Purification Process for Biological Molecules such as Plasmid DNA using Anionic Exchange Chromatography

FIELD OF THE INVENTION

[0001] The present invention provides new, improved methods for the purification or isolation of a biological molecule of interest, such as plasmid DNA (pDNA). The method includes the simple and effective removal of impurities such as RNA, genomic DNA, proteins, cellular fractions, or combinations thereof. The novel methods of the present invention are particularly suitable for large-scale production plants and provide for purified pDNA with excellent quality and good yields, while also allowing for faster processing times and reduced costs.

[0002] The purified biological molecule, in particular the pDNA obtained by the method of the present invention is particularly suitable for applications in areas such as synthetic transient production, ex vivo gene therapy, RNA therapeutics, DNA vaccines, DNA antibodies, non-viral gene therapy, and viral vectors. The purified biological molecules obtained via the method of the present invention may also be used, for example, in vaccines, viral therapy, gene and cell therapy, production of molecules such as RNA and polypeptides in vivo or in vitro, or in chimeric antigen receptor (CAR) T-cell therapy.

[0003] The present invention also describes the novel use of a tulip-shaped vessel which may be favorably used during the clarification of a sample comprising the biological molecule of interest. The tulip-shaped vessel or tank may be advantageously used during phase separation for separating solid components from the mixture containing the biological molecule. More specifically, the present invention provides for the novel use of a tulip-shaped vessel or tank for harvesting the lower (i.e. denser) phase of the mixture containing the biological molecule during phase separation.

BACKGROUND OF THE INVENTION

[0004] Plasmid DNA is a small molecule physically separated from chromosomal DNA and can replicate independently. Plasmid DNA typically exists in three different conformations: super-coiled pDNA, open-circular pDNA, and linear pDNA. Given the various therapeutic applications of pDNA, including but not limited to, synthetic transient protein production, ex vivo gene therapy, RNA therapeutics, DNA vaccines, DNA antibodies, non-viral gene therapy, and viral vectors, it will be appreciated by those of skill in the art that improved methods of purification or isolation of pDNA are highly desirable.

[0005] Having regard to the high negative charge of pDNA, anion exchange chromatography is frequently used in methods for purifying or isolating pDNA. The anion exchange material contains positively charged groups. Thus, anion exchange chromatography separates molecules such as proteins contained in solution according to their charges through the use of an anion exchange material. As those of skill in the art will appreciate, the concentration of the salts used during anion exchange chromatography will vary depending upon the substance that is to be eluted. Examples of typical anion exchange materials include various types of anion exchange membranes, beads, and resins. The proteins bound to the anion exchange material are then eluted through the use of a gradient of increasing concentrations of salt included with an appropriate buffer solution. Thus, in known anion exchange chromatography methods, the loading of the sample is typically carried out with a buffer having a low salt concentration, and the salt concentration is subsequently increased during the elution step.

[0006] When purifying pDNA using anion exchange chromatography, particularly on an industrial scale, there are several upstream steps that typically occur prior to loading the solution containing pDNA on the anion exchange column. These steps normally include fed-batch fermentation, cell harvesting and washing, continuous cell lysis, neutralization, RNA removal, flocculate removal depth filtration, plasmid concentration and diafiltration. Subsequently, the pDNA purification process typically includes the use of anion exchange chromatography (AEX), which includes binding the pDNA on the anion exchange material using a salt having low conductivity, followed by elution of the pDNA with a salt having higher conductivity, tangential flow filtration with buffer exchange, and then subjecting the pDNA containing fractions to a further purification step to further enrich super-coiled pDNA, e.g. by PlasmidSelect Xtra® (PSX) chromatography (available, e.g. from GE Healthcare Technology), often followed by a further tangential flow filtration step with a buffer exchange using an end fill buffer. In these known methods for purifying or isolating pDNA using anion exchange chromatography, the elution step is typically achieved by gradually increasing the salt concentration in the elution buffer.

[0007] Following the initial binding and elution steps, further steps are required in order to obtain the pDNA in acceptable purity and yield, as mentioned above. Even further, existing methods of purifying pDNA are known to result in poor selectivity and poor recovery. The known methods are thus unable to provide efficient and cost-effective separation of the pDNA. It is also worth noting that many of these known methods suffer from the disadvantage of using PEG or other additives, which may not be desired in the preparation of plasmid DNA, as they require additional separation, disposal and quality control steps. These can be difficult, more time consuming and more expensive. As these additional steps add costs to the purification process and increase the time necessary for purification, there exists a need for a method for purification or isolation of pDNA that eliminates the need for many of the additional steps during purification, thereby reducing the amount of time required for purification, without compromising the purity or yield of the pDNA.

[0008] As will be appreciated by those of skill in the art, yet another drawback to known processes for the purification of biomolecules (such as pDNA) is the difficulty to apply such methods in large-scale production, particularly large-scale production of pharmaceutical-grade pDNA. There are many existing constraints on large-scale production, for example personal safety issues and hazardous waste considerations. Further, the application of bench-scale purification processes of pDNA on a larger scale almost always results in decreased yield of the purified pDNA. Given these limitations, it would be desirable to provide for a method of purification or isolation of pDNA that not only provides pDNA with improved purity and in high yield, but that may also be used for large-scale production.

SUMMARY

[0008a] In an aspect, there is provided a method for isolating or purifying a plasmid DNA (pDNA) of interest from a mixture, the method comprising:

Contacting the mixture with an anion exchange material in the presence of a solution comprising a kosmotropic salt at a concentration that allows selective binding of the pDNA of interest to the anion exchange material;

wherein the kosmotropic salt is selected from the group consisting of: ammonium sulfate, ammonium citrate, ammonium phosphate, potassium phosphate, sodium citrate, sodium phosphate, and mixtures thereof;

wherein the concentration of the kosmotropic salt is greater than 0.5M; and

eluting the pDNA of interest with an eluent comprising a chaotropic salt at a concentration that provides an eluant containing the pDNA;

optionally wherein

(i) the fraction(s) comprising said pDNA in the eluant is/are collected and/or

(ii) said pDNA of interest is isolated from any one or all of the collected fractions; and/or

(iii) the method is conducted in the absence of organic solvents, detergents, glycols, hexamine cobalt, spermidine, and/or polyvinylpyrrolidone.

[0009] An example of the present disclosure relates to a method for isolating or purifying a biological molecule of interest from a mixture, the method comprising contacting the mixture comprising the biological molecule of interest with an anion exchange (AEX) material in the presence of a solution comprising a salt at a concentration that allows selective binding of the biological molecule of interest to the anion exchange material; and eluting the biological

molecule of interest with an eluent comprising a salt at a concentration that provides an eluant containing the purified biological molecule. In some examples, the fraction or fractions comprising said biological molecule of interest in the eluant is/are optionally collected. The purified biological molecule of interest may in some instances be subsequently isolated from any one or all of the collected fractions.

[0010] In some examples, the salt used at a concentration that allows selective binding of the biomolecule of interest to the AEX material is a kosmotropic salt, for example ammonium citrate, ammonium sulfate, ammonium phosphate, potassium phosphate, sodium citrate, sodium phosphate, or mixtures of said salts.

[0011] In some examples, the salt that is present in the eluent at a concentration that provides an eluant containing the purified biological molecule is a chaotropic salt, such as ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, guanidinium hydrochloride, or mixtures thereof.

[0012] The biological molecule to be purified by the methods of the disclosure is typically a highly polar / charged biomolecule such as a nucleic acid. It was found that the novel method described herein is particularly suitable for a fast and highly effective purification of plasmid DNA, in particular pharmaceutical-grade super-coiled plasmid DNA (sc pDNA), which must be free from bacterial genomic DNA, RNA, protein and endotoxins.

[0013] In another example, the present disclosure provides an advantageous sample preparation step that can be advantageously used before contacting the mixture containing the biomolecule of interest with the AEX material.

