

METHOD FOR DETERMINING THE LOADING STATE OF AN AAV PARTICLE BY NUCLEAR MAGNETIC RESONANCE RELAXOMETRY

The current invention is in the field of gene therapy. In more detail, herein is reported a method for the determination of the loading state of a viral particle, that is the ratio between full and empty viral particles. To achieve this aim nuclear magnetic resonance is used.

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Background of the Invention

Gene therapy refers broadly to the therapeutic administration of genetic material to modify gene expression of living cells and thereby alter their biological properties. After decades of research, gene therapies have progressed to the market and are expected to become increasingly important. In general, gene therapy can be divided into either in vivo or ex vivo approaches.

Today, most in vivo therapies rely on DNA delivery with recombinant adeno-associated viral (rAAV) vectors. An AAV is a small, naturally occurring, non-pathogenic parvovirus, which is composed of a non-enveloped icosahedral capsid. It contains a linear, single stranded DNA genome of approximately 4.7 kb. The genome of wild-type AAV vectors carries two genes, rep and cap, which are flanked by inverted terminal repeats (ITRs). ITRs are necessary in cis for viral replication and packaging. The rep gene encodes for four different proteins, whose expression is driven by two alternative promoters, P5 and P19. Additionally different forms are generated by alternative splicing. The Rep proteins have multiple functions, such as, e.g., DNA binding, endonuclease and helicase activity. They play a role in gene regulation, site-specific integration, excision, replication and packaging. The cap gene codes for three capsid proteins and one assembly-activating protein. Differential expression of these proteins is accomplished by alternative splicing and alternative start codon usage and driven by a single promoter, P40, which is located in the coding region of the rep gene.

In engineered, therapeutic rAAV vectors, the viral genes are replaced with a transgene expression cassette, which remains flanked by the viral ITRs, but encodes a gene of interest under the control of a promoter of choice. Unlike the wild-type virus, the engineered rAAV vector does not undergo site-specific

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integration into the host genome, remaining predominantly episomal in the nucleus of transduced cells.

5 An AAV is not replication competent by itself but requires the function of helper genes. These are provided in nature by co-infected helper viruses, such as, e.g., adenovirus or herpes simplex virus. For instance, five adenoviral genes, i.e. E1A, E1B, E2A, E4 and VA, are known to be essential for AAV replication. In contrast to the other helper genes, which code for proteins, VA is a small RNA gene.

10 For the production of rAAV vectors, the therapeutic DNA carrying the transgene flanked by ITRs is introduced into a packaging host cell line, which also comprise rep and cap genes as well as the required helper genes to produce rAAV particles. As the packaging efficiency, i.e. the efficiency of introducing the DNA into the viral capsid during the rAAV particle generation inside the packaging cell line, is less than 100 % rAAV, the obtained rAAV particle preparations are mixtures of full, i.e. DNA containing, and empty, i.e. without DNA, viral capsids. As the
15 therapeutic effect is depending on the successful transfer of the therapeutic DNA, the composition of the mixture has a direct effect.

Thus, there is the need for methods for determining the fraction as well as the ratio of full and empty capsids in a rAAV preparation.

20 Nuclear magnetic resonance (NMR) is a technique that has been used in a wide range of applications to study biomolecules. Conclusions regarding different aspects of biomolecules in a sample may be based on NMR signals of the biomolecules, e.g. proteins or peptides, themselves or on NMR signals derived from the solution comprising the biomolecule. E.g., Shigemitsu et al. (Anal. Biochem. 498 (2016) 59-67) describe the measurement of NMR signals of amyloid
25 beta peptide monomers and dimers. Metz and Mäder (Int. J. Pharmaceut. 364 (2008) 170-175) show NMR experiments for the characterization of emulsions and lipid ingredients that may specifically be used in the pharmaceutical field, in particular, in drug delivery.

30 In WO 2014/169229, a method of using NMR relaxation rates of water molecules as an indicator of the extent of aggregation of biopharmaceutical formulations is reported.

In WO 2018/102681, a method of using the transverse relaxation rates of solvent NMR signal to noninvasively assess particle-containing products formulated as suspension or emulsion in solvent(s) is reported.

5 Hills et al. (Mol. Phys. 67 (1989) 903-918) reported the transverse water proton relaxation in solutions of native bovine serum albumin (BSA).

Feng et al. (Chem. Commun. 51 (2015) 6804) reported that the transverse relaxation rate of water protons can be used to quantify protein aggregation and surfactant micellization in water.

10 Using human insulin preparations, Taraban et al. (J. Pharm. Sci. 104 (2015) 4132-4141) demonstrate that the transverse relaxation rate of water protons can serve as a reliable and sensitive indicator to detect and quantify both visible and sub-visible protein aggregates.

15 Taraban et al. (Anal. Chem. 89 (2017) 5494-5502) reported differences in the sensitivity of water NMR and conventional techniques, such as size-exclusion chromatography, microflow imaging and dynamic light scattering, toward detection of the presence of monoclonal antibody aggregates generated by different stresses.

20 Taraban et al. reported that in-line measurement of water NMR is possible and dependent on flow system configuration, flow rate, protein concentration, and protein aggregation (7th Annual PANIC Conference, 2019, Poster presentation P35: "Water Flow-NMR - A Prospective Contact-Free In-Line Analytical Tool for Continuous Biomanufacturing").

Summary of the Invention

25 Herein is reported a method for the determination of the loading status of viral particles in a sample.

30 The current invention is based, at least in part, on the finding that the transverse nuclear magnetic spin relaxation time T_2 and the transverse nuclear magnetic spin relaxation rate R_2 , respectively, of protons of water molecules in an aqueous solution comprising viral particles depends on the loading status of the viral particle.

Thus, one aspect of the current invention is a method for determining the ratio/fraction/concentration of loaded viral particles in a sample,

the method comprising the following steps:

- 5 - determining a nuclear magnetic resonance (NMR) parameter related to the protons of the water molecules present in an aqueous solution comprising a mixture of loaded and empty viral particles by applying an NMR measurement to the solution, and
- 10 - determining the ratio/fraction/concentration of loaded viral particles with the NMR parameter determined in the previous step using/based on a calibration function.

In one embodiment, the NMR parameter is indicative of the transverse nuclear magnetic spin relaxation of water in the solution or fraction thereof, specifically of the protons of the water molecules in the aqueous solution.

15 In one embodiment, the NMR parameter is the transverse nuclear magnetic spin relaxation time T_2 or the transverse nuclear magnetic spin relaxation rate R_2 of the protons of the water molecules in the aqueous solution.

In one embodiment, the isolated, (monomeric) viral particle has a molecular weight in the range of 5,000 kDa to 6,000 kDa. In one preferred embodiment, the isolated, (monomeric) viral particle has a molecular weight of 5,000 kDa to 5,250 kDa.

20 In one embodiment, the viral particle is a virus, a virus-like particle (VLP), or a liposome. In a further embodiment, the viral particle is a virus or a VLP. In another embodiment, the viral particle is a non-enveloped virus or a VLP of a non-enveloped virus. In one preferred embodiment, the viral particle is an adeno-associated virus particle (AAV particle).

25 In one embodiment, the loaded viral particles are DNA-containing viral particles. In one preferred embodiment, the loaded viral particles are DNA-containing AAV particles.

