

PHARMACEUTICAL FORMULATIONS OF GENE DELIVERY VEHICLES

TECHNICAL FIELD

5 The invention relates to the field of gene therapy. In addition, the invention relates to the field of interfering RNA and/or microRNA (miRNA). In particular, the invention relates to gene therapy involving such miRNA and more in particular to pharmaceutical formulations with improved stability for the treatment of diseases including neurodegenerative diseases such as spinocerebellar ataxias type 3.

Gene therapy

10 The elucidation of DNA as the carrier of genetic information, and therefore also as the source of inherited diseases, has led to envisaged therapies in which mutant, damaged genes could be replaced or at least silenced. Many genes and/or other nucleic acid sequences have now been identified to play a role in (genetic) disease. If the mutant gene(s) could be replaced by a healthy one, or if the genes expressing aberrant (sometimes toxic) products could be silenced, the disease could be treated at the
15 molecular level and potentially be cured. Gene therapy provides a promising concept, in particular for diseases caused by mutations in a single gene.

However, the delivery of the desired nucleic acid to the cells that need to be targeted is not an easy task. Numerous (viral) delivery systems have been investigated, all of them with their advantages and
20 drawbacks. One of the viral delivery vehicles that are used for gene therapy is the adeno-associated virus (AAV).

AAV

AAV has a single-stranded DNA genome of approximately 4.8 kilobases (kb). AAV belongs to the
25 parvovirus family and is dependent for replication on co-infection with other viruses, in particular adenoviruses. The genome comprises Rep (Replication) and Cap (Capsid) genes. These coding sequences are flanked by inverted terminal repeats (ITRs) that are required for genome replication and packaging. The Rep gene encodes four proteins (Rep78, Rep68, Rep52, and Rep40), replicates the viral genome, and facilitates packaging, while Cap expression gives rise to the viral capsid proteins
30 (VP; VP1 VP2 VP3), which form the outer capsid shell.

For gene therapy, the viral DNA of the AAV is almost completely removed. Recombinant AAV (rAAV) for gene therapy is formed by a protein capsid containing a desired nucleic acid, the transgene, that is to be delivered to target cells. The desired nucleic acid is flanked by the ITR's of AAV. ITR -flanked
35 transgenes encoded by rAAV can form circular concatemers remaining in the nucleus of transduced cells as episomes. As the episome remains largely episomal, the expression of AAV delivered nucleic acid sequences may be diluted over time if and when the target cell replicates. This dilution may not generally apply to post-mitotic cells such as neurons, which are the target cells for many

neurodegenerative diseases. A review on AAV vectors for gene therapy is provided in Naso et al, Biodrugs 2017 (p.317-334).

miRNA

5 RNA interference (RNAi) is a naturally occurring mechanism that involves sequence-specific down-regulation of messenger RNA (mRNA). The down-regulation of mRNA results in a reduction of the amount of protein that is expressed. RNA interference is triggered by double-stranded RNA. One of the strands of the double-stranded RNA is substantially or completely complementary to its target, the mRNA. This strand is termed the guide strand. The mechanism of RNA interference involves the
10 incorporation of the guide strand in the RNA-induced silencing complex (RISC). This complex is a multiple turnover complex that via complementary base pairing of the guide strand can bind to its target mRNA. Once bound to its target mRNA it can either cleave the mRNA or reduce translation efficiency. RNA interference has since its discovery been widely used to knock down specific target genes. The triggers for inducing RNA interference that have been employed involve the use of small interfering
15 RNAs (siRNAs) or short hairpin RNAs (shRNAs). In addition, molecules that can naturally trigger RNAi, the so-called microRNAs (miRNAs), have been used to make artificial (engineered) miRNAs that mimic their naturally occurring counterparts. These strategies have in common that they provide for substantially double-stranded RNA molecules that are designed to target an mRNA of choice. RNAi-based therapeutic approaches that utilise the sequence-specific modality of RNAi are under
20 development and several are currently in clinical trials (see i.a. Davidson and McCray, Nature Reviews - Genetics, 2011; Vol.12; 329-340).

Spinocerebellar Ataxia Type 3 (SCA3)

Spinocerebellar ataxia type 3 (SCA3), or Machado-Joseph disease (MJD), is an autosomal dominant
25 monogenic, fatal disorder. The disorder is characterized by progressive degeneration of brain areas, which is caused by a CAG expansion in the human ataxin-3 gene, also referred to as the ATXN3 gene (OMIM: 607047, reference sequence Homo sapiens ataxin 3 (ATXN3) on chromosome 14, NCBI Reference Sequence: NG_008198.2). In the 3' regions of the gene, a cytosine-adenine-guanine (CAG) repeat region is present. Said CAG region is in the frame and results in an ataxin-3 protein comprising
30 a polyQ region, a repetitive sequence of glutamines. Healthy, or non-symptomatic, individuals may have up to 44 CAG-repeats in the ATXN3 gene. Diseased individuals have expansions and it has been shown that they may have between 52 and 86 or more CAG repeats. Individuals having between 45-51 CAG repeats are to have symptoms with incomplete penetrance of the disease. Said expansion results in an ataxin-3 protein that has extended polyQ regions and the length of the CAG repeats, and thus polyQ
35 regions within ataxin-3, can be correlated with disease progression, i.e. the longer the region usually the more progressive the disease.

The ataxin-3 protein with the expanded polyQ tract acquires toxic properties (gain of toxic function) and the formation of neuronal aggregates in the brain is the neuropathological hallmark. Neuropathological studies have detected a widespread neuronal loss in various areas, including the cerebellum, thalamus,

midbrain, pons, medulla oblongata, and spinal cord of SCA3 patients (Riess et al., Cerebellum 2008). Although widespread pathology is reported, the consensus is that the main pathology is in the cerebellum and the brainstem (Eichler et al. AJNR Am J Neuroradiol, 2011). The disease has full penetration, which means that if a person has an expansion of 52 or more CAGs, they will inevitably develop the disease and have a 50% chance to pass it on to their offspring.

Formulation and administration challenges

Using AAV vectors to deliver micro-RNAs directly to a patient's brain for knockdown of the ATXN3 gene could represent a highly innovative and promising approach for treating SCA3.

However, administration directly to a patient's brain or spine presents special challenges for the formulation and stability of a suitable pharmaceutical formulation, such as the need to avoid undesirable formation of aggregated or agglomerated particles in the formulation that may be detrimental to a patient. For example, such formulations may be stored frozen and then thawed before use. Aggregates or agglomerates could problematically form during or after the thaw of the formulation, and also during the time following thawing but before administration of the formulation. It would therefore be highly desirable to provide an innovative formulation of an AAV vector for treating spinocerebellar ataxia type 3, or other diseases, that eliminates or substantially eliminates aggregate or agglomerate formation for an extended in-use shelf life.

SUMMARY OF INVENTION

In a first aspect there is provided an isotonic pharmaceutical formulation comprising:

a buffer

an adeno-associated viral vector with a transgene encoding a microRNA;

a tonicity agent;

a cryoprotectant; and

a surfactant.

In some embodiments, the formulation has an osmolarity of from 250 to 330 mOsm/kg and a pH value of from 5 to 8.

In some embodiments, the formulation is substantially free of visible particles.

In some embodiments, the adeno-associated viral vector with a transgene encoding microRNA targets ATXN mRNA, for example, ATXN3 mRNA.

In some embodiments, the adeno-associated viral vector comprises an AAV2 serotype, an AAV5 serotype, an AAV9 serotype, a hybrid AAV serotype, or a combination thereof, preferably the adeno-associated viral vector comprises an AAV5 serotype or an AAV9 serotype, more preferably, an AAV5 serotype.

In some embodiments, the concentration of the adeno-associated viral vector is at least 1E13 gc/ml, such as at least 3E13 gc/ml. In some embodiments, the concentration of the adeno-associated viral vector is between 0.5E13 and 6E13 gc/ml, such as between 1E13 and 5E13 gc/ml.

- 5 In some embodiments, the cryoprotectant is a carbohydrate selected from the group consisting of trehalose, sucrose, dextrose, dextran, and combinations thereof; and present in an amount of up to 8% w/v.

- 10 In some embodiments, the tonicity agent is selected from the group consisting of NaCl, KCl, MgCl₂, CaCl₂, and combinations thereof; and is present in an amount of from 5 mM to 150 mM. In some embodiments, the surfactant is a non-ionic surfactant; and is present in an amount of from 0.001% v/v to 0.1% v/v, preferably from 0.003% v/v to 0.08% v/v, more preferably from 0.005% v/v to 0.02% v/v, for example about 0.01% v/v.

- 15 In some embodiments, the buffer is selected from the group consisting of an artificial cerebrospinal fluid (aCSF), acetate buffer, citrate buffer, phosphate buffer, HEPES, Tris, and combinations thereof.

- 20 In some embodiments, the formulation remains stable at room temperature (15°C-25°C) without substantial formation of aggregates or agglomerates for at least 12 hours after thawing.

- In a second aspect, there is provided the formulation of the invention for use in treating Spinocerebellar Ataxia (SCA).

- 25 In some embodiments, the formulation is administered by intra-CSF (cerebral spinal fluid) delivery.

- In some embodiments, the formulation is administered to a patient's cisterna magna.

DETAILED DESCRIPTION OF INVENTION

- 30 The present invention seeks to provide a new pharmaceutical formulation that has reduced aggregate or agglomerate formation during processing and is essentially free of visible particles for an extended time at room temperature after thawing. The present invention also seeks to provide a pharmaceutical formulation comprising one or more excipients that is substantially isotonic to human cerebrospinal fluid (CSF) for improved administration to a patient's brain. The present invention also seeks to provide a method of treating spinocerebellar ataxia type 3 (SCA3) by administering the formulation of the invention to a patient's brain.
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In one aspect of the invention, the present invention provides an isotonic pharmaceutical formulation comprising a buffer, an adeno-associated viral vector with a transgene encoding a microRNA, a tonicity agent, a cryoprotectant, and a surfactant.

In this way, it has been surprisingly found that the addition of a cryoprotectant and a surfactant to the formulation helps to improve the stability of the drug product, in particular under high concentration. The formulation may remain stable without substantial formation of aggregates or agglomerates for at least about 6 months, preferably at least about 12 months, more preferably at least about 24 months, for example at least about 36 months, at a storage temperature of $\leq -65^{\circ}\text{C}$. The formulation may also remain stable at room temperature (15°C - 25°C) without substantial formation of aggregates or agglomerates for an extended time, preferably at least about 12 hours, more preferably at least about 24 hours, after thawing.