[0014] In this example, solid components (such as cell debris or other materials insoluble in the buffer system) of the mixture comprising the biological molecule of interest are conveniently separated by phase separation and/or filtration. In some examples, the removal of the solid components is achieved via a simple two-phase separation, wherein a buffer is added to increase the density of the mixture to be greater than about 1.1 kg/L. The increase in the density of the mixture causes the solid components to float on top, and the liquid part of the mixture (comprising the biological molecule of interest) can then be conveniently drained off, without requiring a filtration step (or at least offering a higher filtration capacity due to the significantly reduced solid material content, thereby allowing scalability of the process, i.e., making it useful for large-scale production). This sample preparation step can in some examples be advantageously combined with the novel purification method described herein.

[0015] Another, related example of the present disclosure relates to the use of a tulip-shaped vessel or tank for the above phase separation step. The tulip-shaped vessel was found to be particularly advantageous for the convenient and effective separation of the solid components from the liquid part of the mixture comprising the biological molecule of interest (such as plasmid DNA).

[0015a] In another aspect, there is provided a use of a tulip-shaped vessel for the method as defined above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] **Figure 1** depicts a schematic view of plasmid vector pUC19 used as a sample biomolecule to be purified in the working examples.

[0017] **Figure 2A - D** shows agarose gel electrophoresis (AGE) data on the results of a binding buffer screening using Sartobind Q as AEX material.

[0018] **Figure 3A - D** shows agarose gel electrophoresis (AGE) data on the results of an elution buffer screening using Sartobind Q as AEX material.

[0019] **Figure 4** depicts a chromatography profile of the AEX chromatography unit (Sartobind Q) zoomed in on the elution gradient.

[0020] **Figure 5** shows agarose gel electrophoresis data of fractions from the AEX chromatography step (Sartobind Q).

[0021] **Figure 6** shows AEX-HPLC traces from the Sartobind Q elution pool 1 as depicted in Figure 3 (dashed line) and the standard (solid line).

[0022] **Figure 7** shows SDS-PAGE analysis data of the load and elution pool from the Sartobind Q chromatography step.

[0023] **Figure 8** includes a schematic representation of the primary recovery process including the AEX chromatography step.

[0024] **Figure 9** depicts a chromatography profile of the AEX unit using Sartobind Q.

DETAILED DESCRIPTION OF THE INVENTION

[0025] As will be appreciated by those of skill in the art, in view of the various therapeutic applications for plasmid DNA, developing a simple, and scalable method for purifying plasmid DNA with excellent purity and in high quantity is highly desirable, particularly since many known isolation/purification steps that are typically more suited for small-scale production, such as laboratory and bench-scale production, cannot adequately be translated on a larger scale.

[0026] The present invention provides methods for isolation or purification of a biological molecule, for example plasmid DNA. In particular, the present invention includes methods of purification or isolation of plasmid DNA, and even more specifically super-coiled plasmid DNA free from impurities, including open-circular pDNA, linear pDNA, endotoxins, RNA, lipopolysaccharides, genomic DNA, proteins, and/or combinations thereof via use of anion exchange chromatography under defined, unusual conditions. The methods of the present invention are particularly suitable for large-scale isolation or purification of biological molecules, such as plasmid DNA (in particular super-coiled plasmid DNA), in a limited number of steps, with high purity and excellent yields. In particular, the purification methods of the present invention can advantageously be used for large-scale production for volumes up to 10,000 liters fermentation working volume.

[0027] It will be appreciated that the terms “biological molecule of interest”, “biomolecule of interest”, “target molecule” are all used synonymously, referring to the molecule that is to be purified from other components or impurities present in the mixture subjected to the purification method.

[0028] Impurities include, but are not limited to, host cell proteins, endotoxin, host cell DNA and/or RNA. It will be appreciated that what is considered an impurity can depend on the context in which the methods of the invention are practiced. An "impurity" may or may not be host cell derived, i.e., it may or may not be a host cell impurity.

[0029] "Isolating" or "purifying" a biological molecule of interest (such as plasmid DNA) means enrichment of the target molecule from other components which the target molecule is initially associated with. Extents of desired and/or obtainable purification are provided

herein. Preferably, the methods of the invention result in an about five-fold enrichment, preferably an about 10-fold enrichment, preferably an about 20 fold enrichment, preferably an about 50 fold enrichment, preferably an about 100 fold enrichment, preferably an about 200 fold enrichment, preferably an about 500 fold enrichment, preferably an about 1000 fold enrichment. Alternatively, the degree of purification may be expressed as a percentage of the first component with respect to another component, or with respect to the resultant preparation. Examples of such percentages are provided herein.

[0030] The inventors surprisingly found that it is possible to purify biological molecules of interest, such as (super-coiled) plasmid DNA, in a quick, simple and straightforward manner. This is achieved by contacting the mixture comprising the biomolecule of interest with an anion exchange (AEX) chromatography material under conditions that yield highly selective binding of the biological molecule to be purified, while the other components in the mixture do not bind to the AEX material.

[0031] In this regard, the inventors found that loading the mixture comprising the biomolecule to be purified with the AEX material in a solution having a rather high concentration of certain salts results in a highly specific binding of the desired biomolecule to the AEX material, i.e. most components in the solution are not bound to the AEX material. These loading conditions are believed to be rather unusual for AEX chromatography, wherein the sample to be purified is typically contacted with the AEX material under conditions of low ionic strength, and subsequently eluted by (gradually or stepwise) increasing salt concentration/ionic strength of the elution buffer. In other words, the specific loading conditions identified by the present inventors allow an effective enrichment of the targeting molecule already during the loading phase of the AEX chromatography step, with further purification possible during properly selected elution conditions, as will be described in detail herein below.

[0032] Thus, the AEX chromatography step, when employed under the conditions identified by the present inventors, allows separating the desired biomolecule from essentially all unwanted contaminants in a single purification step. In fact, the conditions found by the inventors even allow to dispense with a number of preparatory, pre-purification steps commonly applied in the purification of the biomolecule of interest due to its effective, and highly selective binding of the target molecule to the AEX material, as many of the contaminants simply do not bind under those carefully selected conditions, as will be explained in greater detail herein below.

[0033] The present invention generally relates to a method for isolating or purifying a biological molecule of interest from a mixture, wherein the method comprises contacting the mixture containing the biological molecule of interest with an anion exchange (AEX) material in the presence of a solution comprising a salt at a concentration that allows selective binding of the biological molecule of interest to the anion exchange material; and eluting the

biological molecule of interest from the AEX material with an eluent comprising a salt at a concentration that provides an eluant containing the purified biological molecule of interest.

[0034] "Selective binding" in the context of the present invention means that the large majority of the other components in the mixture do not bind to the AEX material when contacted in a solution comprising the salt at the appropriate concentration. One of skill in the art will appreciate that in biology (and chemistry) binding, elution, etc., will never be 100% exclusive, but for the purposes of the present invention, "selective binding" means that at least 70%, 80%, 85%, 90%, or even 95% of the other components ("contaminants") in the presence of the salt solution will not bind to the AEX material upon contact. For the sake of convenience, the contacting of the mixture with the AEX material may also be referred herein as a "loading step" (as commonly used in column chromatography, although the present method is of course not limited to column chromatography), and the salt solution including the mixture to be purified is also referred as "loading buffer" or "loading solution". The salt solution itself may also be referred to herein as "binding buffer".

[0035] Typically, the fraction or fractions comprising the purified biological molecule of interest in the eluant is/are collected, although this is not always necessary (for example in analytical purification runs where the concentration / purity of the molecule of interest can be measured directly in solution, e.g. by UV spectroscopy). Likewise, in most cases, the biological molecule of interest is then isolated from any one or all of the collected fractions for further use, as outlined herein below.

[0036] For most biomolecules, isolation does not necessarily mean precipitation and drying, but rather keeping the purified molecule in a, typically buffered, solution that ensures stability against degradation, e.g. in long term storage. The isolation may thus also include concentration, i.e. removal of part of the solvent to increase the concentration of the target molecule. As is well-known in the art, solutions comprising a biomolecule (be it proteins or nucleic acid based biomolecules) can in many cases be advantageously stored in a frozen solution, although in some instances, solvent may also be removed, e.g. by lyophilization.