30 In one embodiment, the total concentration of the viral particles in the aqueous solution is at least 1×10^{12} vp/mL (viral particles per mL), in one embodiment at least 5×10^{12} vp/mL, in one preferred embodiment at least 1×10^{13} vp/mL, in one embodiment at least 2×10^{13} vp/mL.

In one embodiment, the aqueous solution is a phosphate buffered saline solution. In one preferred embodiment, the aqueous solution is a phosphate buffered saline solution comprising a detergent and optionally citrate.

5 In one embodiment, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises up to 80 % of DNA-containing viral particles, in one preferred embodiment up to 80 % of DNA-containing AAV particles, and the calibration function is a linear function.

In one embodiment, the calibration function is a second order polynomial function.

10 In one embodiment, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises up to 100 % of DNA-containing viral particles, in one preferred embodiment up to 100 % of DNA-containing AAV particles, and the calibration function is a second order polynomial function.

15 In one embodiment, the calibration function is based on the same NMR parameter determined for at least two (calibration) samples with different ratios of DNA-containing and empty AAV particles.

20 The method according to the current invention is in certain embodiments an in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate, e.g., to producing an aqueous solution comprising (a mixture of) DNA-containing and empty AAV particles, and/or the steps indicated elsewhere herein. Moreover, one or more of said steps may be performed by automated equipment.

25 The present invention also relates to a use of the method according to the present invention, for a purpose selected from the group consisting of producing a recombinant AAV particle and release of recombinant AAV particle preparations for therapeutic application, especially real-time release.

Thus, one aspect of the current invention is the use of the transverse nuclear magnetic spin relaxation time T_2 for the determination of the loading status of viral particles, in one preferred embodiment of AAV particles.

30 In one embodiment, the loading status is the ratio of DNA-containing viral particles to empty viral particles.

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In one embodiment, the loading status is the fraction of DNA-containing viral particles of the total viral particles.

In one embodiment, the loading status is the concentration of DNA-containing viral particles.

5 In one embodiment, the loading status is determined in a sample.

In one embodiment, the determination is in an aqueous solution and the transverse nuclear magnetic spin relaxation time T_2 is the transverse nuclear magnetic spin relaxation time T_2 of the protons of the water molecules in the aqueous solution.

In one embodiment, the use is an in vitro use.

10 In addition to the various embodiments depicted and claimed, the disclosed subject matter is also directed to other embodiments having other combinations of the features disclosed and claimed herein. As such, the particular features presented herein, especially presented as aspects or embodiments, can be combined with each other in other manners within the scope of the disclosed subject matter such that
15 the disclosed subject matter includes any suitable combination of the features disclosed herein. The description of specific embodiments of the disclosed subject matter has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the disclosed subject matter to those embodiments disclosed.

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Detailed Description of the Invention

Herein is reported a method for determining the ratio/fraction/concentration of DNA-containing AAV particles in a sample, wherein the method comprises the steps of determining the water proton transverse relaxation time (T_2) or the water
25 proton transverse relaxation rate (R_2), of the protons of the water molecules in an aqueous solution comprising a mixture of DNA-containing and empty AAV particles, by applying an NMR measurement to the solution and thereafter determining the ratio/fraction/concentration of DNA-containing AAV particles with the determined nuclear magnetic spin relaxation based on a calibration
30 function.

5 The current invention is based, at least in part, on the finding that the transverse nuclear magnetic spin relaxation time T_2 and the transverse nuclear magnetic spin relaxation rate R_2 , respectively, of protons of water molecules in an aqueous solution comprising higher order protein structures, such as DNA-containing AAV particles, is affected by the internal composition of the higher order protein structure.

10 The current invention is based, at least in part, on the finding that the transverse nuclear magnetic spin relaxation time T_2 and the transverse nuclear magnetic spin relaxation rate R_2 , respectively, of protons of water molecules in an aqueous solution comprising AAV particles depends on the DNA-loading of the AAV particle.

DEFINITIONS

15 Useful methods and techniques for carrying out the current invention are described in e.g. Ausubel, F.M. (ed.), Current Protocols in Molecular Biology, Volumes I to III (1997); Glover, N.D., and Hames, B.D., ed., DNA Cloning: A Practical Approach, Volumes I and II (1985), Oxford University Press; Freshney, R.I. (ed.), Animal Cell Culture – a practical approach, IRL Press Limited (1986); Watson, J.D., et al., Recombinant DNA, Second Edition, CHSL Press (1992); Winnacker, 20 E.L., From Genes to Clones; N.Y., VCH Publishers (1987); Celis, J., ed., Cell Biology, Second Edition, Academic Press (1998); Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique, second edition, Alan R. Liss, Inc., N.Y. (1987).

25 The use of recombinant DNA technology enables the generation of derivatives of a nucleic acid. Such derivatives can, for example, be modified in individual or several nucleotide positions by substitution, alteration, exchange, deletion or insertion. The modification or derivatization can, for example, be carried out by means of site directed mutagenesis. Such modifications can easily be carried out by a person skilled in the art (see e.g. Sambrook, J., et al., Molecular Cloning: A 30 laboratory manual (1999) Cold Spring Harbor Laboratory Press, New York, USA; Hames, B.D., and Higgins, S.G., Nucleic acid hybridization – a practical approach (1985) IRL Press, Oxford, England).

As used herein, the term "standard conditions", if not otherwise noted, relates to IUPAC standard ambient temperature and pressure (SATP) conditions, i.e. preferably, a temperature of 25 °C and an absolute pressure of 100 kPa; also preferably, standard conditions include a pH value of 7.

5 It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and equivalents thereof known to those skilled in the art, and so forth. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably
10 herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably.

The term "about" denotes a range of +/- 20 % of the thereafter following numerical value. In one embodiment, the term about denotes a range of +/- 10 % of the thereafter following numerical value. In one embodiment, the term about denotes a
15 range of +/- 5 % of the thereafter following numerical value.

The term "aqueous solution", as used herein, relates to any liquid preparation wherein the concentration of water (H₂O) in the solvent is at least 50 % (w/v), in an embodiment at least 75 % (w/v), in a further embodiment at least 90 % (w/v), in a further embodiment at least 95 % (w/v), in a further embodiment at least 98 %
20 (w/v), in a further embodiment at least 99 % (w/v), in a further embodiment is at least 99.5 % (w/v). Thus, the term aqueous solution encompasses liquid preparations comprising up to 50 % (w/v) D₂O or PEG (poly ethylene glycol).

Further, as used herein, the term "solution" indicates that at least a fraction of the compounds (solutes) in the solution is dissolved in the solvent. Methods for
25 preparing solutions are known in the art. Thus, the term aqueous solution, in an embodiment, relates to a liquid preparation comprising viral particles, such as AAV particles, which are, at least partially, dissolved in a solvent comprising, in an embodiment consisting of, water. In one embodiment, the aqueous solution is a phosphate buffered saline solution. In one preferred embodiment, the aqueous
30 solution is a phosphate buffered saline solution comprising about 0.001 % (w/v) of a detergent and optionally about 100 mM citrate. In one embodiment, the detergent is selected from poloxamers and polysorbates. Poloxamers are commercially available under the tradenames Pluronic® or Kolliphor® or Synperonic®. Polysorbates are commercially available under the tradenames Tween®, Scattics®,

Alkest® and Canarcel®. In one embodiment, the detergent is poloxamer 188 (tradename: Pluronic F-68) and the optional citrate is sodium citrate. A phosphate buffered saline solution comprises about 137 mM sodium chloride, about 2.7 mM potassium chloride and about 12 mM phosphate as a mixture of hydrogen phosphate and dihydrogen phosphate, adjusted to a pH value of 7.4.