In some embodiments, the adeno-associated viral vector with a transgene encoding a microRNA may target ATXN mRNA, which may enable the formulation to treat spinocerebellar ataxia. For example, the adeno-associated viral vector with a transgene encoding a microRNA may target ATXN3, thereby lowering ATXN3 mRNA and protein levels in the treatment of spinocerebellar ataxia type 3. Therefore, in some embodiments, the adeno-associated viral vector with a transgene encoding microRNA targets ATXN mRNA. In further embodiments, the adeno-associated viral vector with a transgene encoding microRNA targets ATXN3 mRNA.

In some embodiments, the adeno-associated viral vector comprises an AAV2 serotype, an AAV5 serotype, an AAV9 serotype, a hybrid AAV serotype, or a combination thereof. Said serotypes have been found particularly preferred for the treatment of spinocerebellar ataxia type 3. In some embodiments, the adeno-associated viral vector comprises an AAV9 serotype.

In some embodiments, the adeno-associated viral vector comprises an AAV5 serotype.

In some embodiments, the adeno-associated viral vector comprises a hybrid AAV serotype. By way of example, the hybrid AAV serotype may be a hybrid AAV2/AAV5; AAV2/AAV9; or AAV5/AAV9 serotype.

An example of a method and means to deliver miRNA to target cells has been provided in WO2020/104469 A1, which is incorporated herein by reference.

In some embodiments, the concentration of the adeno-associated viral vector is at least $1\text{E}13$ gc/mL; preferably at least $2\text{E}13$ gc/mL; more preferably at least $1\text{E}14$ gc/mL; for example at least $3\text{E}14$ gc/mL. In some embodiments, the concentration of the adeno-associated viral vector is at least $6\text{E}14$ gc/mL. In some embodiments, the concentration of the adeno-associated viral vector is between $4\text{E}14$ gc/mL and $5\text{E}14$ gc/mL.

In some embodiments, the formulation comprises an AAV5 with a transgene encoding a microRNA, and the concentration of AAV5 is at least $6\text{E}14$ gc/mL.

In one example, the formulation comprises an AAV5 with a transgene encoding a microRNA, and the concentration of AAV5 is 3×10^{14} gc/mL.

5 In another example, the formulation comprises an AAV5 with a transgene encoding a microRNA, and the concentration of AAV5 is 6×10^{14} gc/mL.

In yet another example, the formulation comprises an AAV5 with a transgene encoding a microRNA, and the concentration of AAV5 is 7.5×10^{14} gc/mL.

10 The stable, high concentration formulation may be particularly useful in delivering sufficient genome copies of the drug product when the dosage volume is limited. An example of such application is when injection into the cisterna magna (CM, whose average volume is approximately 1 ml only) is required.

15 In some embodiments, the formulation comprises an AAV9 with a transgene encoding a microRNA, and the concentration of AAV9 is at least 6×10^{14} gc/mL.

In one example, the formulation comprises an AAV9 with a transgene encoding a microRNA, and the concentration of AAV9 is 3×10^{14} gc/mL.

20 In another example, the formulation comprises an AAV9 with a transgene encoding a microRNA, and the concentration of AAV9 is 6×10^{14} gc/mL.

In yet another example, the formulation comprises an AAV9 with a transgene encoding a microRNA, and the concentration of AAV9 is 7.5×10^{14} gc/mL.

25 The formulation may also have a pH value compatible with human cerebrospinal fluid. For example, the formulation may have a pH of about 5 or higher, preferably about 7 or higher. In some embodiments, the formulation may have a pH value of from 5 to 8, preferably from 6 to 8, more preferably from 6.5 to 8, even more preferably from 7 to 8, for example from 7.1 to 7.7. In some embodiments of the present invention, the formulation has a pH value of 7.3.

30 Tonicity is a measure for the effective osmotic pressure that a liquid formulation can exert, and depends primarily on the number of dissolved particles in solution. Osmotic pressure is an important factor affecting biological cells. Hypertonicity is the presence of a solution that causes cells to shrink. Hypotonicity is the presence of a solution that causes cells to swell. Isotonicity is the presence of a solution that produces no change in cell volume. When a biological cell is in a hypotonic environment, the cell interior accumulates water, water flows across the cell membrane into the cell, causing it to expand. For mammalian cells this can lead to cytolysis, and tonicity is therefore important when fragile cells such as nerve cells or brain cells are to be exposed to a composition. Tonicity agents are therefore added to injectable preparations to prevent osmotic shock at the site of injection upon administration,

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and thereby reduce local irritation or even damage to the CNS. Typical tonicity agents are thus excipients used for tonicity adjustment, and are known in the art.

5 In some embodiments, the tonicity agent comprises a periodic group 1 or group 2 metal salt, preferably a periodic group 1 or group 2 metal chloride salt, preferably selected from the group consisting of NaCl, KCl, CaCl₂, MgCl₂, and combinations thereof. Preferably, the tonicity agent comprises NaCl. In one example of the invention, the tonicity agent comprises a combination of NaCl and KCl. Tonicity agents help to stabilize the formulation. Preferably, the tonicity agent is present in an amount of from 5 mM to 150 mM, preferably from 25 mM to 125 mM, more preferably from 75 mM to 125 mM, for example, 90
10 mM. For example, the tonicity agent may comprise at least 75 mM NaCl. In some embodiments, the tonicity agent is present in an amount of 90 mM. For example, the tonicity agent may comprise a combination of NaCl and KCl and be present in an amount of 90 mM. In some embodiments, the tonicity agent comprises at least 80 mM NaCl and at least 2 mM KCl. For example, the tonicity agent may comprise 3 mM KCl and 87 mM NaCl or 82 mM NaCl. Therefore, in some embodiments the tonicity
15 agent is selected from the group consisting of NaCl, KCl, MgCl₂, CaCl₂, and combinations thereof; and is present in an amount of from 5 mM to 150 mM.

The tonicity agent is primarily used to establish a desired osmolality for the formulation. Preferably, the formulation is substantially isotonic to human cerebrospinal fluid. In particular, the formulation may have
20 an osmolality of from 250 to 330 mOsm/kg. In some preferred embodiments, the formulation has an osmolality of from 270 to 320 mOsm/kg. For example, the formulation has an osmolality of 280 mOsm/kg. Therefore, in one embodiment, the formulation has an osmolality of from 250 to 330 mOsm/kg and a pH value of from 5 to 8.

Cryoprotectants are additives that help minimize polypeptide/biopharmaceutical denaturation when
25 polypeptides are present in the composition. Cryoprotectants have been found to be particularly advantageous in providing a stable drug product that eliminates or substantially eliminates the formation of aggregates or agglomerates. In preferred embodiments, the cryoprotectant is a carbohydrate or a polymer. The term "carbohydrates" as used herein refers to both naturally occurring saccharides as well as to polyols that can be derived from saccharides. Examples of suitable carbohydrates are trehalose,
30 sucrose, dextrose, dextran, and combinations thereof. Therefore, in some embodiments, the cryoprotectant is a carbohydrate selected from the group consisting of trehalose, sucrose, dextrose, dextran, and combinations thereof. Preferably, the cryoprotectant is trehalose or dextran. Trehalose is a disaccharide formed by a 1,1-glycosidic bond between two α -glucose units, and sucrose is a disaccharide that is O- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside. Optionally other disaccharides
35 could be used in their place. Dextrose is D-glucose, and optionally other monosaccharides could be used in its place. In a more preferred embodiment of the invention, the cryoprotectant is trehalose. In another embodiment of the invention, the cryoprotectant is dextran, for example, dextran 6000. In some embodiments, the cryoprotectant is present in the formulation in an amount of up to 8% w/v, preferably from 2% w/v to 5% w/v, for example from 2% w/v to 3% w/v. In some embodiments, the cryoprotectant

is trehalose and is present in the formulation in an amount of from 2% w/v to 6% w/v, for example, 3% w/v. In some embodiments, the cryoprotectant is dextran, for example, dextran 6000, and is present in an amount of from 1% w/v to 3% w/v, for example from 2% w/v to 2.5% w/v. Therefore, in some embodiments, the cryoprotectant is a carbohydrate selected from the group consisting of trehalose, sucrose, dextrose, dextran, and combinations thereof; and present in an amount of up to 8% w/v.

Surfactants and their characteristics are well known, and surfactants generally comprise at least one polar head group and at least one apolar or hydrophobic tail. The surfactants used here preferably comprise a single tail and preferably comprise a single head group. They are preferably charge neutral, which means that the surfactants do not have a net charge at the conditions for their use. Alkylated saccharides are most preferred for use in this invention, particularly polysorbates, more particularly polysorbate 20. In some embodiments, the surfactant is a non-ionic surfactant. Preferably, the surfactant is a polyethoxylated non-ionic surfactant such as a polysorbate. In a preferred embodiment of the invention, the surfactant is Polysorbate-20.

The surfactant has been found to be particularly advantageous in improving the stability and reducing the formation of aggregated or agglomerated particles in the formulation. Optionally, the surfactant may be present in an amount of from 0.001% v/v to 0.1% v/v, preferably from 0.03% v/v to 0.08% v/v, more preferably from 0.005% v/v to 0.02% v/v. In some embodiments, the surfactant is present in the formulation in an amount of from 0.005 to 0.015% v/v. Therefore, in some embodiments, the surfactant is a non-ionic surfactant and is present in an amount of from 0.0001% v/v to 0.1% v/v, preferably from 0.03% v/v to 0.08% v/v, more preferably from 0.005% v/v to 0.02% v/v. In some embodiments, the surfactant is present in the formulation in an amount of from 0.005 to 0.015% v/v, for example about 0.01% v/v. For example, the surfactant is polysorbate-20 and is present in an amount of about 0.01% v/v.

Buffering agents are known in the art, and help maintain the pH of the composition stable within a given range. A buffering agent is often a buffer salt. The buffer may be selected from the group consisting of an artificial cerebrospinal fluid (aCSF), phosphate buffer, acetate buffer, citrate buffer, HEPES buffer, Tris buffer, and combinations thereof.

In some embodiments, the buffer may be a phosphate buffer, for example, a phosphate buffer at a pH from 6.5 to 7.5. In some embodiments, the buffer may be an acetate buffer, for example, an acetate buffer at a pH from 5 to 5.5. In some embodiments, the buffer may be a citrate buffer, for example, a citrate buffer at a pH from 5.5 to 6.5. In some embodiments, the buffer may be a Tris buffer, for example, a Tris buffer at a pH from 7.5 to 8. In some embodiments, the buffer may be a HEPES buffer, for example, a HEPES buffer at a pH from 7 to 8. In some embodiments, the buffer may be aCSF for example, a aCSF at a pH from 7.0 to 7.6. For example, the aCSF may have the following final ion concentration (in mM): Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155. Preferably, the buffer is a phosphate buffer or TRIS; for example, the buffer is a 10mM phosphate buffer at pH 7.3.