[0037] AEX chromatography is typically characterized by loading the mixture to be purified onto the AEX material in a buffer comprising a salt/buffer with low ionic strength, i.e. at rather low salt concentrations. These conditions allow highly polar and/or charged molecules to bind to the charged groups on the AEX material, while the components having lower polarity / hydrophilicity will not bind under such conditions. The molecule of interest is then typically eluted from the column by successively increasing the concentration of the salt/buffer so that the increased number of ions compete with the binding of the molecules still bound to the AEX material. In order to achieve the desired high degree of purity, a gradient increasing the ionic strength of the elution buffer is typically used for purifications via AEX chromatography. In many cases, however, conventional AEX chromatography does not achieve sufficiently high purity because too many contaminants (especially charged / highly polar contaminants)

also bind to the AEX material during the loading phase, and are then eluted at the same time as the molecule of interest.

[0038] The inventors have surprisingly found that it is possible to carry out the loading step (i.e. the initial contact of the mixture with the AEX material) in solutions comprising certain salts at relatively high concentrations. In some embodiments, suitable salts to be used in said loading phase can be characterized as kosmotropic salts, i.e. they contribute to the stability and structure of water-water interactions ("order-making") by causing water molecules to favorably interact, which also leads to a stabilization of intramolecular interactions in macromolecules such as proteins. Ionic kosmotropes are characterized by strong solvation energy which typically leads to an increase of the overall cohesiveness of the solution (as often indicated by an increase of the viscosity and density of the solution). In contrast, chaotropic agents/salts (disorder-makers) have the opposite effect, disrupting water structure, increasing the solubility of nonpolar solvent particles, and destabilizing solute aggregates.

[0039] The inventors have found that using such a kosmotropic salt in the solution contacted with the AEX material at a sufficiently high concentration leads to a highly selective binding of certain biomolecules (particularly biomolecules having a high charge density such as plasmid DNA), while preventing other components from binding to said AEX material. Thus, in some instances, the "flow-through" of the loading step in a solution comprising a kosmotropic salt at a sufficiently high concentration will already contain most, if not essentially all contaminants from the mixture comprising the biomolecule of interest.

[0040] It will be appreciated that the property of being kosmotropic or chaotropic is usually ascribed to the specific ion and not a salt (which comprises a counter ion). Thus, it may well be that a salt may comprise an anion that is known to be kosmotropic, while the counter ion is rather known to be chaotropic. Such salts may (or may not) work in selectively binding the target molecule to the AEX material. However, those of skilled in the art will be able to find out whether a given salt will, at high concentrations, ensure the selective binding of the target molecule on the AEX material by simply carrying out routine experiments.

[0041] In most embodiments, elution of the target molecule from the AEX material can then be achieved by an eluant (i.e. a solution or buffer used to detach the components bound to the AEX material) comprising a chaotropic salt in a concentration sufficiently high to elute the biological molecule of interest. The elution phase is thus in many embodiments not materially different from conventional AEX chromatography, but due to the higher specificity in binding to the AEX material during the loading phase, the purity of the eluant is, often significantly, increased.

[0042] In some embodiments, the biological molecule to be purified by the method of the present invention is a nucleic acid (including DNA and RNA). For example, favorable results have been achieved with the method of the present invention when the biological molecule of

interest is plasmid DNA. Plasmid DNA often exists in different conformations: apart from the "native" super-coiled (SC) conformation, plasmid DNA may also be present in open circular (OC), or even in linear form. Since super-coiled plasmid DNA (sc pDNA) typically represents the desired (and commercially relevant) conformation for plasmid DNA, the biomolecule of interest in some embodiments of this aspect of the present invention is sc pDNA.

[0043] The plasmid DNA to be purified/isolated may typically comprise mammalian DNA, bacterial DNA, non-coding DNA, or viral DNA. In some instances, the plasmid DNA will comprise DNA capable of expressing a polypeptide of interest. The purification method will generally not depend on the size of the plasmid DNA, i.e. minivectors with only 350 bp as well as large plasmid vectors comprising up to 20 genes (35 kbp), and anything in-between may be effectively purified by the method described herein.

[0044] The method of the present invention allows the effective purification of plasmid DNA from other contaminants. Accordingly, in certain embodiments, the resulting **purity** of the plasmid DNA is at least 90%, or at least 93%, or at least 95%, or at least 97%, or at least 98% or at least 99%.

[0045] In some embodiments, the method even accomplishes the fractionation of super-coiled plasmid DNA from other plasmid DNA forms, such as linear plasmid DNA, open-circular plasmid DNA, and, optionally, other nucleic acid molecules.

[0046] Since the biomolecule of interest is typically obtained by biological processes (such as cell culture/fermentation processes), the mixture comprising the target molecule will in many embodiments include plasmid DNA (including super-coiled pDNA, open-circular pDNA, and linear pDNA), genomic DNA, RNA, lipopolysaccharides, endotoxins, proteins, or any combination of the foregoing components.

[0047] It will be appreciated that the concentration of the salt allowing selective binding of the biological molecule of interest to the AEX material during the loading step typically depends on the nature of the target molecule and the chosen AEX material. In some embodiments, highly selective binding of the target molecule has been achieved with salt concentrations of greater than about 0.5 M, or greater than 0.6 M, or greater than 0.8 M. In certain embodiments, the salt concentration of the solution allowing the selective binding of the target molecule is between about 0.5 M and about 4.0 M, or between about 0.8 M and about 2.0 M, or between about 1.0 M and about 2.0 M, such as 1.5 M.

[0048] The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 10%.

[0049] As noted earlier, kosmotropic salts at the above concentrations are preferred for the loading step of the present purification method. In any event, the ideal concentration of said salt for the loading step is dependent on the actual properties of the target molecule and the AEX material, but can be easily determined by one of skill in the art through routine experiments. Once a suitable concentration has been determined, it may be useful for consistent and reproducible binding to equilibrate the AEX material with a buffer comprising the kosmotropic salt at the same concentration as in the loading solution comprising the target molecule to be purified.

[0050] In any event, examples of suitable kosmotropic salts include, but are not limited to ammonium sulfate, ammonium citrate, ammonium phosphate, potassium phosphate, potassium citrate, sodium citrate, sodium phosphate, or mixtures thereof. For example, it was found that ammonium sulfate is a particularly useful kosmotropic salt for the loading phase when the target molecule to be purified is plasmid DNA.

[0051] As can be seen from Example 1, loading the mixture onto an optionally pre-equilibrated AEX membrane in a solution comprising high concentrations (> 0.5 M) of ammonium sulfate ensured highly selective binding of the pDNA to the AEX material, while RNA and other contaminants did not bind to the AEX membrane (cf. Fig. 2A and 2B). Accordingly, a useful concentration of ammonium sulfate in the loading solution is in certain embodiments about 0.5 M to about 2.0 M.

[0052] One of skill in the art will appreciate that other factors may exert an influence on the binding behavior of the components in the mixture. One of these factors is the pH of the solution. For biomolecules, the available pH range for purifications is naturally rather limited. In order to avoid pH conditions that may negatively affect the stability of the target molecule (e.g. by promoting hydrolysis of the biomolecule), pH values during the loading and elution of the biomolecule of interest typically range between pH 2 and pH 11, although pH values closer to neutral will generally be preferred, especially for biomolecules that are sensitive to degradation upon acidic or alkaline conditions. For example, in some embodiments, the pH of the solution comprising the kosmotropic salt contacted with the AEX material (i.e. the "loading buffer") is between pH 4 and pH 9, or between pH 5 and pH 8, or between pH 6 and pH 10, or between pH 6.0 to 8.0, e.g. around pH 7.0. Those of skill in the art will appreciate that optimal pH conditions may be determined by one of skill in the art by routine experiments.

