 2019). These strategies have resulted in many remarkable improvements such as the generation of retrograde AAVs (AAV2-retro; MMN004/008 (Tervo et al., 2016; Davidsson et al., 2019) and AAVs that can cross the Blood Brain Barrier (PHPB) (Deverman et al., 2016). Larger cargo capacity can be achieved through the addition of viral capsid protein units or by splitting the delivery over several vectors. Stronger and faster transgene expression as well as cell type specificity can be achieved by novel combinations of promoter-enhancer elements (Blankvoort et al., 2020), codon optimization of the transgene, transcription-termination sequences (e.g., WPRE) as well as the introduction of protein stabilizing sequences (e.g., V5, P7). Another approach for faster and increased expression was the development of a double-stranded AAV vector (deWitt et al., 2020). Although it reduces the cargo capacity by approximately half, the transgene expression does not have to go through the second strand synthesis, which is a rate-limiting process in the transgene expression. Improvements in production methods are mainly focussing on vector yield to accommodate for the increased demand in high gc-titer AAVs for preclinical and clinical approaches. Large volumes are needed for several approaches, ranging from iv injections for circuit tracing (PHPB), preclinical primates as well as clinical trials, where titers in the range of 1 × 10^13 gc/mL per kg of body weight are becoming the norm (Li and Samulski, 2020). AAV production methods rely on a transfection-based method by which the two or three necessary plasmids are provided separately to a producer cell line. There are currently four different production systems: HEK 293T transfection, stable cell lines, the HSV system, and the baculovirus system using Sf9 cells. The main challenge is the scalability of the given approaches, as most are based on HEK vector systems and produce small to medium quantities. The HEK293T system is the most commonly used approach and relies on the delivery of pAAVrep-cap, pAd-helper, and the pAAV-transgene (containing the ITRs) in three separate plasmids. A two plasmid system where both helper plasmids are merged into one plasmid has been developed as well (Grimm et al., 2003), which increases the likelihood of co-transfection. Traditionally, T175 flasks or comparable cell culture dishes are used for production and scaled up approaches use roller-bottles or fixed bed reactor systems (in combination with stable cell lines). Production methods using suspension culture could be scaled to using large volume bioreactors (Davidsson et al., 2019) for clinical trials. There are several interesting developments such as the generation of producer cell lines, packaging cell lines, and novel cell lines for free-floating cell culture. Although the HSV system can be used to produce AAV vectors, the low risk of replication competent HSV through preferred production method
recombination cannot be completely excluded, therefore an additional step of inactivation/ removal/validation must be taken. The baculovirus/insect cell system is based on Sf9 cells which are grown in suspension cultures where different baculoviruses deliver the essential genes for AAV production. Currently, production of AAV-vectors is based on either a form of density gradient using iodixanol or cesium chloride, different versions of chromatography purifications, or on our current protocol using chloroform precipitation for purification. (Davidsson et al., 2020; Negrini et al., 2020). Although large volume batches of AAV vectors are needed for preclinical and clinical trials, current production methods are not very economical or time saving, when testing several different variants. When testing e.g. different promoters, only small volumes are needed to compare which promoter achieves the desired expression levels in the target tissue. The iodixanol-gradient purification results in volumes of about 200 µl AAV vector, taking days to complete, and requires specialized equipment such as ultracentrifuges and pumps, which are costly infrastructure that might not be available. Our novel PEG precipitation and chloroform extraction method relies on standard equipment and does not necessitate the use of ultracentrifugation. The methods differ mainly in the time of purification, yield, and purity of final AAVs. In our recently published protocol, we show a fast and efficient in-house production method that can be carried out in any normally equipped lab (with the appropriate safety classification). We have shown that this method, compared to standard iodixanol precipitation, produced vectors with comparable purity and packaging efficacy (>90% transduction efficiency) in a shorter time. The method also opens the field for many research groups that do not have access to specialized equipment. Gene therapy based on AAV vectors and non-viral nanoparticles will continue to provide unique opportunities to correct hereditary diseases and improve human health at a rapid pace. Our excitement by this approach is not only justified by the current technological state of the art, but also by the promises that the future holds. Precise control over genes such as base and prime editing has the potential to revolutionize personalized medicine. Our method helps with that process, and could speed up experimental therapeutic options, that hopefully can transition into successful clinical therapeutic modalities.

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