The buffering agent, tonicity agent, cryoprotectant, and any other functionally defined components of the composition, such as a surfactant, can be individual substances, but can also be mixtures. For instance, the buffering agent can be a single phosphate salt such as Na_2HPO_4 , but it can also be a mixture of substances such as a mixture of Na_2HPO_4 and KH_2PO_4 . Similarly the tonicity agent can be a single substance such as NaCl , but it can also be a mixture such as a combination of NaCl and KCl . Throughout this document, when only a single substance is specified for a functional definition, preferably no other substances are present for that same function. The same holds for when a plurality is specified, in which case only the specified substances are comprised for that same function.

Some compounds, such as for example phosphate salts, can act as a buffering agent but can also act as a tonicity agent. As used herein, when a compound is present as one functionally defined component of the composition, then it preferably is not considered as also satisfying any requirements for further functionally defined components. Accordingly, when one or more of acetate salts, citrate salts, HEPES, TRIS, or phosphate salts are present, these are preferably considered as buffering agent only. When one or more of NaCl , KCl , MgCl_2 , CaCl_2 , or a mixture thereof are present, these are preferably considered as tonicity agent only. When one or more of trehalose, dextran, sucrose, dextrose, or poly(ethylene glycol) are present, these are preferably considered as cryoprotectant only. When one or more non-ionic surfactants are present, these are preferably considered as surfactants only.

In a particularly preferred embodiment, the formulation comprises, consists, or consists essentially of:

- an adeno-associated viral vector with a transgene encoding a microRNA targeting ATXN3 mRNA;
- about 5-30 mM Phosphate buffer at a pH from 7 to 8;
- at least about 75 mM NaCl ;
- about 2-5 mM KCl ;
- about 2% w/v to 4% w/v trehalose;
- about 0.005% v/v to 0.015% v/v PS-20.

For example, the formulation may comprise, consist or consist essentially of:

- an adeno-associated viral vector with a transgene encoding a microRNA targeting ATXN3 mRNA;
- about 9 mM Tris at a pH of about 7.3;
- about 82 mM NaCl ;
- about 3 mM KCl ;
- about 3% w/v trehalose;
- about 0.01% v/v PS-20 (Tween 20)

For example, the formulation may comprise, consist or consist essentially of:

- an adeno-associated viral vector with a transgene encoding a microRNA targeting ATXN3 mRNA;
- about 9 mM Tris at a pH of about 7.3;
- about 87 mM NaCl;
- 5 • about 3 mM KCl;
- about 3% w/v trehalose;
- about 0.01% v/v PS-20 (Tween 20)

10 In some embodiments, the formulation is substantially free of visible particles. Thus, the formulation is essentially free of particles visible to the naked human eye, as determined and characterized according to U.S. Pharmacopeial Convention (USP) Chapter 790 "VISIBLE PARTICULATES IN INJECTIONS" guidance of 2014 regarding parenteral medical products being "essentially free" of visible particulate matter (USP 790), which is incorporated herein by reference. Visible particles may be a sign of aggregation, agglomeration, and/or degradation of AAV particles, such that a lack of visible particles
15 may be advantageous to indicate a particularly stable formulation free from substantial aggregation or agglomeration. Preferably, the formulation also comprises limited subvisible particles, as determined and characterized according to U.S. Pharmacopeial Convention (USP) Chapter 787 "SUBVISIBLE PARTICULATE MATTER IN THERAPEUTIC PROTEIN INJECTIONS" (USP 787), which is incorporated herein by reference. For example, the number of particles having a diameter of $\geq 25 \mu\text{m}$
20 is no more than 600 per vial, and the number of particles having a diameter of $\geq 10 \mu\text{m}$ is no more than 6000 per vial, in the formulation. Optionally, the formulation has a Dv90 of less than $50 \mu\text{m}$, preferably less than $25 \mu\text{m}$, more preferably less than $10 \mu\text{m}$. The Dv90 values represent the percent (90% in this case) of the formulation that has a size no larger than the specified value.

25 In some embodiments, the formulation of the invention may be used in treating spinocerebellar ataxia (SCA), for example, spinocerebellar ataxia type 3 (SCA3). In some embodiments, the formulation is be administered by intra-CSF (cerebral spinal fluid) delivery. For example, the formulation is administered by intra-CSF delivery such as intracisternal delivery. Optionally, the formulation may be administered by direct injection to the brain, or by repeat injection at the cisterna magna, or by slow infusion at the
30 cisterna magna, or by a combination of direct injection to the brain and injection(s) at the cisterna magna. In some embodiments, the formulation may be administered by injection at the cisterna magna. In some embodiments, the formulation may be administered by a single injection at the cisterna magna. For example, the formulation may be administered by (a single) injection (optionally CT-guided) into the cisterna magna over 5 minutes.

35 The "cisterna magna" or "cerebellomedullary cistern" is one of three principal openings in the subarachnoid space between the arachnoid and pia mater layers of the meninges surrounding the brain. The openings are collectively referred to as cisterns. The cisterna magna is located between the

cerebellum and the dorsal surface of the medulla oblongata. Cerebrospinal fluid produced in the fourth ventricle drains into the cisterna magna via the lateral apertures and median aperture.

5 In some embodiments, the formulation is administered at 5 mL per subject at a concentration of the adeno-associated viral vector of 3×10^{14} gc/mL. In some specific embodiments, the formulation is administered at 5 mL per injection site at a concentration of the adeno-associated viral vector of 3×10^{14} gc/mL. In some embodiments, the formulation is administered at a dosage of from 4×10^{14} to 5×10^{15} genome copies, preferably from 8×10^{14} to 2×10^{15} genome copies, more preferably from 1×10^{15} to 2×10^{15} genome copies, per subject. For example, the formulation is administered at a dosage of 1.5×10^{15} genome copies per subject.

In some embodiments, the formulation of the invention may be administered together with a contrast agent visualisable by magnetic resonance imaging (MRI). This may be advantageous when it is desired to monitor the patient's brain by MRI.

15 Alternatively, or in addition as another product of interest, the nucleotide sequence comprising the transgene encoding a microRNA as defined herein above may further comprise a nucleotide sequence encoding a polypeptide that serves as a selection marker protein to assess cell transformation and expression. Suitable marker proteins for this purpose are e.g. the fluorescent protein GFP, and the selectable marker genes HSV thymidine kinase (for selection on HAT medium), bacterial hygromycin B phosphotransferase (for selection on hygromycin B), Tn5 aminoglycoside phosphotransferase (for selection on G418), and dihydrofolate reductase (DHFR) (for selection on methotrexate), CD20, the low affinity nerve growth factor gene. Sources for obtaining these marker genes and methods for their use are provided in Sambrook and Russel, see below.

25 Furthermore, the nucleotide sequence comprising the transgene as defined herein above may comprise a further nucleotide sequence encoding a polypeptide that may serve as a fail-safe mechanism that allows to cure a subject from cells transduced with an AAV vector of the invention, if deemed necessary. Such a nucleotide sequence, often referred to as a suicide gene, encodes a protein that is capable of converting a prodrug into a toxic substance that is capable of killing the transgenic cells in which the protein is expressed. Suitable examples of such suicide genes include e.g. the *E.coli* cytosine deaminase gene or one of the thymidine kinase genes from Herpes Simplex Virus, Cytomegalovirus and Varicella-Zoster virus, in which case ganciclovir may be used as prodrug to kill the transgenic cells in the subject (see e.g. Clair *et al.*, 1987, Antimicrob. Agents Chemother. 31: 844-849).

35 In one aspect the invention provides a method for preparing a drug product for administration to the central nervous system, the method comprising the steps of:

- i) providing a formulation according to the invention; and
- ii) aliquoting the formulation into a suitable dosage form.

The prepared drug product is suitable for administration to the central nervous system, for instance as described elsewhere herein. Accordingly, the method is suitable for preparing formulations for use according to the invention, preferably wherein the composition is for intra-cerebrospinal fluid administration. Therefore, in one embodiment the formulation for use according to the invention is administered to a patient's cisterna magna.

In yet another aspect of the invention, the present invention provides a method of treating SCA, in particular SCA3, by administering the formulation of the invention to a patient's brain.

10 DEFINITIONS

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, the verb "to consist" may be replaced by "to consist essentially of" meaning that a combination or a composition as defined herein may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristic of the invention. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements.

20 The indefinite article "a" or "an" thus usually means "at least one".

Whenever a parameter of a substance is discussed in the context of this invention, it is assumed that unless otherwise specified, the parameter is determined, measured, or manifested under physiological conditions. Physiological conditions are known to a person skilled in the art, and comprise aqueous solvent systems, atmospheric pressure, pH-values between 6 and 8, a temperature ranging from room temperature to about 37° C (from about 20° C to about 40° C), and a suitable concentration of buffer salts or other components.

30 In the context of this invention, a decrease or increase of a parameter to be assessed means a change of at least 5% of the value corresponding to that parameter. More preferably, a decrease or increase of the value means a change of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, or 100%. In this latter case, it can be the case that there is no longer a detectable value associated with the parameter.

35 The word "about" or "approximately" when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 10% of the value, optionally more or less 5%.

Each embodiment as identified herein may be combined together unless otherwise indicated. The invention has been described above with reference to a number of embodiments. A skilled person could envision trivial variations for some elements of the embodiments. These are included in the scope of protection as defined in the appended claims. All patent and literature references cited are hereby
5 incorporated by reference in their entirety.

In addition, the following terms are used herein and defined as follows.

- “*Capsid*” – A protein shell surrounding viral DNA, RNA, or microRNA that helps target the genetic material to specific cell types.
- 10 • “*Gene cassette*” – A small fragment of DNA, RNA, or microRNA containing the therapeutic gene and instructions for the cell on how to use the gene.
- “*Vector*” – This may refer to the DNA, RNA, or microRNA molecule itself or the carrier construct containing the DNA, RNA, or microRNA to be delivered to a cell.
- “*microRNA*” (also termed “*miRNA*” or “*siRNA*”) – a small single-stranded non-coding RNA
15 molecule, typically containing a guide strand as described above.
- “*SCA*” – spinocerebellar ataxia.
- “*substantially*” – the term “substantially” as used herein is a broad term, and is to be given its ordinary and customary meaning to a person of ordinary skill in the art (and is not to be limited to a special or customized meaning), and refers without limitation to being largely but not
20 necessarily wholly that which is specified.
- “*PS-20*” – polysorbate-20.
- “*iPSCs*” - induced pluripotent stem cells.
- “*ITR*” – inverted terminal repeat.
- “*MRI*” – magnetic resonance imaging.