The term “comprising” also encompasses the term “consisting of”.

The term "determining" is used as understood by the skilled person and relates to determining a value of a relevant parameter, in particular an NMR parameter with a suitable method.

10 In some embodiments, the determination is an off-line determination. Thus, in certain embodiments, fractions or aliquots are transferred to a measurement device.

In other embodiments, the determination is a continuous in-line determination. Thus, in certain embodiments, the aliquots are virtual aliquots. Thus, in certain embodiments, the aliquots are generated as a continuous stream of liquid. In further
15 embodiments, the concentration, the NMR parameter and/or any other parameters(s) optionally determined is/are determined in a flow-through cell, in particular, in case of the concentration, in a flow-through cuvette. The parameters may be determined simultaneously or sequentially, in specific embodiments, the parameters are determined sequentially.

20 The terms "empty capsid" and "empty particle", refer to a viral particle, such as an AAV particle that includes a viral protein shell but that lacks in whole or part a nucleic acid that encodes a protein or is transcribed into a transcript of interest. Accordingly, the empty capsid does not function to transfer a nucleic acid that encodes a protein or is transcribed into a transcript of interest into the host cell.

25 The term “endogenous” denotes that something is naturally occurring within a cell; naturally produced by a cell; endogenous gene locus/cell-endogenous gene locus: naturally occurring locus in a cell.

As used herein, the term “exogenous” indicates that a nucleotide sequence does not originate from a specific cell and is introduced into said cell by DNA delivery
30 methods, e.g., by transfection, electroporation, or transduction by viral vectors. Thus, an exogenous nucleotide sequence is an artificial sequence wherein the artificiality can originate, e.g., from the combination of subsequences of different

origin (e.g. a combination of a recombinase recognition sequence with an SV40 promoter and a coding sequence of green fluorescent protein is an artificial nucleic acid) or from the deletion of parts of a sequence (e.g. a sequence coding only the extracellular domain of a membrane-bound receptor or a cDNA) or the mutation of nucleobases. The term “endogenous” refers to a nucleotide sequence originating from a cell. An “exogenous” nucleotide sequence can have an “endogenous” counterpart that is identical in base compositions, but where the “exogenous” sequence is introduced into the cell, e.g., via recombinant DNA technology.

As used herein, the term "impurities" denotes all compounds decreasing purity of a viral particle preparation, such as an AAV particle preparation, especially including empty viral particles. Thus, in certain embodiments, impurities are AAV particle-specific by-products, especially empty AAV particles, and host cell protein by-products.

An "isolated" composition is one, which has been separated from a component of its natural environment. In some embodiments, a composition is purified to greater than 95 % or 99 % purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis, CE-SDS) or chromatographic (e.g., size exclusion chromatography or ion exchange or reverse phase HPLC) or amplificative (PCR) methods. For review of methods for assessment of e.g. antibody purity, see, e.g., Flatman, S. et al., J. Chrom. B 848 (2007) 79-87.

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

An "isolated" polypeptide or antibody refers to a polypeptide molecule or antibody molecule that has been separated from a component of its natural environment.

The term "nuclear magnetic resonance", abbreviated as “NMR”, has the meaning as understood by the skilled person. In accordance, the term "nuclear magnetic resonance parameter", abbreviated as "NMR parameter", relates, in principle, to any parameter, which can be determined by applying a nuclear magnetic resonance measurement to a sample, in particular to an aqueous solution comprising (a mixture of) DNA-containing and empty viral particles, such as AAV particles, and

indicating a nuclear magnetic spin relaxation. In specific embodiments, the NMR parameter is a parameter derivable from water proton NMR ($^1\text{H}_2\text{O}$ NMR). In specific embodiments, the NMR parameter comprises or is a relaxation time or a relaxation rate determined in an NMR measurement. In specific embodiments, the NMR parameter comprises or is a relaxation time or a relaxation rate determined in an $^1\text{H}_2\text{O}$ NMR measurement. In further embodiments, the NMR parameter comprises or is at least one of a transverse nuclear magnetic spin relaxation time T_2 and a transverse nuclear magnetic spin relaxation rate R_2 of water in an aqueous solution or aliquot thereof. In further embodiments, the NMR parameter is indicative of a transverse nuclear magnetic spin relaxation of water in an aqueous solution or aliquot thereof, specifically of protons in water in solution or aliquot thereof. As the skilled person understands, the relaxation time T is the reciprocal of the corresponding relaxation rate R , i.e. $T = 1/R$. In certain embodiments, the NMR parameter is a transverse relaxation time (T_2) or a transverse relaxation rate (R_2) determined in an NMR measurement. In certain embodiments, the NMR parameter is the transverse relaxation time (T_2) or the transverse relaxation rate (R_2) determined in an $^1\text{H}_2\text{O}$ NMR measurement. In one preferred embodiment, the NMR parameter is the water proton transverse relaxation time (T_2) or the water proton transverse relaxation rate (R_2) of the protons of water in the aqueous solution or aliquot thereof. In certain embodiments, the magnetic field strength during the determination is of from 0.1 Tesla (T) to 24 T, in certain embodiments of from 0.2 T to 10 T, in further embodiments of from 0.3 T to 5 T, in further embodiments of from 0.4 T to 2 T. In one embodiment, the magnetic field strength is in the range of 0.45 to 0.50 T. In one preferred embodiment, the magnetic field strength is about 0.47 T. In certain embodiments, the resonance frequency is of from 5 MHz to 500 MHz, in further embodiments of from 7.5 MHz to 200 MHz, in further embodiments of from 10 MHz to 100 MHz, in further embodiments of from 15 MHz to 50 MHz. In one preferred embodiment, the resonance frequency is about 20 MHz.

In certain embodiments, the NMR parameter is corrected for changes not caused by viral particles, such as AAV particles, in particular for solute effects, which may in certain embodiments be based on pH value and/or ion concentration. In certain embodiments, such correction is provided by performing a pre-run using a sample with known viral particle content and otherwise identical composition as the samples to be tested.

Thus, in certain embodiments, the ratio/fraction/concentration of DNA-containing viral particles, such as DNA-containing AAV particles, is directly proportional to the NMR parameter being the transverse relaxation time (T_2) of the protons of the water molecules in the aqueous solution.