[0053] It will be further appreciated that a pH change, for example during the elution step, may change the binding behavior/strength of the biomolecules bound to the AEX material, and may therefore be used in some embodiments for the controlled and selective release of the target molecule from the AEX material. However, in other instances, the binding of the target molecule to the AEX material will be essentially independent of the pH. For example, the binding of plasmid DNA did not change within a wide pH range (as low as pH 2).

[0054] With regard to the conditions selected for elution of the target molecule, the concentration of the salt, preferably a chaotropic salt, that provides an eluant containing the purified biological molecule of interest is typically about 0.25 M to about 4.0 M, or about 0.4 M to about 3.0 M, or about 0.5 M to 2.0 M. As is well-known in the art, the elution step may include a gradient elution by varying the concentration of the (preferably chaotropic) salt in the eluent, which typically involves increasing (continuously or step-wise) the concentration of the salt in the eluent.

[0055] In some embodiments, the chaotropic salt for the elution step is selected from the group consisting of: ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, guanidinium hydrochloride, and mixtures thereof. A particularly useful salt, especially for the purification of plasmid DNA, is magnesium sulfate. In these embodiments, the concentration of chaotropic salt, e.g., magnesium sulfate or sodium chloride is typically at least about 0.5M, and may in some instances range from 0.5 M to about 2.0 M or from about 0.5 M to about 1.0 M.

[0056] Again, as in the case of the kosmotropic salt contained in the loading buffer, the chaotropic salt and its optimal concentration for the elution of the target molecule depends on the nature of the biological molecule of interest and the specific AEX material chosen for the purification, and can be easily determined by one of skill in the art through routine experimentation (see Examples). Since the AEX material can typically be reused for another purification run, it may be useful to add a high salt concentration elution at the end (e.g. with about 2.0 to 4.0 M NaCl) which will strip essentially any bound material from the AEX material.

[0057] In principle, any available AEX materials may be used in the context of the method of the present invention. For example, the method can be applied with weak and with strong AEX set-ups, at high and low ligand density, with different bead chemistries and/or linkers. Suitable AEX materials are for example available as an anion exchange membrane, an anion exchange resin, a three-dimensional microporous hydrogel structure, a packed bed of superporous beads, macroporous beads, a monolith, agarose beads, cross-linked agarose, silica beads, large pore gels, methacrylate-based beads, polystyrene-based beads, cellulose-based beads, dextran-based beads, bisacrylamide-based beads, polyvinylether-based beads, ceramic-based beads, or polymer-based beads. Examples of commercially available AEX materials include, among others, Sartobind Q®, Mustang Q®, Mustang E®, Poros XQ®, Poros HQ®, Nuvia Q®, Capto Q®, Bakerbond PolyQuat® , DEAE Sepharose®, NH2-750F®, Q Sepharose®, Giga Cap® Q-650M, Fractogel® EMD COO, NatriFlo® HD-Q, and 3M™ Emphaze™ AEX Hybrid Purifier (all registered trademark names).

[0058] Particularly for larger, industrial scale purifications, the method may be carried out using AEX membrane chromatography or resin bed chromatography. In some

embodiments, the anion exchange membrane has an average pore size of about 3.0 μm to about 5.0 μm, preferably the average pore size is about 3.0 μm.

[0059] Alternatively, the method may be carried out using AEX column chromatography. In these embodiments, the anion exchange resin has preferably an average particle diameter from about 30 μm to about 300 μm.

[0060] In any event, it will be appreciated by those of skill in the art that the method described herein does not require the AEX material to be in a specific form, i.e. other AEX materials in whatever form, such as the other alternatives mentioned above, may also be employed as the purification agent in the purification method described herein.

[0061] Since the method provides excellent purification abilities, it may typically be carried out in the absence of any organic solvents, detergents, glycols, hexamine cobalt, spermidine, and / or polyvinylpyrrolidone, thereby no longer necessitating removal of such agents before providing the final target molecule in isolated form.

[0062] While the AEX purification step represents the main aspect of the present invention, it will be appreciated that a purification method for a biomolecule of interest will typically include further method steps, including steps carried out prior to the AEX chromatography step, but also, optionally, additional steps carried out subsequent to the AEX chromatography step.

[0063] As noted earlier, biological molecules of interest are often obtained from cells grown in a so-called cell culture. The biological molecule of interest may in certain cases be obtained from the cell culture in a rather straightforward way when the molecule is secreted by the cells into the surrounding cell culture medium. However, in most cases the target molecule will need to be released from the cells by destroying the cells via cell lysis, which may be accomplished by physical and/or chemical means, as is well-known in the art. Cell lysis will typically yield the target molecule together with a large number of host cell derived water-soluble and insoluble contaminants, such as cell membranes, cell organelles, genomic DNA, RNA and host cell proteins. Thus, a purification of a target molecule will typically require removal of these contaminants, where in particular the solid (i.e. non-soluble) contaminants are removed prior to the AEX chromatography step, typically by mechanical means.

[0064] Accordingly, in some embodiments of the present invention the method further comprises separating solid components from the mixture comprising the biological molecule of interest prior to contacting the mixture with an AEX material.

[0065] The removal of solid components from the mixture to be purified may for example be achieved by filtration or phase separation. In some instances, this method step achieves

removal of solid components via a two-phase separation, such as a solid-liquid phase separation.

[0066] In order to achieve a better separation, the mixture comprising solid components may in some embodiments be modified, for example by increasing the density of the mixture, for example to a density of greater than about 1.1 kg / L. This increase in the density of the mixture results in the solid components (such as cell debris or other precipitated matter) floating on top of the mixture. The solution comprising the biological molecule of interest, such as plasmid DNA, can then be conveniently drained off from the bottom part of the vessel / tank containing the mixture. The adjustment of the density, such as to at least about 1.1 kg / L, therefore yields a mixture where no filtration is required to obtain a clarified mixture that can subsequently be subjected to AEX chromatography. The density of the solution can generally be increased by a variety of operations. For example, a water-soluble salt, a carbohydrate (e.g. glucose, sucrose, glycerol, etc), urea, or other components that increase the density of an aqueous solution. In particular in the context of the purification method of the present invention, it may however be advantageous to employ a kosmotropic salt that is also suitable for the loading step of the AEX chromatography step, such as any of the exemplary kosmotropic salts described herein above.

[0067] Alternatively, phase separation techniques to remove insoluble contaminants may also be achieved by modifying the density of the mixture in other ways. For example, by adding alcohols (e.g. ethanol, methanol, isopropanol, etc) to the mixture the density would be decreased, causing the solid components to sediment to the bottom of the vessel or tank (optionally sedimentation could be assisted with centrifugation). Another option would be to add liquid non-aqueous compounds to the mixture so as to establish a liquid/liquid two-phase system, e.g., a PEG/salt two-phase system where the insoluble particles will be in the PEG phase and the target molecule (such as plasmid DNA) in the salt phase.

[0068] Furthermore, it will be understood that a light filtration, for example a depth filtration, may in certain cases still be applied for either the liquid, drained-off part of the mixture and/or for the final remaining volume of the mixture (to maximize yields). The optional additional filtration step ensures removal any remaining solid particles in the liquid part of the mixture. In any event, it will be appreciated that such a filtration will be much quicker and will not clog the filter due to the much lower solid content in the mixture. Depth filtration devices are commercially available, for example under the name Clarisolve® depth filter from Merck-Millipore.

[0069] Since this method step can also be used in the preparatory phase of other purification methods, another aspect of the present disclosure relates to the above two phase separation per se, i.e., not or not necessarily followed by the AEX chromatography step of the present invention.

[0070] The inventors have also found that a tulip-shaped vessel or tank is particularly useful for the removal of solid components in the mixture, particularly when the density is adjusted to at least about 1.1 kg / L which ensures that the solid components will float on top of the solid/liquid two phase mixture, i.e. where the diameter of the vessel/tank is greater that in the bottom part of the vessel from which the liquid phase is drained (see, for example the tulip-shaped vessel in **Figure 8**). Accordingly, the use of a tulip-shaped tank or vessel for the above-described two phase separation represents another aspect of the present disclosure.