25

Specific embodiments of the present invention are described in the following paragraphs.

1. An isotonic pharmaceutical formulation comprising:
a buffer
an adeno-associated viral vector with a transgene encoding a microRNA;
30 a tonicity agent;
a cryoprotectant; and
a surfactant.
2. The formulation of paragraph 1, wherein the formulation has an osmolarity of from 250 to 330
35 mOsm/kg, preferably from 260 to 310 mOsm/kg, for example from 270 to 300 mOsm/kg.
3. The formulation of paragraph 1 or 2, wherein formulation has a pH value of from 5 to 8, preferably from 6 to 8, more preferably from 6.5 to 8, for example from 7 to 8.

4. The formulation of any one of the preceding paragraphs, wherein the formulation is substantially free of visible particles.
5. The formulation of any one of the preceding paragraphs, wherein the adeno-associated viral vector with a transgene encoding microRNA targets ATXN mRNA, for example, ATXN3 mRNA.
6. The formulation of any one of the preceding paragraphs, wherein the adeno-associated viral vector comprises an AAV2 serotype, an AAV5 serotype, an AAV9 serotype, a hybrid AAV serotype, or a combination thereof, preferably the adeno-associated viral vector comprises an AAV5 serotype or an AAV9 serotype, for example, an AAV5 serotype.
7. The formulation of any one of the preceding paragraphs, wherein the concentration of the adeno-associated viral vector is at least 1E13 gc/ml, such as at least 3E13 gc/ml. In some embodiments, the concentration of the adeno-associated viral vector is between 0.5E13 and 6E13 gc/ml, such as between 1E13 and 5E13 gc/ml.
8. The formulation of any one of the preceding paragraphs, wherein the cryoprotectant is a sugar.
9. The formulation of paragraph 8, where the cryoprotectant is a sugar selected from the group consisting of trehalose, sucrose, dextrose, dextran, and combinations thereof; for example, the cryoprotectant is trehalose; alternatively, the cryoprotectant is a dextran, such as a dextran 6000.
10. The formulation of any one of the preceding paragraphs, wherein the cryoprotectant is present in an amount of up to 8% w/v preferably from 2% w/v to 5% w/v, for example from 2% w/v to 3% w/v.
11. The formulation of any one of the preceding paragraphs, wherein the cryoprotectant is trehalose and is present in an amount of 3% w/v.
12. The formulation of any one of the preceding paragraphs, wherein the tonicity agent is selected from the group consisting of NaCl, KCl, MgCl₂, CaCl₂, and combinations thereof.
13. The formulation of paragraph 12, wherein the tonicity agent is a combination of NaCl and KCl,
14. The formulation of any one of the preceding paragraphs, wherein the tonicity agent is present in an amount of from 5 mM to 150 mM, preferably from 25 mM to 125 mM, more preferably from 75 mM to 125 mM, for example, 90 mM.

15. The formulation of paragraph 14, wherein the tonicity agent comprises at least 75 mM NaCl.
16. The formulation of any one of the preceding paragraphs, wherein the surfactant is a non-ionic surfactant, preferably a polyethoxylated non-ionic surfactant such as a polysorbate, for example, polysorbate-20.
17. The formulation of any one of the preceding paragraphs, wherein the surfactant is present in an amount of from 0.001% v/v to 0.1% v/v, preferably from 0.003% v/v to 0.08% v/v, more preferably from 0.005% v/v to 0.02% v/v, for example about 0.01% v/v.
18. The formulation of any one of the preceding paragraphs, wherein the surfactant is polysorbate 20 and is present in an amount of 0.01% v/v.
19. The formulation of any one of the preceding paragraphs, wherein the buffer is selected from the group consisting of an artificial cerebrospinal fluid (aCSF), acetate buffer, citrate buffer, phosphate buffer, HEPES, Tris, and combinations thereof; preferably, the buffer is a phosphate buffer or TRIS; for example, the buffer is a 9-10 mM phosphate buffer at pH 7.3.
20. The formulation of any one of the preceding paragraphs, wherein the formulation remains stable at room temperature without substantial formation of aggregates or agglomerates for at least 12 hours, preferably at least 18 hours, for example at least 24 hours, after thawing.
21. The formulation of any one of the preceding paragraphs for use in treating Spinocerebellar Ataxia (SCA), for example, SCA type 1, SCA type 2, or SCA type 3, preferably SCA type 3.
22. The formulation for use of paragraph 21, wherein the formulation is administered by intra-CSF (cerebral spinal fluid) delivery; preferably, the formulation is administered by intracisternal delivery.
23. The formulation for use of paragraph 21 or 22, wherein the formulation is administered to a patient's cisterna magna.
24. The formulation for use of any one of paragraphs 21 to 23, wherein the formulation is administered together with contrast agent for MRI monitoring of the administration.
25. A method of treating Spinocerebellar Ataxia by administering the formulation of any one of the preceding paragraphs to a patient.

26. The method of paragraph 25, wherein the formulation is administered by a single dosage delivered by intracisternal injection into the patient.

5 27. The method of paragraph 25 or 26, wherein the formulation is administered together with a contrast agent visualisable by MRI.

FIGURES

Specific embodiments of the present invention will be described by way of example, with reference to the accompanying drawings in which:

10

Figure 1 shows results of Example 1. T_m (melting temperature of the capsid as determined by iDSF approach) as a function of the accelerated stress conditions and duration.

15

Figure 2 shows protein content and fluorescence as a function of the stress conditions and durations for the different salt concentrations in Example 1. The NaCl concentrations are in different greyscale shades. The different buffers are pooled. UV280 content and Trp_Fluo:280,335 are in arbitrary units. For the storage at 25°C and 40°C the scale is in days, 7 and 2 respectively, while for the shaking is for 24 hours.

20

Figure 3 is a Production profiler from a DoE experiment based on the absorbance and fluorescence response on the full dataset of all timepoints and all stresses.

25

Figure 4 is a Production profiler from a DoE experiment based on the absorbance and fluorescence response on the full dataset of all timepoints and all stresses.

30

Figure 5 shows poly-dispersity index (PDI) measurements by dynamic light scattering as a function of fold concentration.

Figure 6 shows z-average measurements by dynamic light scattering as a function of fold concentration.

Figure 7 shows z-average measurements by dynamic light scattering of accelerated- and long-term stability.

35

Figure 8 A/B show a subvisible particle analysis of accelerated- and long-term stability.

Figure 9 is a visual inspection overview of AAV9 DP in five formulation buffers at different stress conditions. No fill: no visible particulates present; Diagonal line fill: one-two visible particulates present; Dense diagonal line fill: few visible particulates present.

Figure 10 shows luciferase activity in cell lysates of Huh-7 cells transduced with AAV9 (1489)-luciferase formulated in different buffers.

EXAMPLES

5 *Abbreviations*

AAV is Adeno Associated Virus

AC is Affinity Chromatography

aCSF is Artificial Cerebrospinal Fluid

AIM is Analytical Investigational Method

10 CSF is Cerebrospinal Fluid

DLS is Dynamic Light Scattering

DoE is Design of Experiments

DP is Drug Product

DPD is Drug Product Development

15 DS is Drug Substance

F/T is Freeze/Thaw

Gc is Genome Copies

GMP is Good Manufacturing Practice

iDSF is Intrinsic Differential Scanning Fluorimetry

20 IEX is Ion Exchange

MW is Molecular weight

N/A is Not Applicable

NF is Nanofiltration

PD is Process Development

25 PDI is Polydispersity index

PEG is Poly-ethylene Glycol

R.U. is Arbitrary units

SOP is Standard Operational Procedure

STR is Stirring Tank Reactor

30 Tp is Total Particles

UF/DF is Ultrafiltration/Diafiltration

VI is Visual Inspection

Materials and Methods

35 The following materials were used in the examples and are available as specified below in Table 1.

Table 1: Materials used in the examples and their suppliers

Raw Materials	Supplier
Acetic Acid	Merck glacial 100%,
Citric acid	Merck
Dextran 20000	Sigma
Dextran 55000	Sigma
Dextran 6000	Sigma
Dextran 70000	Sigma
Dextrose	Sigma
Ethanol 96%	Fisher Chemical
Formulation Buffer PS-20	J.T.Baker
HCl (37%)	Merck
Hepes	Sigma
Hepes Sodium Salt	Sigma
K ₂ HPO ₄ (salt)	MP Biomedicals
KCl	Merck
KH ₂ PO ₄ (acid)	Merck
L-Histidine	Sigma
MgCl ₂ ·6H ₂ O	Sigma
NaCl	Merck
PEG 200	Sigma
PEG 20000	Sigma
PEG 35000	Sigma
PEG 4000	Sigma
Polysorbate 20 (PS-20)	J.T.Baker
Sodium Acetate	Merck
Sodium citrate tribasic dihydrate	Sigma-Aldrich
Sodium phosphate dibasic	Merck
Sucrose	Merck
Trehalose	Sigma
Tris	Merck

MEASUREMENTS

The read-outs obtained were as follows:

5

- Absorbance at 260 nm, which correlates with DNA concentration.
- Absorbance at 280 nm, which correlates with protein concentration.
- Absorbance at 350 nm (turbidity), which correlates with protein aggregation and/or particle formation.

- Absorbance at 900 nm, which correlates with the background signal from the plastic of the wells and seal.
- Absorbance at 975 nm measures the water absorbance, thus, the path length.
- Tryptophan fluorescence at 280/335 nm, measured from the top and the bottom of the micro-well plate. This measurement correlates with protein and/or capsid unfolding and denaturation. The top read-out was used in the figures.

The protein and DNA concentrations were obtained as follows:

According to the Lambert-Beer law, the absorbance is proportional to the concentration and the light path length. A constant named the extinction coefficient turns this into an equation. $A = cc \cdot L \cdot \epsilon$, where A is the absorbance, cc is the concentration, L is the light path length and ϵ is the extinction coefficient.

The light pathlength is measured by the water absorbance measured at 975 nm (A_{975}) minus the plastic background measured at 900 nm (A_{900}) thus:
 $L = A_{975} - A_{900}$

The protein concentration is measured by measuring the absorbance at 280 nm (A_{280}), thus:

$$A_{280} = cc(\text{protein}) \cdot L \cdot \epsilon(\text{protein})$$

The background from the precipitated and aggregated proteins or turbidity is measured by the absorbance at 350 nm (A_{350}). It is assumed to be similar to the contribution of turbidity at 280 nm, and thus, deducted from the absorbance at 280 nm. Then:

$$cc(\text{protein}) \cdot \epsilon(\text{protein}) = (A_{280} - A_{350}) / (A_{975} - A_{900})$$

Since $\epsilon(\text{protein})$ is a constant, we can conclude that $cc(\text{protein}) \cdot \epsilon(\text{protein})$, is proportional to the protein concentration, which is computed from the different absorbance values measured (A_{280} , A_{350} , A_{975} & A_{900}). For the DNA concentration at 260 nm, similar reasoning was applied.