5 The terms "nuclear magnetic resonance measurement device" and "NMR measurement device", as used herein, equally include any and all devices configured for determining an NMR parameter by applying an NMR measurement to an aqueous solution or an aliquot thereof. In certain embodiments, the NMR measurement device is configured to perform measurement of a nuclear magnetic
10 spin relaxation value, in certain embodiments of the water proton nuclear magnetic spin relaxation value. In certain embodiments, the NMR measurement device is configured for in-line measurement, in further embodiments for continuous in-line measurement. Suitable devices are known in the art, e.g. from Metz & Mäder (Int. J. Pharmaceut. 364 (2008) 170) and from Taraban et al. (Anal. Chem. 89 (2017) 5494; 7th Annual PANIC Conference, 2019, Poster presentation P35: "Water Flow-NMR - A Prospective Contact-Free In-Line Analytical Tool for Continuous
15 Biomanufacturing").

A "recombinant AAV vector" is derived from the wild-type genome of a virus, such as AAV by using molecular methods to remove the wild type genome from
20 the virus (e.g., AAV), and replacing it with a non-native nucleic acid, such as a nucleic acid transcribed into a transcript or that encodes a protein. Typically, for AAV one or both inverted terminal repeat (ITR) sequences of AAV genome are retained in the AAV vector. A "recombinant" AAV vector is distinguished from a naturally occurring viral AAV genome, since all or a part of the viral genome has
25 been replaced with a non-native (i.e., heterologous) sequence with respect to the natural viral genomic nucleic acid. Incorporation of a non-native sequence therefore defines the viral vector (e.g., AAV) as a "recombinant" vector, which in the case of AAV can be referred to as a "rAAV vector."

A recombinant viral vector sequence (e.g., an AAV vector sequence) can be
30 packaged - referred to herein as a "particle" - for subsequent infection (transduction) of a cell, ex vivo, in vitro or in vivo. Where a recombinant vector sequence is encapsulated or packaged into an AAV particle, the particle can also be referred to as an "AAV particle" or a "rAAV particle". Such particles include proteins that encapsulated or package the vector genome. Particular examples

include viral envelope proteins, and in the case of AAV, capsid proteins, such as AAV VP1, VP2 and VP3.

As used herein, the term "serotype" is a distinction used to refer to an AAV having a capsid that is serologically distinct from other AAV serotypes. Serologic distinctiveness is determined based on the lack of cross-reactivity between antibodies to one AAV as compared to another AAV. Such cross-reactivity differences are usually due to differences in capsid protein sequences/antigenic determinants (e.g., due to VP1, VP2, and/or VP3 sequence differences of AAV serotypes). Despite the possibility that AAV variants including capsid variants may not be serologically distinct from a reference AAV or other AAV serotype, they differ by at least one nucleotide or amino acid residue compared to the reference or other AAV serotype.

Under the traditional definition, a serotype means that the virus of interest has been tested against serum specific for all existing and characterized serotypes for neutralizing activity and no antibodies have been found that neutralize the virus of interest. As more naturally occurring virus isolates are discovered and/or capsid mutants generated, there may or may not be serological differences with any of the currently existing serotypes. Thus, in cases where the new virus (e.g., AAV) has no serological difference, this new virus (e.g., AAV) would be a subgroup or variant of the corresponding serotype. In many cases, serology testing for neutralizing activity has yet to be performed on mutant viruses with capsid sequence modifications to determine if they are of another serotype according to the traditional definition of serotype. Accordingly, for the sake of convenience and to avoid repetition, the term "serotype" broadly refers to both serologically distinct viruses (e.g., AAV) as well as viruses (e.g., AAV) that are not serologically distinct that may be within a subgroup or a variant of a given serotype.

The terms "transduce" and "transfect" refer to introduction of a molecule such as a nucleic acid (plasmid) into a cell. A cell has been "transduced" or "transfected" when exogenous nucleic acid has been introduced inside the cell membrane. Accordingly, a "transduced cell" is a cell into which a "nucleic acid" or "polynucleotide" has been introduced, or a progeny thereof in which an exogenous nucleic acid has been introduced. In particular embodiments, a "transduced" cell (e.g., in a mammal, such as a cell or tissue or organ cell) is a genetic change in a cell following incorporation of an exogenous molecule, for example, a nucleic acid

(e.g., a transgene). A "transduced" cell(s) can be propagated and the introduced nucleic acid transcribed and/or protein expressed.

5 In a "transduced" or "transfected" cell, the nucleic acid (plasmid) may or may not be integrated into genomic nucleic acid. If an introduced nucleic acid becomes integrated into the nucleic acid (genomic DNA) of the recipient cell or organism, it can be stably maintained in that cell or organism and further passed on to or inherited by progeny cells or organisms of the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism extrachromosomally, or only transiently. A number of techniques are known, see, 10 e.g., Graham et al. (1973) Virology, 52:456; Sambrook et al. (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York; Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier; and Chu et al. (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

15 A "transgene" is used herein to conveniently refer to a nucleic acid that is intended or has been introduced into a cell or organism. Transgenes include any nucleic acid, such as a gene that is transcribed into a transcript or that encodes a polypeptide or protein.

20 A "vector" refers to the portion of the recombinant plasmid sequence ultimately packaged or encapsulated, either directly or in form of a single- or double-stranded DNA or RNA, to form a viral (e.g., AAV) particle. In cases recombinant plasmids are used to construct or manufacture recombinant viral particles, the viral particle does not include the portion of the "plasmid" that does not correspond to the vector sequence of the recombinant plasmid. This non-vector portion of the recombinant 25 plasmid is referred to as the "plasmid backbone", which is important for cloning and amplification of the plasmid, a process that is needed for propagation and recombinant virus production, but is not itself packaged or encapsulated into virus (e.g., AAV) particles. Thus, a "vector" refers to the nucleic acid that is packaged or encapsulated by a virus particle (e.g., AAV).

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SPECIFIC EMBODIMENTS OF THE INVENTION

The current invention is based, at least in part, on the finding that the transverse nuclear magnetic spin relaxation time T_2 and the transverse nuclear magnetic spin

relaxation rate R_2 , respectively, of protons of water molecules in an aqueous solution comprising viral particles depends on the loading status (full vs. empty) of the viral particle.

Thus, one aspect of the current invention is a method for determining the
5 ratio/fraction/concentration of loaded viral particles in a sample,

the method comprising the following steps:

- determining a nuclear magnetic resonance (NMR) parameter related to the protons of the water molecules present in an aqueous solution comprising a mixture of loaded and empty viral particles by applying an NMR
10 measurement to the solution; and
- determining the ratio/fraction/concentration of loaded viral particles with the NMR parameter determined in the previous step based on a calibration function.

In certain embodiments, the NMR parameter is indicative of a transverse nuclear
15 magnetic spin relaxation of water in the solution or fraction thereof, specifically of protons in water in solution or fraction thereof.

In certain embodiments, the NMR parameter is the transverse nuclear magnetic spin relaxation time T_2 or the transverse nuclear magnetic spin relaxation rate R_2 of the protons of the water molecules in the aqueous solution.

20 In certain embodiments, the viral particle is a virus, a virus-like particle (VLP), or a liposome. In a further embodiment, the viral particle is a virus or a VLP. In another embodiment, the viral particle is a non-enveloped virus or a VLP of a non-enveloped virus. In one preferred embodiment, the viral particle is an adeno-associated virus particle (AAV particle).

25 In certain embodiments, the loaded viral particles are DNA-containing viral particles. In one preferred embodiment, the loaded viral particles are DNA-containing AAV particles.