[0071] The above-described two-phase separation represents a highly effective, fast, reproducible and economic method to prepare the solution comprising the biological molecule of interest for any subsequent purification step, such as the AEX chromatography step of the present invention. Particularly when the density adjustment is already accomplished with a relatively high concentration of the kosmotropic salt as used in the subsequent AEX chromatography step (e.g., ammonium sulfate), the two-phase separation may be the only step (possibly combined with a depth filtration prior to contacting the mixture with the AEX material) required after harvesting the cell supernatant. Since some of the kosmotropic salts act as a buffer, another advantage of adjusting the density of the mixture with said kosmotropic salts is that less neutralization buffer (to prevent possible degradation of the target molecule) is required immediately after cell lysis.

[0072] Moreover, the convenient removal of the solid components by simple draining off of the denser solution containing the target molecule is highly advantageous for the subsequent (though optional) depth filtration, not the least since the required filter area is greatly reduced as only a small fraction of the insoluble particles are processed over the filter.

[0073] The AEX chromatography method of the present invention may in some embodiments contain further steps after elution of the purified biological molecule of interest. For example, in some cases, the method further comprises the step of precipitating the biological molecule of interest from the eluant. Precipitation may be achieved by a variety of of methods generally known to the art, such as changing the pH of the eluant fractions comprising the target molecule, or adding antisolvents or other additives to the eluant, thereby causing the target molecule to precipitate from the eluant.

[0074] After precipitation, the method may in some instances further comprise filtering the eluant by tangential-flow filtration to isolate the precipitated biological molecule. For example, in some cases, the average pore size of the filtration membrane used in the tangential-flow filtration step will be ≤ 0.45 µm or even ≤ 0.2 µm.

[0075] In some instances, the method may further include the lyophilization of the purified biological molecule of interest, optionally in the presence of a carbohydrate that may stabilize the plasmid DNA in lyophilized form during storage. Many mono- or disaccharides can be used for said purpose as is generally known in the art.

[0076] In other embodiments, the method will further include dilution, concentration, or buffer exchange of the fraction(s) comprising the purified target molecule. In certain cases, the target molecule may be modified chemically (e.g., biotinylation, methylation, acetylation), or cut into smaller pieces (e.g. digestion by restriction enzymes in the case of nucleic acids).

[0077] Depending on the specific purification problem, and source for the target molecule, it may in some cases be useful to further collect the flow-through from the loading step (i.e. the step of contacting the mixture with an AEX material in the presence of the solution comprising the salt at a concentration that allows selective binding of the biological molecule to the anion exchange material).

[0078] As discussed herein, the AEX chromatography step is capable of selectively binding a biomolecule of interest to the AEX material, i.e. most of the unwanted components in the mixture will not bind to the AEX material under these loading conditions. For example, for purification of plasmid DNA from a cell culture, the flow-through may comprise RNA, genomic DNA, proteins, cellular fractions, or combinations thereof. In some embodiments, the flow through will comprise substantially all (i.e. at least 80%, at least 90%, or even at least 95%) RNA that was present in the mixture contacted with the AEX material.

[0079] Purification may in some instances be improved by further including a washing step. Thus, in some embodiments the method will further comprise washing the AEX material with a washing buffer solution prior to the elution of the biological molecule of interest. In some instances, washing may be continued with additional clean “loading” buffer (i.e. typically comprising a kosmotropic salt at relatively high concentration). In other instances, a washing buffer solution may comprise a chaotropic salt at a concentration lower than the concentration required for the elution of the biological molecule of interest.

[0080] In these cases, the washing buffer may also still comprise the kosmotropic salt (possibly in lower concentration than in the loading buffer), or it may only comprise the chaotropic salt. The chaotropic salt for the washing buffer is preferably selected from the group consisting of: ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, and mixtures thereof. In particular when the chaotropic salt has no buffer capacity, the washing buffer may further comprise a suitable buffer substance to maintain the pH at the desired level. For simplification, the washing buffer will in many cases comprise the same chaotropic salt as in the eluent, although this may not be required in terms of purification efficiency.

[0081] It will be further appreciated that, particularly when the purification of the target molecule must yield the product at a very high level of purity, the method of the present invention may further comprise an additional purification step, which typically follows the step of eluting and optionally isolating the biological molecule from the AEX chromatography step. In some embodiments where the target molecule to be purified is super-coiled plasmid DNA, the second purification step may therefore involve the use of a thiophilic aromatic adsorption

chromatography medium, available for example from GE Healthcare Life Sciences under the trade name PlasmidSelect Xtra®, or in short "PSX"). This particular resin is known to have excellent selectivity to separate super-coiled plasmid DNA from open circular and/or linear plasmid DNA, and may therefore be used for the final enrichment of the super-coiled conformation of pharmaceutical-grade DNA.

[0082] In some embodiments, the method will further comprise, after purification and isolation of a nucleic acid, such as plasmid DNA, a further step of using said nucleic acid for the expression of a polypeptide of interest encoded by said nucleic acid. The nucleic acid is in these instances preferably plasmid DNA. The polypeptide produced via the nucleic acid, preferably plasmid DNA, will typically be harvested and, optionally, purified. Finally, the method may in some cases even include the formulation of the polypeptide (obtained via expression of the purified nucleic acid) into a pharmaceutical composition which may optionally comprise one or more pharmaceutically acceptable excipients.

[0083] Alternatively, when the nucleic acid, such as the plasmid DNA, is intended for pharmaceutical use directly, the method may include the formulation of the nucleic acid into a pharmaceutical composition, which may again optionally comprise one or more pharmaceutically acceptable excipients. Plasmid DNA purified by the method of the present invention may be used as DNA-based vaccine or be used as a "prodrug" wherein the plasmid will then involve the cell`s transcription and translation apparatus to biosynthesize the therapeutic entity in situ.

[0084] In yet other embodiments, the method will further comprise the use of the purified plasma DNA for the production of RNA, including RNA-based drugs, for the production of shorter oligonucleotides such as siRNA or aptamers (short single-stranded nucleic acid segments (typically 20-60 nucleotides), or for the production of DNAzymes (ribozyme analogs with an RNA backbone replaced by DNA motifs that confer improved biological stability).

[0085] A preferred variant of the purification method involving AEX chromatography comprises, prior to said AEX chromatography step, the steps of cell harvesting and washing, cell lysis, neutralization, and flocculate removal prior to contacting the solution comprising the biological molecule of interest with the AEX material.

[0086] As noted above, the conditions identified for the AEX loading step allows for selective binding of plasmid DNA to the AEX material. Thus, RNA, which is typically also bound to the AEX material under standard, low salt conditions, does not or not substantially bind to the AEX material under the loading conditions described herein, and can thus be quantitatively removed from the target molecule in a single step.

[0087] The method described herein thus has the great advantage that RNA does not need to be removed by a calcium chloride ($CaCl_2$) precipitation step. Avoiding additional

purification steps are of course beneficial in terms of yield, costs and duration of the overall purification method. Accordingly, the method is in certain embodiments be further characterized in that it does not comprise a calcium chloride ($CaCl_2$) precipitation step.

[0088] The same is true for proteins which also do not bind to the AEX material under the conditions identified herein. Since open-circular pDNA, linear pDNA and genomic DNA all have different interaction strength with AEX ligands compared to super-coiled plasmid DNA, their levels can be greatly reduced during the AEX chromatography step. Accordingly, the method as described and claimed herein allows for the simple, quick, highly effective and economic purification of biomolecules of interest such as plasmid DNA involving essentially only a single chromatographic step involving materials having AEX ligands.

[0089] Many additional steps commonly needed in plasmid DNA purification procedures described in the prior art, such as buffer exchange, (multiple) depth filtrations, $CaCl_2$ precipitation or additional chromatographic steps, such as PSX chromatography, can be avoided (depending on the required content of sc pDNA). Finally, the method described herein allows scaling it for large production plants.