Intrinsic Differential Scanning Fluorimetry (iDSF) measurements using Nanotemper® technology

The denaturation temperature of the capsids was followed using the Nanotemper® technology by which a sample was heated at increasing temperatures and the fluorescence of the capsids followed. As the capsids denature and expose fluorescent amino acids to the solvent environment, the fluorescence diminishes as the environment changes from a rigid one, inside the protein, to a less rigid one. The ratio between the fluorescence at 335 nm and 350 nm is computed to make the measurements independent of the concentrations.

Example 1 - Formulation Study AAV5

The drug product (DP) used in this example was AAV5 with a transgene encoding a microRNA targeting AAXN3 mRNA at a concentration of 4.7E13 gc/mL.

5 rAAV5-miAAXN3s was buffer exchanged in a multi-well system into thirty different formulations comprising different pHs, buffer types, and salt contents using 96 well filter plates (100 kDa cut-off). After the buffer was exchanged and filtered through a 0.2 µm filter, the formulations were aliquoted into four plates according to the plate layout and buffer formulation compositions. The final concentration of the AAV5 in the wells was 1,0E13 gc/mL. Each formulation is prepared in duplicate. The outer rows and columns are filled with WFI to rule out plate location effects. The buffers have overlapping pHs, to
10 differentiate between a buffer and a pH effect.

Each plate was assigned to a specific stress condition: F/T at 80°C, storage at 25°C and 65°C and shaking at RT. The accelerated studies comprised the following:

- 15 • Freeze at -80°C (at least 1h) and thaw at RT (1 hour) for 1, 3 & 10 cycles. The table with the experiments dates show when each F/T cycle was carried out.
- Shaking at RT for 1 and 6 hours. There are two shaking measurements in the experiments due to a deviation. The first shake experiment was not spun down before the measurements, thus, a second shaking plate was added which underwent the spinning down and the shaking at RT for 1 and 6 hours.
- 20 • Storage at 25°C for 2, 7, and 14 days.
- Storage at 65°C for 2, 7, and 14 days.
- For all the above-mentioned conditions a micro-well plate was prepared and initial measurements (T=0) were carried out.

25 The T=0 protein contents as followed by Absorbance at 280 nm (corrected for turbidity and path-length in four multi-well plates; arbitrary units) are shown in Table 2. The T=0 protein contents as followed by Fluorescence at 335 nm (top measurement from four multi-well plates; arbitrary units) are shown in Table 3.

30

Table 2: The T=0 protein contents as followed by Absorbance at 280 nm (A: F/T at -80°C; B: Shaking; C: 25°C; D: 65°C)

A.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	0.8844	0.9551	1.7323	1.6212	1.7374	1.6010	1.7922	1.7200	1.5835	1.6015
	0.075M NaCl	1.4720	1.5909	1.5459	1.6280	1.6263	1.4016	1.6465	1.6179	1.4414	1.7020
	0.15M NaCl	1.5859	1.6733	1.5100	1.6299	1.6987	1.4634	1.7135	1.6517	1.4899	1.5455
B.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	0.7788	0.8592	1.4826	1.4898	1.5025	1.3705	1.3990	1.5253	1.4321	1.5303
	0.075M NaCl	1.4309	1.5458	1.5209	1.4575	1.5794	1.4562	1.4661	1.5478	1.5581	1.5228
	0.15M NaCl	1.4367	1.6224	1.6181	1.4415	1.6447	1.6499	1.5224	1.6433	1.5680	1.4873
C.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	0.7636	0.8480	1.5426	1.5396	1.5550	1.4405	1.4446	1.5819	1.5203	1.5673
	0.075M NaCl	1.4487	1.5833	1.6106	1.4319	1.6252	1.4550	1.4802	1.5818	1.5716	1.5745
	0.15M NaCl	1.5204	1.752	1.8037	1.5347	1.7079	1.7423	1.6641	1.6965	1.6468	1.5618
D.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	0.7850	0.8692	1.5101	1.5354	1.5585	1.4719	1.4643	1.6042	1.5561	1.7406
	0.075M NaCl	1.4500	1.5929	1.5784	1.4394	1.6313	1.4770	1.5101	1.6010	1.5878	1.6010
	0.15M NaCl	1.4968	1.7490	1.7500	1.5500	1.7437	1.7489	1.6600	1.7079	1.6734	1.5718

5 **Table 3:** The T=0 protein contents as followed by Fluorescence at 335 nm (A: F/T at -80°C; B: Shaking; C: 25°C; D: 65°C)

A.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	1001	946	5451	5347	5503	4359	5439	5652	4826	6079
	0.075M NaCl	4682	5341	5153	5938	6029	4715	6160	6010	4404	6001
	0.15M NaCl	5227	5806	4982	5879	6443	4988	6395	6254	5240	5625
B.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	816	1204	4727	4846	5105	4302	4578	5302	4666	5296
	0.075M NaCl	4132	4882	4957	4500	5516	4811	4976	5542	5588	5417
	0.15M NaCl	4212	5261	5437	4621	5887	5815	5171	6012	5536	5334
C.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	859	1264	4906	5262	5560	4789	4886	5744	5271	5540
	0.075M NaCl	4404	5204	5113	4688	5939	4955	5288	5903	5829	5918
	0.15M NaCl	4577	5870	6015	5165	6442	6305	5918	6436	6160	5685
D.	Buffer	Acetate			Citrate			Phosphate			Tris
	pH	5	5.5	5.5	6	6.5	6.5	7	7.5	7.5	8
Salt	WFI	849	1234	4917	5173	5495	4651	4738	5616	5161	5470
	0.075M NaCl	4407	5154	5150	4588	5800	4882	5210	5788	5759	5816
	0.15M NaCl	4548	5839	6001	5176	6364	6270	5865	6359	6009	5633

10 Table 2 clearly shows that the four multi-well plates have similar values, although not identical, for the corrected absorbance at 280 nm (protein concentration) as expected. In addition, for the four plates at T=0, the absorbances are higher in the presence of 150 mM NaCl for almost all of the conditions, therefore showing a higher protein content and a lower degree of aggregation. This can be concluded

since the values measured include the correction for the background. On the whole, it is observed that the effects of the different formulations take place immediately at T=0 during the buffer exchange.

5 In Table 3, it is observed that there is a stabilizing salt effect at both 75 mM and 150 mM NaCl for most of the conditions. Acetate at pH 5 and 5,5 shows mostly destabilizing effects, especially without NaCl. Phosphate at pH 7,5 and citrate at pH 6,5 seem to favour the folded conformations. As above, the fact that the effects are observed at T=0 suggests that these take place during the buffer exchange step.

10 Results of the accelerated studies are shown in Figure 1. These results point out the importance of the pH in the formulations as well as the effects of salt concentrations.

The overall observations are:

- 15 • The effect of all tested buffers (at 20 mM: acetate, citrate, phosphate, and Tris), on the stability of the drug product measured by analytical assays: absorbance, and fluorescence, is seen immediately at T=0.
- The lack of salt and buffer is detrimental to the stability of the drug product.
- Salt (NaCl) addition, especially at a concentration level 75 mM or higher is beneficial for a formulation stabilizing effect.
- 20 • A neutral or slightly basic formulation pH (7.0-7.5) is better for the stability of the drug product.
- Formulations with basic buffer capacity, such as Tris, are more stabilizing when compared with buffered formulations with lower pHs.

25 Formulations with NaCl in buffers at a neutral or slightly basic pH (7.5) are used for further tests and optimization.

Excipient Screening Study

According to the result of above, neutral to slightly basic pHs and the inclusion of salt support the stability of the capsids. Three buffers were chosen: phosphate, Tris, and Hepes, to support a pH equal
30 to 7.5. As a control, artificial cerebrospinal fluid (aCSF) was used. 117 formulations in different buffers, with increasing concentrations of NaCl and defined concentrations of the putative excipients, were prepared; the latter were high enough to impact the viscosity but low enough not to have a large impact on the osmolality. The different excipients tested and their concentrations are shown in Table 4 below, among them surfactants such as various PEGs, and cryoprotectants such as trehalose, sucrose,
35 dextrose, and dextrans. The polymer excipients were chosen to increase the viscosity of the formulations.

Table 4: Excipients and their corresponding concentrations

Excipient	Example 2 (1) % w/v	Example 2 (2) % w/v
Sucrose	4	
Trehalose	4	4 and 8
Dextrose	4	2 and 4
Dextran 6000	3	1.5 and 3
Dextran 20000	3	
Dextran 70000	3	
Dextran 550000	3	
PEG 200	3	
PEG 4000	3	
PEG 20000	3	
PEG 35000	3	

In the first excipient screening four sets of multi-well plates were prepared.

For the second excipients screening 18 formulations were prepared in glass vial containers. These comprise three buffers, as before, Tris, HEPES, and phosphate, and three putative excipients: trehalose, Low MW Dextran, and dextrose.

The plates of the first excipient screening and the vials of the second excipient screening were subject to accelerated studies at different stresses: F/T, shaking, and storage at two different temperatures (25°C and 40°C) which comprised the following durations/cycles as specified in Table 5 below:

Table 5: Accelerated stresses test conditions used

Stress	First Screening Experiment	Second Screening Experiment
Storage at 25°C	2 and 7 days	7 days
Storage at 40°C	2 and 7 days	2 days
Freeze at -80°C (at least 1h) and Thaw at RT (1 hour)	1, 3 & 10 cycles	4 cycles
Shaking	1 and 6 hours on the vortex at maximum speed	24 hours at 200rpm shaker

The protein content and fluorescence data as a function of the stress conditions and duration are shown in Figure 2. A DoE experimental design was also carried out with the JMP® software and the prediction profilers are shown in Figures 3 and 4. The results suggest that higher salts have a positive effect while the effect of the buffers show that the most favourable buffers are phosphate and HEPES.

The overall observations are:

- The buffer effects are immediate, take place at the buffer exchange step, and are observed at T=0;

- High salt concentrations are beneficial for the stability of the samples, as well as slightly alkaline buffers;
- Dextran 6000 and trehalose have a better stabilizing effect than dextrose
- HEPES and phosphate are slightly better than Tris.

5

The following buffer and excipient choices are used for further tests and optimizations.

- HEPES and phosphate as buffers
- Dextran 6000 and trehalose as sugar excipients.