In certain embodiments, the total concentration of viral particles in the aqueous solution is equivalent to at least 1×10^{12} vg/mL, in one embodiment at least
30 5×10^{12} vg/mL, in one preferred embodiment at least 1×10^{13} vg/mL, in one embodiment at least 2×10^{13} vg/mL.

In certain embodiments, the total concentration of viral particles in the aqueous solution is at least 1×10^{12} vp/mL (viral particles per mL), in one embodiment at least 5×10^{12} vp/mL, in one preferred embodiment at least 1×10^{13} vp/mL, in one embodiment at least 2×10^{13} vp/mL.

5 In certain embodiments, the aqueous solution has a pH value in the range from pH 7 to pH 8. In one preferred embodiment, the aqueous sample has a pH value of about 7.4.

10 In certain embodiments, the aqueous solution is a phosphate buffered saline solution. In one preferred embodiment, the aqueous solution is a phosphate buffered saline solution comprising a detergent and optionally citrate, especially about 0.001 % (w/v) detergent and optionally about 100 mM citrate.

15 In certain embodiments, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises up to 100 % of DNA-containing viral particles, in one preferred embodiment up to 100 % of DNA-containing AAV particles, and the calibration function is a linear function.

20 In certain embodiments, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises between 50 % and 100 % of DNA-containing viral particles, in one preferred embodiment between 50 % and 100 % of DNA-containing AAV particles, and the calibration function is a linear function.

In certain embodiments, the calibration function is a second order polynomial function.

25 In certain embodiments, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises up to 100 % of DNA-containing viral particles, in one preferred embodiment up to 100 % of DNA-containing AAV particles, and the calibration function is a second order polynomial function.

30 In certain embodiments, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises between 50 % and 100 % of DNA-containing viral particles, in

one preferred embodiment between 50 % and 100 % of DNA-containing AAV particles, and the calibration function is a second order polynomial function.

5 In certain embodiments, the calibration function is based on the same NMR parameter determined for at least two samples with different ratios of DNA-containing and empty AAV particles.

10 The method according to the current invention is an in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate, e.g., to producing an aqueous solution comprising (a mixture of) DNA-containing and empty AAV particles, and/or the steps indicated elsewhere herein. Moreover, one or more of said steps may be performed by automated equipment.

15 The present invention also relates to a use of the method according to the present invention, for a purpose selected from the group consisting of producing an AAV particle, purifying an AAV particle, removing impurities from a preparation of an AAV particle, and release of recombinant AAV particle preparations for therapeutic application, especially real-time release.

20 The current invention is further based, at least in part, on the finding that the transverse nuclear magnetic spin relaxation time T_2 and the transverse nuclear magnetic spin relaxation rate R_2 , respectively, of protons of water molecules in an aqueous solution comprising AAV particles depends on the loading status (full vs. empty) of the AAV particle.

25 The current invention is based, at least in part, that the transverse nuclear magnetic spin relaxation time T_2 can be used for the determination of the loading status of AAV particles independent of the serotype of the AAV particle, thus is independent of the serotype of the AAV particle.

30 In more detail, it has been found that the fraction/ratio/percentage/concentration of DNA-containing AAV particles can be obtained by determining the transverse nuclear magnetic spin relaxation time T_2 in an aqueous solution comprising DNA-containing and empty AAV particles by water proton NMR ($^1\text{H}_2\text{O}$ NMR) and correlating said time with standard/calibration values. That is, it has been found that the water proton transverse spin relaxation time T_2 correlates with the fraction/ratio/percentage/concentration of DNA-containing AAV particles in an aqueous sample.

Figure 1 shows the dependency of T_2 on the DNA-loading of AAV particles of the serotype 2 (AAV2), i.e. the dependency of T_2 on the concentration of DNA-containing AAV2 particles in an aqueous sample. It can be seen that the absolute difference in the relaxation time T_2 between empty AAV2 particles (0 % full) and completely DNA-containing AAV2 particles (100 % full) at a concentration of $2 \cdot 10^{13}$ particles/mL is 197.1 ± 6.4 ms and the relative difference is 12.6 ± 0.4 %. The data points can be fitted with a second order polynomial function with an R^2 value of 0.996.

Figure 2 shows the dependency of T_2 on the DNA-loading of AAV particles of the serotype 6 (AAV6), i.e. the dependency of T_2 on the concentration of DNA-containing AAV6 particles in an aqueous sample. It can be seen that the absolute difference in the relaxation time T_2 between empty AAV6 particles (0 % full) and completely DNA-containing AAV6 particles (100 % full) at a concentration of $2 \cdot 10^{13}$ particles/mL is 149.8 ± 22.1 ms and the relative difference is 9.2 ± 1.4 %. The data points can be fitted with a second order polynomial function with an R^2 value of 0.9987.

Figure 3 shows the dependency of T_2 on the DNA-loading of AAV particles of the serotype 8 (AAV8), i.e. the dependency of T_2 on the concentration of DNA-containing AAV8 particles in an aqueous sample. It can be seen that the absolute difference in the relaxation time T_2 between empty AAV8 particles (0 % full) and completely DNA-containing AAV8 particles (100 % full) at a concentration of $2 \cdot 10^{13}$ particles/mL is 206.9 ms and the relative difference is 14 %. The data points can be fitted with a second order polynomial function with an R^2 value of 0.9633.

Without being bound by this theory, it is assumed that a relative difference of the transverse nuclear magnetic spin relaxation time T_2 between empty AAV particles (0 % full) and completely DNA-containing AAV particles (100 % full) of at least 5 % is sufficient for performing the method according to the current invention.

Thus, one aspect of the current invention is a method for determining the ratio of DNA-containing AAV particles to empty AAV particles in a sample.

Thus, one aspect of the current invention is a method for determining the fraction of DNA-containing AAV particles with respect to all AAV particles in a sample.

Thus, one aspect of the current invention is a method for determining the concentration of DNA-containing AAV particles in a sample.

The method according to these three aspects of the invention comprises the following steps:

- 5 - determining a nuclear magnetic resonance (NMR) parameter of the protons of the water molecules in (an aliquot of) an aqueous solution comprising a mixture of DNA-containing and empty AAV particles, by applying an NMR measurement to the solution; and
- determining the ratio/fraction/concentration of DNA-containing AAV particles with the NMR parameter determined in the previous step based on a calibration function.

10 In specific embodiments, the NMR parameter is indicative of a transverse nuclear magnetic spin relaxation of water in the solution or aliquot thereof, specifically of protons in water in solution or aliquot thereof.

 In specific embodiments, the NMR parameter is the transverse nuclear magnetic spin relaxation time T_2 or the transverse nuclear magnetic spin relaxation rate R_2 of
15 the protons of the water molecules in the aqueous solution.

 In one preferred embodiment, the NMR parameter is the water proton transverse relaxation time (T_2) of the protons of the water molecules in the aqueous solution.