[0090] Having described the various aspects of the present invention in general terms, it will be apparent to those of skill in the art that many modifications and slight variations are possible without departing from the spirit and scope of the present invention. The present invention is furthermore described by reference to the following, non-limiting numbered embodiments and working examples.

1. A method for isolating or purifying a biological molecule of interest from a mixture, the method comprising:

   contacting the mixture with an anion exchange material in the presence of a solution comprising a salt at a concentration that allows selective binding of the biological molecule of interest to the anion exchange material; and

   eluting the biological molecule of interest with an eluent comprising a salt at a concentration that provides an eluant containing the purified biological molecule;

   optionally wherein the fraction(s) comprising said biological molecule in the eluant is/are collected and, further optionally, wherein said biological molecule of interest is isolated from any one or all of the collected fractions.

2. The method of embodiment 1, wherein the salt at a concentration that allows selective binding of the biological molecule to the anion exchange material is a kosmotropic salt.

3. The method of embodiment 1 or embodiment 2, wherein the salt at a concentration that provides an eluant containing the purified biological molecule is a chaotropic salt.

4. The method of embodiments 1 to 3, wherein the biological molecule to be purified is a nucleic acid.

5. The method of embodiment 4, wherein the nucleic acid is plasmid DNA (pDNA), optionally wherein the plasmid DNA is super-coiled plasmid DNA (sc pDNA).

6. The method of embodiment 5, wherein the plasmid DNA comprises mammalian DNA, bacterial DNA, non-coding DNA, or viral DNA; optionally wherein the plasmid DNA comprises DNA capable of expressing a polypeptide of interest.

7. The method of embodiment 5 or embodiment 6, wherein the purity of the purified and/or isolated plasmid DNA is at least 90%, or at least 95%, or at least 98%.

8. The method of any one of embodiments 1 to 7, wherein the mixture comprises super-coiled plasmid DNA, open-circular plasmid DNA, linear plasmid DNA, genomic DNA, RNA, lipopolysaccharides, endotoxins, proteins, or any combination thereof.

9. The method of any one of the preceding embodiments, wherein the concentration of the salt that allows selective binding of the biological molecule of interest to the anion exchange material is greater than about 0.5 M, optionally wherein the concentration of the salt that allows selective binding of the biological molecule to the anion exchange material is about 0.8 M to about 4.0 M.

10. The method of any one of the preceding embodiments, wherein the concentration of the salt that provides an eluant containing the purified biological molecule is about 0.25 M to about 4.0 M.

11. The method of any one of the preceding embodiments, wherein the elution step comprises a gradient elution by varying the concentration of the chaotropic salt in the eluent; optionally wherein the concentration of the chaotropic salt in the eluent is increased.

12. The method of embodiment 11, wherein the biological molecule is plasmid DNA and wherein the elution step includes fractionation of super-coiled plasmid DNA from other plasmid DNA forms such as linear plasmid DNA and open-circular plasmid DNA, and, optionally, other nucleic acid molecules.

13. The method of any one of embodiments 2 to 12, wherein the kosmotropic salt is selected from the group consisting of: ammonium sulfate, ammonium citrate, ammonium phosphate, potassium phosphate, sodium citrate, sodium phosphate, and mixtures thereof.

14. The method of embodiment 13, wherein the kosmotropic salt is ammonium sulfate.

15. The method of embodiment 14, wherein the concentration of ammonium sulfate is about 0.5 M to about 2.0M.

16. The method of any one of embodiments 2 to 15, wherein the solution comprising the kosmotropic salt has a pH in the range of about 5.0 to about 10.0, or of about 5.0 to about 8.0, or about 6.0 to about 8.0, or about pH 7.

17. The method of any one of embodiments 3 to 16, wherein the chaotropic salt is selected from the group consisting of: ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, guanidinium hydrochloride, and mixtures thereof.

18. The method of embodiment 17, wherein the chaotropic salt is magnesium sulfate.

19. The method of embodiment 18, wherein the concentration of magnesium sulfate is about 0.5M to about 1.0M.

20. The method of any one of the preceding embodiments, wherein the anion exchange material is selected from an anion exchange membrane, an ion exchange resin, a three-dimensional microporous hydrogel structure, a packed bed of superporous beads, macroporous beads, a monolith, agarose beads, cross-linked agarose, silica beads, large pore gels, methacrylate-based beads, polystyrene-based beads, cellulose-based beads, dextran-based beads, bisacrylamide-based beads, polyvinylether-based beads, ceramic-based beads, or polymer-based beads.

21. The method of embodiment 20, wherein the anion exchange material is an anion exchange membrane.

22. The method of embodiment 21, wherein the anion exchange membrane has an average pore size of about 3.0μm to about 5.0μm; preferably wherein the average pore size is about 3.0μm.

23. The method of embodiment 20, wherein the ion exchange resin has an average particle diameter from about 30 μm to about 300μm.

24. The method of any one of the preceding embodiments, wherein the method is conducted in the absence of organic solvents, detergents, glycols, hexamine cobalt, spermidine, polyvinylpyrrolidone.

25. The method of any one of the preceding embodiments, wherein the method is carried out using membrane chromatography.

26. The method of any one of embodiments 1 to 24, wherein the method is carried out using column chromatography.

27. The method of any one of the preceding embodiments, wherein the method further comprises separating solid components from the mixture comprising the biological molecule of interest prior to contacting the mixture with an anion exchange material in the presence of the solution comprising the salt at a concentration that allows selective binding of the biological molecule to the anion exchange material,

optionally wherein said removal of solid components is achieved by filtration or phase separation.

28. The method of embodiment 27, wherein the removal of solid components is achieved via two-phase separation, and wherein a buffer is added to increase the density of the mixture to be greater than about 1.1 kg/L.

29. The method of embodiment 27 or embodiment 28, wherein the buffer comprises a kosmotropic salt, preferably wherein the kosmotropic salt is ammonium sulfate.

30. The method of any one of embodiments 27 to 29, wherein the lower phase of the two-phase mixture having a higher density than the top phase is subjected to a depth filtration.

31. Use of a tulip-shaped vessel for the method of any one of embodiments 26 to 30.

32. The method of any one of the preceding embodiments, wherein the method further comprises the step of precipitating the biological molecule from the eluant.

33. The method of any one of the preceding embodiments, wherein the method further comprises the step of filtering the eluant by tangential-flow filtration to isolate the biological molecule.

34. The method of embodiment 33, wherein the average pore size of the filtration membrane used in the tangential-flow filtration step is ≤ 0.2 µm.

35. The method of any one of the preceding embodiments, wherein the method further comprises the step of lyophilization of the purified biological molecule of interest, optionally wherein the lyophilization is carried out in the presence of a carbohydrate.

36. The method of any one of the preceding embodiments, wherein the method further comprises the step of collecting a flow-through following the step of contacting the mixture with an anion exchange material in the presence of the solution comprising the salt at a concentration that allows selective binding of the biological molecule to the anion exchange material.

37. The method of embodiment 36, wherein the flow-through comprises RNA, genomic DNA, proteins, cellular fractions, or combinations thereof, preferably wherein the flow-through comprises RNA.

38. The method of any one of the preceding embodiments, wherein the method further comprises washing the anion exchange material with a washing buffer solution prior to the elution of the biological molecule of interest.

39. The method of embodiment 38, wherein the washing buffer solution comprises a chaotropic salt at a concentration lower than the concentration required for the elution of the biological molecule of interest, wherein the chaotropic salt is preferably selected from the group consisting of: ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, and mixtures thereof.

40. The method of any one of the preceding embodiments, wherein the method further comprises a further purification step following the step of eluting and optionally isolating the biological molecule;
optionally wherein the second purification step comprises use of a thiophilic aromatic adsorption chromatography medium having a selectivity that allows super-coiled plasmid DNA to be separated from open circular and/or linear DNA.