10 *Point of Aggregation Study*

The four buffers used in the formulations are as specified in Table 6 below;

Table 6: Buffer compositions used

Diafiltration Buffer A	HEPES 10 mM pH 7.3 80 mM NaCl 3 mM KCl 1.4 mM CaCl ₂ 0.8 mM MgCl ₂ 3% w/v Trehalose
Diafiltration Buffer B	HEPES 10 mM pH 7.3 123 mM NaCl 3 mM KCl 1.4 mM CaCl ₂ 0.8 mM MgCl ₂ 2.2% w/v Dextran 6000
Diafiltration Buffer C	Phosphate 10 mM pH 7.3 82 mM NaCl 3 mM KCl 0.8 mM MgCl ₂ 3% w/v Trehalose
Diafiltration Buffer D	Phosphate 10 mM pH 7.3 124 mM NaCl 3 mM KCl 0.8 mM MgCl ₂ 2.2% w/v Dextran 6000

15 These formulations were concentrated stepwise by diafiltration. Samples were collected after each concentration step. Table 7 below shows the concentration folds and expected concentration.

Table 7: Sequential concentration steps applied to the different formulations

Fold during PoA	Concentration	Volume of Retentate (mL)	Expected concentration (Gc/mL)
DF retentate (Start sample)		300	5E13
1,5		200	7.5E13
2		150	1E14
4		75	2E14
6		50	3E14
12		25	6E14
15		20	7.5E14
20		15	1E15

The measurements followed after each concentration step were the following:

- 5
- Genome copies per mL (Gc/mL)
 - Total particles per mL (TP/mL)
 - Visual inspection at T=0 and after 24 hours at 4°C and two freeze and thaw (F/T) cycles.
 - Sub-visible particles by AccuSizer®.
 - Poly-dispersity index by Dynamic light scattering.

10

Table 8 below shows the visible inspection as a function of the fold concentration for the four formulations also after stresses, such as storage for 24hrs at 4°C or after two F/T cycles. It is observed that up to a six-fold concentration the phosphate-containing formulations perform better than the HEPES-containing formulations. It is also observed that the trehalose-containing formulations perform better than the Dextran 6000-containing formulations after a six-fold concentration step.

15

Table 8: Visible particle inspection as a function of the fold concentration after 4°C storage and F/T cycles.

Fold concentration	HEPES/Trehalose			HEPES/Dextran			Phosphate/Trehalose			Phosphate/Dextran		
	T ₀	24H 4°C	F/T	T ₀	24H 4°C	F/T	T ₀	24H 4°C	F/T	T ₀	24H 4°C	F/T
0	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
1.5	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
2	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
4	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
6	clear	clear	clear	clear	clear	collapsed	clear	clear	clear	clear	clear	clear
12	clear	turbid	clear	clear	collapsed	collapsed	clear	clear	clear	clear	clear	collapsed
15							clear	clear	clear	clear	collapsed	collapsed

In Table 9 below, it is observed that the number of particles increases as the different formulations are concentrated. Furthermore, all the formulations show very similar results until the six-fold concentration step, which defines the largest concentration possible as described above. Only on the phosphate-containing formulations concentration steps up to 15-fold were carried out, which show an increment in the number of particles as expected.

Table 9: Sub-visible particles as a function of the fold concentration

Formulation	Fold concentration	Subvisible particles					
		>0,497µm	>1,013µm	>2,067µm	>5,036µm	>10,27µm	>25,027µm
HEPES/Trehalose	0	2585	299	57	5	0	0
	1,5	4945	110	11	0	0	0
	2	2753	111	38	0	0	0
	4	92897	1053	223	79	27	0
	6	45315	955	95	5	0	0
	12	53918	1210	127	22	0	0
HEPES/Dextran	0	2279	185	15	0	0	0
	1,5	863	98	27	0	0	0
	2	7607	157	17	0	0	0
	4	19156	432	34	0	0	0
	6	118089	1849	99	13	0	0
	12	44445	1724	169	38	4	0
Phosphate/Trehalose	0	2022	126	36	0	0	0
	1,5	2054	395	26	0	0	0
	2	2001	256	17	0	0	0
	4	11288	498	117	22	0	0
	6	16078	523	73	21	0	0
	12	21411	667	80	0	0	0
Phosphate/Dextran	0	3261	278	79	12	0	0
	1,5	961	121	17	0	0	0
	2	3844	157	23	0	0	0
	4	7589	192	25	0	0	0
	6	50843	571	49	4	0	0
	12	70765	1615	204	36	4	0
	15	65410	4227	732	157	55	0

Polydispersity index (PDI) and z-average measurements by dynamic light scattering give information regarding the particle size, uniformity, and shape of the formulations under study. The results of PDI and z-average for the formulations are shown in Figures 5 and 6. PDI is a representation of the distribution of size populations within a given sample. The numerical value of PDI ranges from 0.0 (for a perfectly uniform sample concerning the particle size) to 1.0 (for a highly polydisperse sample with multiple particle size populations). Figure 6 shows that all four formulations reach a plateau after a six-fold concentration in agreement with the GC and TP measurements above. Furthermore, the presence of trehalose causes the aggregates to be smaller than those in the presence of Dextran 6000. The same effect can be stated for the phosphate-containing formulations which induce smaller particles than the HEPES counterparts. The behaviour of the z-average is the same as that of the PDI, as described above.

Table 10 below shows the results of the stability test.

Table 10: Stability test as a function of the fold concentration.

Fold Concentration	HEPES/Trehalose			HEPES/Dextran			Phosphate/Trehalose			Phosphate/Dextran		
	T0	24hrs 2-8 C	F/T	T0	24hrs 2-8 C	F/T	T0	24hrs 2-8 C	F/T	T0	24hrs 2-8 C	F/T
4	C	C	C	C	C	C	C	C	C	C	C	C
6	C	C	C	C	C	Co	C	C	C	C	C	C
12	C	T	C	C	Co	Co	C	C	C	C	C	Co
15							C	C	C	C	Co	Co

C = Clear; Co = collapsed; T = turbid.

The overall observations are:

- Highest concentration achievable 6E14 gc/mL (6-fold concentration)
- Formulations in phosphate buffer show better visible particle profiles beyond 6E14 gc/mL
- Phosphate/trehalose shows the narrowest particle size distribution

Formulation Confirmation Study

Two formulations were proposed as a stability-enhancing for further tests and optimizations:

Formulation 1. Phosphate/Trehalose: Phosphate (Na/K) 10 mM; NaCl 82 mM; KCl 3mM; Trehalose 3% (w/v); pH 7.3.

Formulation 2. Phosphate/Dextran: Phosphate (Na/K) 10 mM; NaCl 125 mM; KCl 3mM; Dextran 6000 2.2% (w/v); pH 7.3.

The two formulations were filled in the final volume (1.2 mL) and container closure vials (2ml borosilicate type I vials closed with a siliconized stopper and a flip-off seal). These vials were subject to accelerated studies at different stresses: F/T, shaking, and short-term development stability at 25 and 40°C and one-year development stability (-80 °C and 2-8 °C) and comprised the following:

- Freeze at -80°C (at least 1h) and thaw at RT (1 hour) for 3 cycles (for actual cycles length, look above in the study dates schedule).
- Shaking for 24 hours at 200 rpm.
- Accelerated stability: Storage at 25°C for 2 weeks
- Accelerated stability: Storage at 40°C for 1 week.
- Short-term development stability studies at -20°C for 3 months
- Long-term development stability studies at -80°C (targeted DP storage temperature) and 2-8 °C for 1, 3, 6, 9, and 12 months
- For all the above-mentioned conditions initial measurements (T=0) were carried out.
- Additional time points were added after 1 F/T, after 1 week at 25°C, and after 2 weeks at 40°C, for Appearance and Visual inspection

The measurements followed were as follows:

- Appearance/Visible particles (VI)
- Sub-visible particles
- Genome copies (gc); which correlates with concentration
- 5 • tp: Total particles
- ip: Infective particles
- gc/ip: (genome copies/infective particles); which correlates with the number of active particles
- tp/gc (total particles/genome copies); which correlates with purity
- PDI by DLS (polydispersity index); it is expected to be lower than 0.1 as we were aiming to
- 10 obtain mono-dispersed systems

Full data is shown in Tables 11 to 13 below. Further data is also illustrated in Figures 7 and 8.

Table 11: Full data set for accelerated stressed condition studies.

Formulation Buffer	Assay	Unit	t=0	SHK 24hrs	3x F/T	40°C 1 week	25°C 2 weeks
Formulation 1	Genome Copies	gc/mL	3.4E+14	2.5E+14	3.4E+14	4.0E+14	2,13E14
	Total Particles	tp/mL	3.6E+14	3.1E+14	3.1E+14	2.2E+14	2.8E+14
	TP/GC	tp/gc	1.1	1.2	0.9	0.6	1.3
	Monomeric particle purity	% monomeric	100	100	100	99.8	99.6
		Z-average	26.47	25.94	26.37	29.02	27.08
		Pdl	0.111	0.087	0.113	0.215	0.180
		extra peaks	1	0	0	2	2
	Infectivity	ip/mL	8.6E+12	7.8E+12	7.8E+12	6.7E+12	N/D
		gc/ip	39.5	32.1	43.6	59.7	N/D
Formulation 2	Genome Copies	gc/mL	4.2E+14	3.0E+14	3.1E+14	3,7E14	4,3E14
	Total Particles	tp/mL	4.9E+14	4.1E+14	2.1E+13	7,2E+13	3,8E+14
	TP/GC	tp/gc	1.2	1.4	0.1	0.2	0.9
	Monomeric particle purity	% monomeric	99.8	99.7	99.7	78.9	99.7
		Z-average	30.69	30.66	30.59	33.75	30.87
		Pdl	0.218	0.232	0.236	0.31	0.242
		extra peaks	2	2	1	1	1
	Infectivity	ip/mL	8.7E+12	7.7E+12	6.2E+12	9,2E+11	5,0E+12
		gc/ip	48.3	38.4	49.2	402.2	86.0

Table 12: Full data set for long-term development stability studies at 2-8°C, -80°C & -20°C.