 In specific embodiments, the method according to the current invention is performed at conditions wherein the relative difference of the transverse nuclear magnetic spin relaxation time T_2 determined with 100 % empty AAV particles and determined with 100 % DNA-containing AAV particles is at least 5 %. In one
20 embodiment, the method is performed at conditions wherein the relative difference of the transverse nuclear magnetic spin relaxation time T_2 determined with 100 % empty AAV particles and determined with 100 % DNA-containing AAV particles
25 is at least 8 %. In one preferred embodiment, the method is performed at conditions wherein the relative difference of the transverse nuclear magnetic spin relaxation time T_2 determined with 100 % empty AAV particles and determined with 100 % DNA-containing AAV particles is at least 10 %. In one embodiment, the conditions
30 are a temperature, a total concentration of AAV particles in the aqueous solution, a magnetic field strength, a resonance frequency, a buffer condition, optionally a detergent concentration, and optionally a salt concentration. The smaller of the two transverse nuclear magnetic spin relaxation times T_2 is set to 100 % for the

calculation of the relative difference. The calculation is done according to the following equation:

$$\text{relative difference} = \left(\frac{\text{larger } T_2 \text{ value} - \text{smaller } T_2 \text{ value}}{\text{smaller } T_2 \text{ value}} \right) * 100 \%$$

5 In specific embodiments, the magnetic field strength during the determination of the NMR parameter is of from 0.1 T to 24 T, in certain embodiments of from 0.2 T to 10 T, in further embodiments of from 0.3 T to 5 T, in further embodiments of from 0.4 T to 2 T. In one embodiment, the magnetic field strength is in the range of 0.45 T to 0.50 T. In one preferred embodiment, the magnetic field strength is about 0.47 T.

10 In specific embodiments, the resonance frequency during the determination of the NMR parameter is of from 5 MHz to 500 MHz, in further embodiments of from 7.5 MHz to 200 MHz, in further embodiments of from 10 MHz to 100 MHz, in further embodiments of from 15 MHz to 50 MHz. In one preferred embodiment, the resonance frequency is about 20 MHz.

15 In specific embodiments, the method is performed at about room temperature. In one embodiment, the method is performed at a temperature in the range from 18 °C to 25 °C. In one embodiment, the method is performed at a temperature in the range from 19 °C to 22 °C. In one preferred embodiment, the method is performed at a temperature of about 20 °C.

20 In specific embodiments, the aqueous solution is a phosphate buffered saline solution. In one embodiment, the aqueous solution is a phosphate buffered saline solution comprising a detergent. In one embodiment, the detergent is selected from the groups of poloxamers and polysorbates. In one embodiment, the detergent is selected from the group of detergents consisting of poloxamer 188, poloxamer 407,
25 polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80 and polysorbate 100. In one embodiment, the detergent is present at a concentration of about 0.001 % (w/v). In one preferred embodiment, the detergent is poloxamer 188. In another embodiment, the aqueous solution further comprises a citrate (salt of citric acid). In one embodiment, the citrate is present at a concentration of 100 mM. In
30 one preferred embodiment, the citrate is sodium citrate. A phosphate buffered saline solution comprises about 137 mM sodium chloride, about 2.7 mM potassium chloride and about 12 mM phosphate as a mixture of hydrogen phosphate and dihydrogen phosphate, adjusted to a pH value of 7.4.

5 In specific embodiments, the AAV particle has a serotype selected from the group of AAV serotypes consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 and mixtures as well as chimeras thereof. In one embodiment, the AAV particle has a serotype selected from the group of AAV serotypes consisting of AAV2, AAV4, AAV6, AAV8 and AAV9. In one preferred embodiment, the AAV particle is of the AAV2 serotype, or the AAV6 serotype, or the AAV8 serotype.

10 In specific embodiments, the AAV particle is an AAV8 particle and the aqueous solution is a phosphate buffered saline solution additionally comprising about 0.001 % (w/v) poloxamer 188.

In specific embodiments, the AAV particle is an AAV2 particle and the aqueous solution is a phosphate buffered saline solution additionally comprising about 0.001 % (w/v) poloxamer 188 and about 100 mM sodium citrate.

15 In specific embodiments, the AAV particle is an AAV6 particle and the aqueous solution is a phosphate buffered saline solution additionally comprising about 0.001 % (w/v) poloxamer 188 and about 100 mM sodium citrate.

In specific embodiments, the method comprises as first step the step of

- providing an aqueous solution comprising a mixture of DNA-containing and empty AAV particles.

20 In specific embodiments, the method comprises as second step the step of

- determining the total concentration of AAV particles in the aqueous solution.

In specific embodiments, the calibration function is based on the same NMR parameter determined for at least two samples with different known concentrations and/or ratios of DNA-containing and empty AAV particles.

25 In specific embodiments, the calibration function is a linear function.

In one embodiment, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises up to 80 % of DNA-containing viral particles, in one preferred embodiment up to 80 % of DNA-containing AAV particles, and the calibration function is a linear function.

In specific embodiments, the calibration function is obtained by determining the same NMR parameter for two calibration samples, the first comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 10:90 to 30:70, in one preferred embodiment at about 25:75, and the second comprising
5 DNA-containing AAV particles and empty AAV particles at a ratio in the range of 70:30 to 90:10, in one preferred embodiment at about 75:25, and fitting to a linear function to provide a calibration function.

In specific embodiments, the calibration function is obtained by determining the same NMR parameter for two calibration samples, the first comprising only DNA-
10 containing AAV particles or only empty AAV particles and the second being selected from the group of calibration samples consisting of samples comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 10:90 to 30:70, in one preferred embodiment at about 25:75, samples comprising
15 DNA-containing AAV particles and empty AAV particles at a ratio in the range of 40:60 to 60:40, in one preferred embodiment at about 50:50, and samples comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 70:30 to 90:10, in one preferred embodiment at about 75:25, and fitting to a linear function to provide a calibration function.

In specific embodiments, the calibration function is obtained by determining the same NMR parameter for at least three calibration samples, the first comprising
20 DNA-containing AAV particles and empty AAV particles at a ratio in the range of 0:100 to 30:70, in one preferred embodiment at about 0:100 or 25:75, the second comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 40:60 to 60:40, in one preferred embodiment at about 50:50, and the
25 third comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 70:30 to 100:0, in one preferred embodiment at about 75:25 or 100:0, and fitting to a linear function to provide a calibration function.

In specific embodiments, the calibration function is obtained by determining the same NMR parameter for at least three calibration samples, the first comprising
30 DNA-containing AAV particles and empty AAV particles at a ratio in the range of 0:100 to 30:70, in one preferred embodiment at about 0:100 or 25:75, the second comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 40:60 to 60:40, in one preferred embodiment at about 50:50, and the
35 third comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 70:30 to 100:0, in one preferred embodiment at about 75:25 or

100:0, and fitting the first and the second calibration sample to a first linear calibration function for the ratio from 0:100 up to and including the ratio 50:50 and fitting the second and the third calibration samples to a second linear calibration function for the ratio from and excluding 50:50 to 100:0.

5 In one embodiment, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises between 75 % and 100 % of DNA-containing viral particles, in one preferred embodiment between 50 % and 100 % of DNA-containing AAV particles, and the calibration function is a linear function.

10 In specific embodiments, the calibration function is obtained by determining the same NMR parameter for two calibration samples, the first comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 40:60 to 60:40, in one preferred embodiment at about 50:50, and the second comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of
15 70:30 to 90:10, in one preferred embodiment at about 75:25, and fitting to a linear function to provide a calibration function.