41. The method of any one of embodiments 4 to 40, wherein the method further comprises the step of using the nucleic acid to express a polypeptide of interest; preferably wherein the nucleic acid is plasmid DNA.

42. The method of any one of embodiments 4 to 41, wherein the method further comprises the step of using the nucleic acid to produce and harvest a polypeptide of interest; preferably wherein the nucleic acid is plasmid DNA.

43. The method of embodiment 42, wherein the method further comprises the step of formulating the polypeptide of interest into a pharmaceutical composition.

44. The method of any one of the preceding embodiments, wherein the method includes the steps of cell harvesting and washing, cell lysis, neutralization, and flocculate removal prior to contacting the resulting mixture comprising the biological molecule of interest with the anion exchange material.

45. The method of any one of the preceding embodiments, wherein the method does not comprise a $CaCl_2$ precipitation step to remove RNA.

EXAMPLES

Example 1: Screening of conditions for binding and elution with anion exchange material Sartobind Q for a sample plasmid DNA (pUC19)

[0091] All buffers used throughout the process were filtered through 0.2 µm filters before use. E. coli cells harboring the plasmid pUC19 (a schematic description of this plasmid DNA is found in Fig. 1) were grown, lysed and clarified through filtration methods as described in more detail below.

[0092] Cells were grown in small scale bioreactors at fed-batch condition to an optical density of 65 - 90, measured at 600 nm (OD_{600}). The cells were harvested by centrifugation (4'400 – 4'800, 45 minutes). The cells harvested at the end of cellular growth were resuspended in P1 buffer (50 mM Tris, 10 mM EDTA; pH 7.4). Then P2 buffer (0.2 M NaOH, 1% (w/v) SDS) was added in a 1:1 ratio (v/v), in order to perform alkaline lysis of the cells. The mixture was gently homogenized and left to rest at room temperature for 5 minutes. To stop cell lysis and neutralize the mixture, the appropriate volume of cold P3 buffer (5 M potassium acetate, acetic acid, 4 °C), approximately 25 % in volume of the other buffers, was added. After gentle homogenization, the conditioning buffer containing 3 M Ammonium sulfate was added in a 1:1 ratio (v/v), in order to change the density of the solution, thereby causing the cell debris to float on the upper part of the solution. The solution was then subjected to a final depth filtration (Clarisolve depth filter, Merck-Millipore, with pore sizes from 40 to 0.5 µm) to remove any remaining cell debris, as well as some precipitated genomic DNA and proteins. After filtration, the pH of the filtrated solution was adjusted to pH 7.0 by adding an appropriate volume of 1 M NaOH to the filtrate.

[0093] Screening of the binding and elution conditions were performed on a liquid handling robot (Freedom Evo 150; Tecan) and allowed 96 chromatography steps to be run in parallel. Sartobind Q 96 well-plates (Cat#: 99IEXQ42GC ----- V, Sartorius) were used for the screening. The load conditioning was performed by changing buffer with the help of Zeba Spin desalting plates (Cat# 89808, ThermoScientific) and the respective binding buffer. The Sartobind Q units were equilibrated with binding buffer before 2.0 mL of the load solution were applied. The membrane was washed with 1 mL of the respective binding buffer and 1 mL of wash buffer (20 mM Na-Phosphate, pH 7.5). Material was eluted by applying 1 mL of elution buffer followed by a strip step with 1 mL of 4 M NaCl. Sample analyses were performed by agarose gel electrophoresis (AGE), where quantities and qualities can be estimated.

a) Binding buffer screening

[0094] In a first round, several binding buffers at different concentrations were screened and the elution was performed with an elution buffer comprising 2 M NaCl in 20 mM Na-Phosphate at pH 7.5. Agarose gels were loaded with eluates according to **Table 1** below.

Binding buffer salts listed in the table were used in conjunction with 20 mM sodium phosphate at pH 7.0 unless indicated otherwise.

Table 1: Loading scheme of samples subjected to different binding buffer conditions.

Lane	Binding Buffer salts
1	DNA Ladder 1kb
2	MilliQ-H2O
3	250 mM Tetramethyl-ammonium Sulfate
4	500 mM Tetramethyl-ammonium Sulfate
5	750 mM Tetramethyl-ammonium Sulfate
6	1000 mM Tetramethyl-ammonium Sulfate
7	1250 mM Tetramethyl-ammonium Sulfate
8	250 mM Ammonium Citrate
9	500 mM Ammonium Citrate
10	750 mM Ammonium Citrate
11	1000 mM Ammonium Citrate
12	1250 mM Ammonium Citrate
13	1500 mM Ammonium Citrate
14	1750 mM Ammonium Citrate
15	2000 mM Ammonium Citrate
16	250 mM Ammonium Sulfate, pH 8.0
17	500 mM Ammonium Sulfate, pH 8.0
18	750 mM Ammonium Sulfate, pH 8.0
19	1000 mM Ammonium Sulfate, pH 8.0
20	1250 mM Ammonium Sulfate, pH 8.0
21	1500 mM Ammonium Sulfate, pH 8.0
22	1750 mM Ammonium Sulfate, pH 8.0
23	2000 mM Ammonium Sulfate, pH 8.0
24	250 mM Ammonium Sulfate, pH 7.0
25	500 mM Ammonium Sulfate, pH 7.0
26	DNA Ladder 1kb

Lane	Binding buffer salts
26	DNA Ladder 1kb
27	750 mM Ammonium Sulfate, pH 7.0
28	1000 mM Ammonium Sulfate, pH 7.0
29	1250 mM Ammonium Sulfate, pH 7.0
30	1500 mM Ammonium Sulfate, pH 7.0
31	1750 mM Ammonium Sulfate, pH 7.0
32	2000 mM Ammonium Sulfate, pH 7.0
33	250 mM Ammonium Sulfate, pH 6.0
34	500 mM Ammonium Sulfate, pH 6.0
35	750 mM Ammonium Sulfate, pH 6.0
36	1000 mM Ammonium Sulfate, pH 6.0
37	1250 mM Ammonium Sulfate, pH 6.0
38	1500 mM Ammonium Sulfate, pH 6.0
39	1750 mM Ammonium Sulfate, pH 6.0
40	2000 mM Ammonium Sulfate, pH 6.0
41	250 mM Ammonium Sulfate, pH 5.0
42	500 mM Ammonium Sulfate, pH 5.0
43	750 mM Ammonium Sulfate, pH 5.0
44	1000 mM Ammonium Sulfate, pH 5.0
45	1250 mM Ammonium Sulfate, pH 5.0
46	1500 mM Ammonium Sulfate, pH 5.0
47	1750 mM Ammonium Sulfate, pH 5.0
48	2000 mM Ammonium Sulfate, pH 5.0
49	250 mM Ammonium Phosphate
50	Load
51	DNA Ladder 1kb

Lane	Binding buffer salts
52	DNA Ladder 1kb
53	500 mM Ammonium Phosphate
54	750 mM Ammonium Phosphate
55	1000 mM Ammonium Phosphate
56	1250 mM Ammonium Phosphate
57	1500 mM Ammonium Phosphate
58	1750 mM Ammonium Phosphate
59	2000 mM Ammonium Phosphate
60	250 mM Potassium Citrate
61	500 mM Potassium Citrate
62	750 mM Potassium Citrate
63	1000 mM Potassium Citrate
64	1250 mM Potassium Citrate
65	1500 mM Potassium Citrate
66	1750 mM Potassium Citrate
67	MilliQ-H2O
68	250 mM Potassium Phosphate
69	500 mM Potassium Phosphate
70	750 mM Potassium Phosphate
71	1000 mM Potassium Phosphate
72	1250 mM Potassium Phosphate
73	1500 mM Potassium Phosphate
74	1750 mM Potassium Phosphate
75	2000 mM Potassium Phosphate
76	250 mM Sodium Citrate
77	DNA Ladder 1kb