Formulation Buffer	Assay	Unit	t=0	4°C			-80°C					-20°C
				1 month	3 months	6 months	1 month	3 months	6 months	9 months	12 months	3 months
Formulation 1	Genome Copies	gc/mL	3.4E+14	2.5E+14	2.7E+14	2.7E+14	2.4E+14	2.8E+14	2.9E+14	3.0E+14	2.8E+14	2.7E+14
	Total Particles	tp/mL	3.6E+14	2.9E+14	N/D	2.6E+14	2.9E+14	N/D	2.6E+14	2.6E+14	2.4E+14	N/D
	TP/GC	tp/gc	1.1	1.2	N/D	1.0	1.2	N/D	0.9	0.9	0.9	N/D
	Monomeric particle purity	% monomeric	100	100	99.9	100	100	100	100	100	100	100
		Z-average	26.47	26.42	26.74	26.79	26.45	26.4	26.85	26.75	26.53	26.18
		Pdl	0.111	0.110	0.131	0.115	0.119	0.107	0.124	0.114	0.113	0.108
		extra peaks	1	0	2	0	0	1	0	1	0	0
	Infectivity	ip/mL	8.6E+12	1.9E+13	N/D	7.0E+12	1.5E+13	N/D	7.5E+12	9.9E+12	6.2E+12	N/D
		gc/ip	39.5	13.2	N/D	38.6	16.0	N/D	38.7	30.3	45.2	N/D
Formulation 2	Genome Copies	gc/mL	4.2E+14	3.6E+14	3.5E+14	4.9E+14	3.2E+14	3.5E+14	4.3E+14	4.0E+14	3.8E+14	4.2E+14
	Total Particles	tp/mL	4.9E+14	4.2E+14	N/D	3.5E+14	4.3E+14	N/D	3.5E+14	3.4E+14	3.1E+14	N/D
	TP/GC	tp/gc	1.2	1.2	N/D	0.7	1.3	N/D	0.8	0.9	0.8	N/D
	Monomeric particle purity	% monomeric	99.8	99.7	99.8	99.7	99.6	99.8	99.8	99.5	99.7	99.8
		Z-average	30.69	31.01	30.66	31.0	30.44	30.66	30.64	31.04	30.68	30.08
		Pdl	0.218	0.233	0.235	0.231	0.229	0.235	0.225	0.225	0.217	0.228
		extra peaks	2	1	1	1.0	1	1	1.000	2.000	1.000	1
	Infectivity	ip/mL	8.7E+12	1.5E+13	N/D	8.2E+12	9.7E+12	N/D	7.2E+12	1.0E+13	9.1E+12	N/D
		gc/ip	48.3	24.0	N/D	59.8	33.0	N/D	59.7	40.0	41.8	N/D

5

Table 13: Visual inspection results of the stress tests

stress condition	Trehalose (Formulation 1)									Dextran 6K (Formulation 2)								
	F/T	24hrs	1w	2w	1M	3M	6M	9M	12	F/T	24hrs	1w	2w	1M	3M	6M	9M	12
40 C			Co	Co								Co						
25 C				Co	Co								Co					
2-8 C					Co	Co	Co							Co	Co	Co		
-20 C				T		T							Co		Co			
≤-65					T	T	T	C	C					T	T	T	T	T
1F/T	T									Co								
3F/T	T									Co								
SHK		Co									Co							

C = Clear; Co = collapsed; T = turbid

5 The results confirm that the formulation containing trehalose is preferred to the one containing Dextran 6000. The strongest data supporting this conclusion is the visual inspection analysis, which shows that the formulations with trehalose show much less particles after F/T cycles or (long-term) storage at -80°C. Furthermore, the DLS measurements show that the PDI-value of the samples containing trehalose is almost half of the PDI value for samples containing Dextran 6000, meaning that the samples containing trehalose are more homogeneous than the ones containing Dextran 6000. The overall conclusion from the subvisible particle data is that trehalose shows much better results for the long term storage conditions.

10 The formulation: Phosphate (Na/K) 10mM; NaCl 82 mM; KCl 3mM; Trehalose 3% (W/V); pH 7,3 is recommended for further optimizations.

A further study was performed to select one of the following five different formulations, which contain different DP concentrations, MgCl₂ and/or PS-20 for the drug product. The previous early formulation report suggested a formulation with phosphate (Na/K) 10 mM; NaCl 82 mM; KCl 3 mM; Trehalose 3% w/v; pH 7.3 is recommended as the initial point for further optimizations.

15

The distinct DP materials were formulated as follows:

1. 3E14 gc/mL in trehalose/phosphate buffer;
2. 4E14 gc/mL in trehalose/phosphate buffer;
- 20 3. 3E14 gc/mL in trehalose/phosphate buffer+ 0.8 mM MgCl₂;
4. 3E14 gc/mL in trehalose/phosphate buffer+ 0.005 % v/v PS-20;
5. 3E14 gc/mL in trehalose/phosphate buffer+ 0.01% v/v PS-20.

The trehalose/phosphate buffer comprises 9 mM phosphate buffer, 82 mM NaCl, 3 mM KCl and 3% w/v trehalose. These formulations were placed on stress studies as and the results are shown in Table 14 below.

25

Table 14: Results of the stress tests

C = Clear; Co = collapsed; T = turbid.

Formulation		at T0		Time points				
		gc/mL	tp/mL	Vial	T0	1D SHK	1M 2-8 C	
					1W 40C			
	3.0E+14 gc/mL	2.50E+14	2.60E+14	1	C	T	Co	Co
				2	C	T	C	Co
				3	C	T	C	
	3.0E+14 gc/mL 0.005% PS20	3.10E+14	2.70E+14	1	C	C	C	C
				2	C	C	C	C
				3	C	C	C	
	3.0E+14 gc/mL 0.01% PS20	2.90E+14	2.70E+14	1	C	C	C	C
				2	C	C	C	C
				3	C	C	C	
	3.0E+14 gc/mL 0.8 M MgCl2	2.70E+14	2.70E+14	1	C	T	Co	Co
				2	C	T	Co	Co
				3	C	T	C	
	4.0E+14 gc/mL	3.40E+14	3.60E+14	1	C	T	Co	Co
				2	C	T	C	Co
				3	C	T		

The results shown above confirm that the addition of PS-20 (in particular at 0.01% v/v) has a positive effect on the stability of the formulation.

Conclusion

- Plate-based screening studies highlighted stabilizing effect of NaCl and neutral to slightly alkaline pH
- Studies show stabilizing effect of trehalose and dextran 6k in either phosphate or HEPES buffer
- Phosphate + trehalose/dextran 6k supports concentrations up to 6E14 gc/mL
- Phosphate + trehalose shows superior visible particle profile
- Formulation optimization through the addition of 0.01% v/v PS-20 improves visual appearance & greatly reduces subvisible particles
- Formulation has been optimized for frozen storage at $\leq -65^{\circ}\text{C}$

One particular embodiment of the present invention comprises the compositions shown in Table 15 below, in addition to the adeno-associated viral vector with a transgene encoding a microRNA.

Table 15: Buffers analysed

	Buffer (1)	Buffer (2)	Buffer (3)	Buffer (4)	Buffer (5)
Parameter	Zolgensma	PBS+ 0.001% Poloxamer 188	AMT-150 FB	AMT-130 FB	Zolgensma modified
Buffer/pH	20 mM Tris pH 8.0	10 mM phosphate pH 7.4	9 mM phosphate pH 7.3	20 mM Tris pH 7.5	20 mM Tris pH 8.0
Tonicifier	200 mM NaCl	137 mM NaCl, 2.7 mM KCl	87 mM NaCl, 3mM KCl	135 mM NaCl	135 mM NaCl
Cryoprotectant	X	X	3% Trehalose.2H ₂ O	0.1% Mannitol 0.1% HP- b-BCD	X
Surfactant	0.005% Poloxamer 188	0.001% Poloxamer 188	0.01% PS20	X	0.005% Poloxamer 188
Other ingredients	1 mM MgCl ₂	X	X	2.5 mM His	1 mM MgCl ₂
Theoretical Osmo (mOsm/L)	423	309	286	299	293

Example 2 – Formulation Study AAV9

- 5 The drug product (DP) used in this example was AAV9 with a transgene encoding a luciferase reporter at a concentration of approximately 3E13 gc/mL (see Table 19).

10 The composition of the five formulation buffers used in this study are listed in Table 15. All five buffers were prepared in a 4L volume and they were filtered using a 0.22 µm PES filter. Poloxamer 188 and PS20 surfactants were added freshly to the corresponding formulation buffers on the date of the buffer exchange process.

30 mL of AAV9 formulation per buffer condition was exchanged using dialysis. Three cycles of buffer exchange (1L per cycle) were performed for 16 hours at 2-8 °C.

AAV9 collected volumes after buffer exchange process were filtered using a 0.22µm PVDF syringe filter. AAV9 DP was filled in 2 mL type I glass vials with a fill volume of 1.2 mL DP. Table 16 shows the total number of filled DP vials per collected AAV9 volume in each formulation buffer.

5 **Table 16:** Number of AAV9 DP filled vials per each formulation buffer

DP description	Volume after buffer exchange and filtration	Number of filled (1.2 mL) glass vials
AAV9 in formulation buffer 1	31 mL	25
AAV9 in formulation buffer 2	26.5 mL	21
AAV9 in formulation buffer 3	26 mL	21
AAV9 in formulation buffer 4	31.5 mL	25
AAV9 in formulation buffer 5	27 mL	23

All filled DP vials were visually inspected. DP vials that passed visual inspections were placed under different stress conditions as listed in Table 17 and routed for analysis as described in Table 18.

10 **Table 17:** Number of AAV9 DP vials per each stress condition

Formulation Stress Conditions	AAV9 buffer 1	AAV9 buffer 2	AAV9 buffer 3	AAV9 buffer 4	AAV9 buffer 5
T=0	2	2	2	2	2
SHK, 1Day (200 rpm, RT)	2	2	2	2	2
1xFT	2	2	2	2	2
1xFT+24hrs at RT	2	2	2	2	2
3xFT	2	2	2	2	2
1xFT+1W at RT	2	2	2	2	2

1xFT+4W at 2-8°C	2	2	2	2	2
Total number of DP vials	14	14	14	14	14

Table 18: Overview of the analytical methods

Quality attributes/ Method	Assay format	Volume (mL) per buffer condition
Genome copies / Quantitative Polymerase Chain Reaction (QPCR)	2×3	3×0.25
pH / Potentiometry	1×1	1×0.7
Osmolality / Freezing point depression	1×1	1×0.25
Visible particulates / Visual inspection	200%	2×1.2
Vis and Sub-visible particulates / Background membrane imaging	1×2	2×0.025
Subvisible particulates / Light obscuration	2×1	2×1.2
Purity, % monomer / HPL-SEC	1×2	1×0.25
Hydrodynamic radius and polydispersity index / Dynamic Light Scattering (DLS)	1×3	3×0.03
Temperature of unfolding / Nano Differential Scanning Fluorimetry (nanoDSF)	1×2	2×0.01
DNA, Protein absorbance (UV260, UV280) and Intrinsic Fluorescence (F350 nm)/ Spectroscopy	1×2	2×0.1
Transduction efficiency/ Dual Reporter Luciferase Assay	2×3	2×0.1

Results5 *Genome copies*

Genome copy (GC) measures the amount of AAV particles that have the vector DNA packaged within the particle. GC concentration is determined by quantitative PCR. GC assay was performed for T=0 (after buffer exchange), SHK (1Day, 200 rpm), 1FT+7Days and 1FT+4W at 2-8°C stress conditions. The averaged GC triplicates for two vials per formulation buffer at T=0, are shown in Table 19.