In specific embodiments, the calibration function is a second order polynomial function.

20 In one embodiment, the method is for determining the ratio/fraction/concentration of loaded viral particles in a sample, wherein the sample comprises up to 100 % of DNA-containing viral particles, in one preferred embodiment up to 100 % of DNA-containing AAV particles and the calibration function is a second order polynomial function.

25 In specific embodiments, the calibration function is obtained by determining the same NMR parameter for at least three calibration samples, the first comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 0:100 to 30:70, in one preferred embodiment at about 0:100 or 25:75, the second comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 40:60 to 60:40, in one preferred embodiment at about 50:50, and the
30 third comprising DNA-containing AAV particles and empty AAV particles at a ratio in the range of 70:30 to 100:00, in one preferred embodiment at about 75:25 or 100:0, and fitting to a linear function to provide a calibration function.

The calibration function is then used to obtain the respective ratio/fraction/concentration based on the determined NMR parameter for an unknown sample.

5 In specific embodiments, the total concentration of the viral particles in the aqueous solution is at least 1×10^{12} viral particles per mL (vp/mL). In one embodiment, the total concentration of the viral particles in the aqueous solution is at least 5×10^{12} vp/mL. In one preferred embodiment, the total concentration of the viral particles in the aqueous solution is at least 1×10^{13} vp/mL. In one embodiment, the total concentration of the viral particles in the aqueous solution is at least
10 2×10^{13} vg/mL.

In specific embodiments, the aqueous solution has a volume of at least 900 μ L.

The method according to the current invention is an in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate, e.g., to producing an aqueous solution comprising (a
15 mixture of) DNA-containing and empty AAV particles, and/or the steps indicated elsewhere herein. Moreover, one or more of said steps may be performed by automated equipment.

The present invention also relates to a use of the method according to the present invention, for a purpose selected from the group consisting of producing an AAV
20 particle, purifying an AAV particle, removing impurities from a preparation of an AAV particle, and release of recombinant AAV particle preparations for therapeutic application, especially real-time release.

Specifically, one aspect of the current invention is the use of the transverse nuclear magnetic spin relaxation time T_2 for the determination of the loading status of
25 AAV particles. In one embodiment, the loading status is the ratio of DNA-containing AAV particles to empty AAV particles. In one embodiment, the loading status is the fraction of DNA-containing AAV particles in a sample. In one embodiment, the loading status is the concentration of DNA-containing AAV particles in a sample.

30 Thus, one preferred aspect of the current invention is a method for determining the concentration of DNA-containing AAV particles in a sample,

wherein the method comprises the following steps:

- 25 -

a) determining the transverse nuclear magnetic spin relaxation time T_2 of the protons of the water molecules in an aqueous solution comprising a mixture of DNA-containing and empty AAV particles by nuclear magnetic resonance (NMR), and

5 b) determining the concentration of DNA-containing AAV particles in the aqueous solution with the transverse nuclear magnetic spin relaxation time T_2 determined in step a) based on a calibration function,

wherein the AAV particle is of the AAV2 or AAV6 or AAV8 serotype,

10 wherein the aqueous solution is a phosphate buffered saline solution further comprising about 0.001 % (w/v) of a poloxamer or polysorbate and optionally further comprising about 100 mM of a citrate,

wherein the total concentration of AAV particles in the aqueous sample is at least $5 \cdot 10^{12}$ particles per mL,

15 optionally wherein i) the calibration function is a linear function or second order polynomial function if the aqueous sample comprises up to 80 % of DNA-containing AAV particles, or ii) the calibration function is a linear function or a second order polynomial function if the aqueous solution comprises up to 100 % of DNA-containing AAV particles, or iii) the calibration function is a linear function for aqueous solutions comprising between 0 % and less than 20 80 % DNA-containing AAV particles and a second order polynomial function for aqueous solutions comprising between 80 % and 100 % DNA-containing AAV particles,

25 optionally wherein the determining in step a) is performed at a temperature, a total concentration of AAV particles in the aqueous solution, a magnetic field strength, a resonance frequency, a buffer condition, optionally a detergent concentration, and optionally a salt concentration at which the relative difference of the transverse nuclear magnetic spin relaxation time T_2 determined with 100 % empty AAV particles and determined with 100 % DNA-containing AAV particles is at least 10 %.

30 Thus, one preferred aspect of the current invention is a method for determining the fraction of DNA-containing AAV particles in a sample,

wherein the method comprises the following steps:

- 26 -

a) determining the transverse nuclear magnetic spin relaxation time T_2 of the protons of the water molecules in an aqueous solution comprising a mixture of DNA-containing and empty AAV particles by nuclear magnetic resonance (NMR), and

5 b) determining the fraction of DNA-containing AAV particles of the total number of AAV particles in the aqueous solution with the transverse nuclear magnetic spin relaxation time T_2 determined in step a) based on a calibration function,

wherein the AAV particle is of the AAV2 or AAV6 or AAV8 serotype,

10 wherein the aqueous solution is a phosphate buffered saline solution further comprising about 0.001 % (w/v) of a poloxamer or polysorbate and optionally further comprising about 100 mM of a citrate,

wherein the total concentration of AAV particles in the aqueous sample is at least $5 \cdot 10^{12}$ particles per mL,

15 optionally wherein i) the calibration function is a linear function or second order polynomial function if the aqueous sample comprises up to 80 % of DNA-containing AAV particles, or ii) the calibration function is a linear function or a second order polynomial function if the aqueous solution comprises up to 100 % of DNA-containing AAV particles, or iii) the calibration function is a
20 linear function for aqueous solutions comprising between 0 % and less than 80 % DNA-containing AAV particles and a second order polynomial function for aqueous solutions comprising between 80 % and 100 % DNA-containing AAV particles,

25 optionally wherein the determining in step a) is performed at a temperature, a total concentration of AAV particles in the aqueous solution, a magnetic field strength, a resonance frequency, a buffer condition, optionally a detergent concentration, and optionally a salt concentration at which the relative difference of the transverse nuclear magnetic spin relaxation time T_2 determined with 100 % empty AAV particles and determined with 100 %
30 DNA-containing AAV particles is at least 10 %.

Thus, one preferred aspect of the current invention is a method for determining the ratio of DNA-containing AAV particles to empty AAV particles in a sample,

wherein the method comprises the following steps:

- 5 a) determining the transverse nuclear magnetic spin relaxation time T_2 of the protons of the water molecules in an aqueous solution comprising a mixture of DNA-containing and empty AAV particles by nuclear magnetic resonance (NMR), and
- b) determining the ratio of DNA-containing AAV particles to empty AAV particles in the aqueous solution with the transverse nuclear magnetic spin relaxation time T_2 determined in step a) based on a calibration function,

wherein the AAV particle is of the AAV2 or AAV6 or AAV8 serotype,

- 10 wherein the aqueous solution is a phosphate buffered saline solution further comprising about 0.001 % (w/v) of a poloxamer or polysorbate and optionally further comprising about 100 mM of a citrate,

wherein the total concentration of AAV particles in the aqueous sample is at least $5 \cdot 10^{12}$ particles per mL,

- 15 optionally wherein i) the calibration function is a linear function or second order polynomial function if the aqueous sample comprises up to 80 % of DNA-containing AAV particles, or ii) the calibration function is a linear function or a second order polynomial function if the aqueous solution comprises up to 100 % of DNA-containing AAV particles, or iii) the calibration function is a
- 20 linear function for aqueous solutions comprising between 0 % and less than 80 % DNA-containing AAV particles and a second order polynomial function for aqueous solutions comprising between 80 % and 100 % DNA-containing AAV particles,

- 25 optionally wherein the determining in step a) is performed at a temperature, a total concentration of AAV particles in the aqueous solution, a magnetic field strength, a resonance frequency, a buffer condition, optionally a detergent concentration, and optionally a salt concentration at which the relative difference of the transverse nuclear magnetic spin relaxation time T_2 determined with 100 % empty AAV particles and determined with 100 %
- 30 DNA-containing AAV particles is at least 10 %.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

5

Description of the Figures

- Figure 1** Dependency of T₂ on the DNA-loading of AAV particles of the serotype 2 (AAV2).
- Figure 2** Dependency of T₂ on the DNA-loading of AAV particles of the serotype 6 (AAV6).
- Figure 3** Dependency of T₂ on the DNA-loading of AAV particles of the serotype 8 (AAV8)

10

15

Examples

Materials and Methods

AAV particles

DNA-containing AAV particles as well as empty AAV particles of different serotypes were purchased from Virovek, Hayward, CA, United States of America, at a concentration of 2×10^{13} vg/mL. The concentration of vg/mL (virus genomes per mL) equals the concentration of vp/mL (virus particles per mL) as one genome is packaged in one particle. The DNA of the DNA-containing AAV particles comprised an expression cassette for green fluorescent protein with a CMV promoter (CMV-GFP):

20

AAV8-empty (Lot 19-564E) / AAV8-CMV-GFP (Lot 18-737) in 1x PBS buffer containing 0.001 % (w/v) Pluronic F-68, and 0.22 µm filter sterilized.

25

AAV2-empty (Lot 19-604E) / AAV2-CMV-GFP (Lot 17-600) in 1x PBS buffer containing 0.001 % (w/v) Pluronic F-68, 100 mM sodium citrate and 0.22 µm filter sterilized.

AAV6-empty (Lot 19-540E) / AAV2-CMV-GFP (Lot 19-718) in 1x PBS buffer containing 0.001 % (w/v) Pluronic F-68, 100 mM sodium citrate and 0.22 µm filter sterilized.

NMR

5 Transverse relaxation rates (R_2) or times (T_2) were recorded with a Bruker minispec mq20 spectrometer (20 MHz; Bruker BioSpin GmbH, Rheinstetten, Germany). The spectrometer was equipped with a 0.47 T magnet and a H2O-10-25AVGX4 probe. At least 4 acquisitions were measured for each sample with sample volumes of 900 µl at 20 °C. To determine T_2 or R_2 , signal decay was
10 followed for at least 5 sec.

Preparation of AAV samples

The samples were generated by mixing full and empty AAV2, AAV6 and AAV8, respectively, having the same concentration (2×10^{13} vg/ml) dissolved in the same aqueous solution comprising 1x PBS buffer containing 0.001 % (w/v) Pluronic F-
15 68 (for AAV8), and containing 0.001 % (w/v) Pluronic F-68 as well as 100 mM sodium citrate (for AAV2 and AAV6) at different ratios. Samples were measured without further longtime storage, e.g. $<0^\circ$ C.

Example 1

**Determination of the transverse relaxation times for different ratios of DNA-
20 containing and empty AAV particles**

DNA-containing AAV particles and empty AAV particles were mixed in aqueous solution (AAV2 and AAV6: 1x PBS buffer containing 0.001 % (w/v) Pluronic F-68, 100 mM sodium citrate; AAV8 without sodium citrate) to result in different full/empty ratios spanning the range from 0 % to 100 % DNA-containing AAV
25 particle. This has been done for AAV particles of the serotypes 2, 6 and 8. For the individual samples, the transverse relaxation times (T_2) were recorded as outlined in the Materials and Methods section above. The results are presented in the following Tables.

Table 1: T₂ values for differently DNA-containing AAV2 particle samples.

ratio full/empty	concentration of DNA-containing AAV2 particles	fraction	transverse relaxation time [ms]			
			experiment 1	experiment 2	average	standard deviation
0:100	0 vp/mL	0.00	1559.9	1566.9	1563.4	3.5
25:75	0.5*10 ¹³ vp/mL	0.25	1605.8	1568.0	1586.9	18.9
50:50	1*10 ¹³ vp/mL	0.5	1627.0	1602.4	1614.7	12.3
75:25	1.5*10 ¹³ vp/mL	0.75	1677.6	1662.4	1670.0	7.6
100:0	2*10 ¹³ vp/mL	1.00	1750.6	1770.3	1760.5	9.9

Table 2: T₂ values for differently DNA-containing AAV6 particle samples.

ratio full/empty	concentration of DNA-containing AAV6 particles	fraction	transverse relaxation time [ms]			
			experiment 1	experiment 2	average	standard deviation
0:100	0 vp/mL	0.00	1629.9	1614.2	1622.1	7.9
25:75	0.5*10 ¹³ vp/mL	0.25	1664.6	1629.1	1646.9	17.8
50:50	1*10 ¹³ vp/mL	0.5	1700.7	1673.8	1687.3	13.5
75:25	1.5*10 ¹³ vp/mL	0.75	1688.5	1688.5	1688.5	0.0
100:0	2*10 ¹³ vp/mL	1.00	1757.5	1786.1	1771.8	14.3

The value for the 75:25 ratio was excluded for the fitting calculation due to being deemed to be an experimental error.

5 **Table 3:** T₂ values for differently DNA-containing AAV8 particle samples.

ratio full/empty	concentration of DNA-containing AAV8 particles	fraction	transverse relaxation time [ms]
0:100	0 vp/mL	0.00	1704.2
25:75	0.5*10 ¹³ vp/mL	0.25	1668.0
50:50	1*10 ¹³ vp/mL	0.5	1635.6
75:25	1.5*10 ¹³ vp/mL	0.75	1608.9
100:0	2*10 ¹³ vp/mL	1.00	1497.3

The obtained transverse relaxation times were fitted using linear and second order polynomial functions. The results are presented in the following Table 4.

- 31 -

Table 4: Fitting results. For the serotype 6 the fitting is shown including (in brackets) and excluding the data for the 75:25 ratio.

AAV serotype	linear fitting	
	function	R ² value
2	$1.9088 \cdot x + 1543.7$	0.9208
6	$1.5298 \cdot x + 1615.1$ ($1.3646 \cdot x + 1615.1$)	0.9905 (0.8984)
8	$-1.8916 \cdot x + 1717.4$	0.9026

AAV serotype	2 nd order polynomial fitting	
	function	R ² value
2	$0.0184 \cdot x^2 + 0.0642 \cdot x + 1566.7$	0.996
6	$0.0047 \cdot x^2 + 1.0479 \cdot x + 1620.9$ ($0.0089 \cdot x^2 + 0.4749 \cdot x + 1626.2$)	0.9987 (0.9318)
8	$-0.0166 \cdot x^2 - 0.2333 \cdot x + 1696.7$	0.9633