Table 19: Genome copies of AAV9 DP in different formulation buffers after DP fill

Description	Genome copies (gc/mL)
AAV9 Buffer 1	3.17E+13
AAV9 Buffer 2	2.98E+13
AAV9 Buffer 3	4.00E+13
AAV9 Buffer 4	3.27E+13
AAV9 Buffer 5	2.67E+13

The AAV9 GC was around 3E13 gc/ml among all tested formulations taking in consideration the 20% GC assay variation. A slightly higher concentration of AAV9, 4E13 gc/ml, was measured in formulation buffer 3. No observable loss of GC was detected in either of the tested samples under the different stress conditions.

pH and Osmolality

pH and osmolality data are close to the theoretical values for each formulation buffer (see Table 20), which indicates that buffer exchange was performed correctly.

Table 20: pH and osmolality of AAV9 DP in different formulation buffers

Description	pH		Osmolarity	Osmolality
	targeted	measured	Theoretical mOsm/L	Measured mOsm/kg
AAV9 NF starting material	8.3	8.5	600	687
AAV9 Buffer 1	8.0	7.9	423	398.0
AAV9 Buffer 2	7.4	7.3	309	282.5
AAV9 Buffer 3	7.3	7.7	286	268.5
AAV9 Buffer 4	7.5	7.6	299	287.5
AAV9 Buffer 5	8.0	7.8	293	278.0

Before buffer exchange, measured AAV9 buffer osmolality was 687 mOsm/kg and pH 8.5, buffer composition being 0.1M Glycine, 0.5M Tris, pH 8.3. After buffer exchange, all formulations were close to the targeted pH and theoretical osmolality. With the exception of buffer 4, Zolgesma's formulation buffer an hypertonic formulation, all other formulations are isotonic formulations.

Visual and sub-visible particle analysis

Visual inspection acceptance criteria was aligned to the USP <790> specification and defined as colourless liquid, clear, free of visible particles. Visual inspection was performed against a white and black background. All AAV9 DP vials were visually inspected after fill and after applied stresses. DP vials containing extrinsic particles after filling and those with low filling volumes were rejected. The results are shown in Table 21 and Figure 9.

Table 21. Visual inspection results after DP Fill and finish, T=0

Type of formulation	Total Vials	Clarity	Color of Liquid	Description of Particulates	No. of rejected vials	Remaining vials
AAV9 Buffer 1	25	Clear	Colorless	One vial with particle	1	24
AAV9 Buffer 2	22	Clear	Colorless	One vial with low volume	1	21
AAV9 Buffer 3	21	Clear	Colorless	One vial with particle	1	20
AAV9 Buffer 4	26	Clear	Colorless	One vial with low volume	1	25
AAV9 Buffer 5	23	Clear	Colorless	One vial with low volume	1	22

Two vials per formulation buffer and stress condition were visually inspected. AAV9 DP was most stable in formulation buffer 3. In this formulation, AAV9 DP remained free of particles after agitation for one day at RT, after three freeze/thaw cycles, and after one week after thawing when stored at RT or two weeks after thawing when stored at 2-8 °C respectively (Figure 9). Two vials per stress condition and formulation buffer were analyzed for the subvisible particles using the light obscuration technique AccuSizer FX.

Under all stress conditions, subvisible particles remained within the acceptance criteria according to USP<787>: 6000 particles/mL above 10 µm and 600 particles/mL above 25 µm. A similar aggregation profile was observed for all tested formulation buffers in the 0.3-1 µm region. AAV9 aggregation was accelerated upon freeze-thaw stresses, while agitation stress condition had less of an impact on aggregation.

It is generally observed that the number of subvisible particles larger than 2 µm of AAV9 in formulation buffer 3 is less when compared to the other formulations under all applied stress conditions.

Background Membrane Imaging (BMI)

Particles from 2 µm to 4 µm were visualized with Aura system that uses BMI technology, a form of automated membrane microscopy that enables particle counting, sizing, and morphology in a 96 well format. AAV9 DP in formulation buffer 4 after 1FT+4 weeks at 2-8°C contains the highest number of particles > 2 µm when compared to the other formulations in agreement with the results from visual inspection related to visible particles and sub-visible particle analysis by light obscuration technique.

AAV9 transduction into target cells

Potential effect of the different formulations on AAV9 transduction efficiency has been evaluated *in vitro* in a cellular assay. For each buffer condition, AAV9 transduction efficiency was tested in human hepatoma-derived carcinoma (Huh7) cells. Huh7 cells were chosen as an *in vitro* model cell line based on previously published data by Pietersz et al., 2021 (Pietersz KL, Plessis FD, Pouw SM, Liefhebber JM, van Deventer SJ, Martens GJM, Konstantinova PS, Blits B. PhP.B Enhanced Adeno-Associated Virus Mediated-Expression Following Systemic Delivery or Direct Brain Administration. Front Bioeng Biotechnol. 2021 Aug 3;9:679483. doi: 10.3389/fbioe.2021.679483. PMID: 34414171; PMCID: PMC8370029).

Huh7 cells were co-transduced with the differently formulated AAV9 DPs and *wild-type* Adeno5, Ad5 ARM adenovirus, at three different AAV9 doses (GC/cell) also known as multiplicity of infections (MOI). In addition to the untreated AAV9 in the different formulation buffers, 50 µL of AAV9 per buffer condition was heat-inactivated by incubation at 95°C for 10 min as a negative control for each condition. The ready out was performed using Dual-Luciferase Reporter Assay System.

As shown in Figure 10, from the luciferase activity in the cell lysates of Huh-7 cells transduced with AAV9, it can be concluded that the different buffer formulations did not significantly impact the transduction efficiency of AAV9 regardless of the MOI used.

Conclusion

A platform formulation fit study was carried out to select the most suitable formulation from already developed formulations for brain administration in which AAV9-luciferase proves to be most stable under selected stress conditions.

Overall, AAV9 DP was most stable in formulation buffer 3 under applied stress conditions. No visible particles and less sub-visible particles above 2 µm were observed in formulation buffer 3 when compared to the other selected formulations upon all applied stress conditions. The subvisible particles remained within the acceptance criteria according to USP<787>: 6000 particles/mL above 10 µm and 600 particles/mL above 25 µm. The other quality attributes, genome copies, SEC purity (% monomer), hydrodynamic radius and polydispersity index and temperature of unfolding, showed no significant changes in response to the applied stresses when compared to T=0. In addition, AAV9 transduction into the target cells was not impacted by the formulation buffers regardless of multiplicity of infection used.

Therefore buffer 3 is considered the most suitable formulation for preserving the quality of AAV9-luciferase product and should be considered for subsequent studies. Furthermore, this formulation is an isotonic formulation, with a similar tonicity and pH as cerebrospinal fluid: 280-310 mOsm/kg and pH 7.3-7.4 (Salvador L, Valero R, Carrero E, Caral L, Fernández S, Marín JL, Ferrer E, Fábregas N.

Cerebrospinal fluid composition modifications after neuroendoscopic procedures. Minim Invasive Neurosurg. 2007 Feb;50(1):51-5. doi: 10.1055/s-2007-973823. PMID: 17546545).

Table 22: Formulation of one particular embodiment of the present invention (in addition to the adeno-associated viral vector)

Formulation buffer	Cryoprotectant	Tonicity agent	Surfactant
10 mM Na/K-phosphate	3 % (w/v) trehalose	3 mM KCl 87 mM NaCl	0.01% (v/v) PS-20

Table 23: Formulation of one particular embodiment of the present invention (in addition to the adeno-associated viral vector)

Formulation buffer	Cryoprotectant	Tonicity agent	Surfactant
10 mM Na/K-phosphate	3 % (w/v) trehalose	3 mM KCl 82 mM NaCl	0.01% (v/v) PS-20

CLAIMS

1. An isotonic pharmaceutical formulation comprising:
a buffer
5 an adeno-associated viral vector with a transgene encoding a microRNA;
a tonicity agent;
a cryoprotectant; and
a surfactant.
- 10 2. The formulation of claim 1, wherein the formulation has an osmolarity of from 250 to 330 mOsm/kg and a pH value of from 5 to 8.
3. The formulation of any preceding claim, wherein the formulation is substantially free of visible particles.
- 15 4. The formulation of any preceding claim, wherein the adeno-associated viral vector with a transgene encoding microRNA targets ATXN mRNA, for example, ATXN3 mRNA.
5. The formulation of any preceding claim, wherein the adeno-associated viral vector comprises
20 an AAV2 serotype, an AAV5 serotype, an AAV9 serotype, a hybrid AAV serotype, or a combination thereof, preferably the adeno-associated viral vector comprises an AAV5 serotype or an AAV9 serotype, more preferably, an AAV5 serotype.
6. The formulation of any preceding claim, wherein the concentration of the adeno-associated viral
25 vector is at least 1×10^{13} gc/ml, such as at least 3×10^{13} gc/ml.
7. The formulation of any preceding claim, wherein the cryoprotectant is a carbohydrate selected from the group consisting of trehalose, sucrose, dextrose, dextran, and combinations thereof; and present in an amount of up to 8% w/v.
- 30 8. The formulation of any preceding claim, wherein the tonicity agent is selected from the group consisting of NaCl, KCl, $MgCl_2$, $CaCl_2$, and combinations thereof; and is present in an amount of from 5 mM to 150 mM.
- 35 9. The formulation of any preceding claim, wherein the surfactant is a non-ionic surfactant; and is present in an amount of from 0.001% v/v to 0.1% v/v, preferably from 0.003% v/v to 0.08% v/v, more preferably from 0.005% v/v to 0.02% v/v, for example about 0.01% v/v.

10. The formulation of any preceding claim, wherein the buffer is selected from the group consisting of an artificial cerebrospinal fluid (aCSF), acetate buffer, citrate buffer, phosphate buffer, HEPES, Tris, and combinations thereof.
- 5 11. The formulation of any preceding claim, wherein the formulation remains stable at room temperature (15°C-25°C) without substantial formation of aggregates or agglomerates for at least 12 hours after thawing.
- 10 12. The formulation of any preceding claim for use in treating Spinocerebellar Ataxia (SCA).
13. The formulation for use according to claim 12, wherein the formulation is administered by intra-CSF (cerebral spinal fluid) delivery.
- 15 14. The formulation for use according to claim 12 or claim 13, wherein the formulation is administered to a patient's cisterna magna.