Lane	Binding buffer salts
77	DNA Ladder 1kb
78	500 mM Sodium Citrate
79	750 mM Sodium Citrate
80	1000 mM Sodium Citrate
81	1250 mM Sodium Citrate
82	1500 mM Sodium Citrate
83	250 mM Sodium Phosphate
84	500 mM Sodium Phosphate
85	250 mM Ammonium Chloride
86	500 mM Ammonium Chloride
87	750 mM Ammonium Chloride
88	1000 mM Ammonium Chloride
89	1250 mM Ammonium Chloride
90	1500 mM Ammonium Chloride
91	1750 mM Ammonium Chloride
92	2000 mM Ammonium Chloride
93	250 mM Sodium Chloride
94	500 mM Sodium Chloride
95	750 mM Sodium Chloride
96	1000 mM Sodium Chloride
97	1250 mM Sodium Chloride
98	1500 mM Sodium Chloride
99	1750 mM Sodium Chloride
100	2000 mM Sodium Chloride
101	Load
102	DNA Ladder 1kb

[0095] Results of the binding buffer screening effort are shown in **Figures 2A to 2D**, wherein the upper and lower panel show the same gel after different exposure times (upper panel = shorter exposure time; lower panel = longer exposure time). The labeling on the right side of the gel depicts the bands of open circular (oc) and super coiled (sc) pUC19 plasmid DNA and RNA impurities within the agarose gel.

[0096] The results suggest that binding at high salt concentrations is feasible, particularly when using kosmotropic salts (e.g. ammonium citrate, ammonium sulfate, ammonium phosphate, potassium citrate, potassium phosphate, sodium citrate, sodium phosphate, etc.), and is essentially independent of the pH of the binding buffer. The results also show that, particularly at higher salt concentrations, binding of RNA is markedly reduced as compared to the load (cf., lanes 50 and 101) or does not bind at all (e.g., lanes 10 - 15).

b) Elution buffer screening

[0097] In a second round, several elution buffers were screened at different concentrations and the binding of the sample mixture was accomplished with a binding buffer comprising 20 mM Na-Phosphate and 1.5 M Ammonium Sulfate at pH 7.0. Agarose gels were loaded with eluates according to **Table 2** below. Elution buffer salts listed in the table were used together with 20 mM Na-Phosphate at pH 7.0 unless indicated otherwise.

Table 2: Loading scheme of samples subjected to different elution buffer conditions.

Lane	Elution buffer salts
1	DNA Ladder 1kb
2	250 mM Sodium Acetate
3	500 mM Sodium Acetate
4	750 mM Sodium Acetate
5	1000 mM Sodium Acetate
6	1250 mM Sodium Acetate
7	1500 mM Sodium Acetate
8	1750 mM Sodium Acetate
9	2000 mM Sodium Acetate
10	250 mM Ammonium Chloride
11	500 mM Ammonium Chloride
12	750 mM Ammonium Chloride
13	1000 mM Ammonium Chloride
14	1250 mM Ammonium Chloride
15	1500 mM Ammonium Chloride
16	1750 mM Ammonium Chloride
17	2000 mM Ammonium Chloride
18	250 mM Potassium Chloride
19	500 mM Potassium Chloride
20	750 mM Potassium Chloride
21	1000 mM Potassium Chloride
22	1250 mM Potassium Chloride
23	1500 mM Potassium Chloride
24	1750 mM Potassium Chloride
25	2000 mM Potassium Chloride
26	DNA Ladder 1kb

Lane	Elution buffer salts
26	DNA Ladder 1kb
27	250 mM Sodium Chloride
28	500 mM Sodium Chloride
29	750 mM Sodium Chloride
30	1000 mM Sodium Chloride
31	1250 mM Sodium Chloride
32	1500 mM Sodium Chloride
33	1750 mM Sodium Chloride
34	2000 mM Sodium Chloride
35	250 mM Magnesium Sulfate
36	500 mM Magnesium Sulfate
37	750 mM Magnesium Sulfate
38	1000 mM Magnesium Sulfate
39	1250 mM Magnesium Sulfate
40	1500 mM Magnesium Sulfate
41	1750 mM Magnesium Sulfate
42	2000 mM Magnesium Sulfate
43	250 mM Magnesium Chloride
44	500 mM Magnesium Chloride
45	750 mM Magnesium Chloride
46	1000 mM Magnesium Chloride
47	1250 mM Magnesium Chloride
48	1500 mM Magnesium Chloride
49	1750 mM Magnesium Chloride
50	Load
51	DNA Ladder 1kb

Lane	Elution buffer salts
52	DNA Ladder 1kb
53	2000 mM Magnesium Chloride
54	250 mM Magnesium Nitrate
55	500 mM Magnesium Nitrate
56	750 mM Magnesium Nitrate
57	1000 mM Magnesium Nitrate
58	1250 mM Magnesium Nitrate
59	1500 mM Magnesium Nitrate
60	MilliQ-H2O
61	20 mM Sodium Citrate, pH 2.0
62	20 mM Sodium Citrate, pH 2.5
63	20 mM Sodium Citrate, pH 3.0
64	20 mM Sodium Citrate, pH 3.5
65	20 mM Sodium Citrate, pH 4.0
66	20 mM Sodium Citrate, pH 4.5
67	20 mM Sodium Citrate, pH 5.0
68	20 mM Sodium Citrate, pH 5.5
69	50 mM Glycine, pH 2.0
70	50 mM Glycine, pH 2.5
71	50 mM Glycine, pH 3.0
72	20mM Sodium Acetate, pH 3.5
73	20mM Sodium Acetate, pH 4.0
74	20mM Sodium Acetate, pH 4.5
75	20mM Sodium Acetate, pH 5.0
76	50 mM Sodium Formate, pH 3.0
77	DNA Ladder 1kb

Lane	Elution buffer salts
77	DNA Ladder 1kb
78	50 mM Sodium Formate, pH 3.5
79	50 mM Sodium Formate, pH 4.0
80	50 mM Sodium Formate, pH 4.5
81	50 mM Na-Phosphate, pH 5.5
82	50 mM Na-Phosphate, pH 6.0
83	50 mM Na-Phosphate, pH 6.5
84	50 mM Na-Phosphate, pH 7.0
85	1% Tween 20
86	0.1% Tween 20
87	1% CTAB
88	0.1% CTAB
89	1% SDS
90	0.1% SDS
91	1% SDS
92	0.1% SDS
93	30% EtOH
94	30% IPA
95	15% DMSO
96	7 M urea
97	6 M GuHCl
98	0.8 M Arginine
99	0.8 M Lysine
100	0.25 M Histidine
101	Load
102	DNA Ladder 1kb

[0098] Results of the elution buffer screening effort are shown in Figure 3A to 3D, wherein the upper and lower panel show the same gel after different exposure times (upper panel = shorter exposure time; lower panel = longer exposure time). The labeling on the right side of the gel depicts the bands of open circular (oc) and super coiled (sc) pUC19 plasmid DNA and RNA impurities within the agarose gel.

[0099] The results show that plasmid DNA can be eluted from the AEX resin when using chaotropic salts (e.g. ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, etc.) even in cases where the conductivity of the elution buffer is significantly lower than the conductivity of the binding buffer. Moreover, most of the tested elution buffers elute sc pDNA better than oc pDNA, and do not elute RNA. However, it was observed that certain chaotropic salts, e.g. magnesium nitrate, preferentially eluted oc pDNA over sc pDNA (cf. Fig. 3C).

Example 2: pUC19 purification using Sartobind Q and magnesium sulfate for the elution with a phosphate buffer system

a) Purification procedure

[0100] All buffers used throughout the process were filtered through 0.2 µm filters. E. coli cells harboring the plasmid pUC19 were grown and lysed as described in Example 1.

[0101] The 150 mL solution containing pUC19 was conditioned with 150 mL conditioning buffer (40 mM Na-Phosphate, pH 7.0, 3 M Ammonium Sulfate). The chromatography unit was equilibrated with 20 column volumes (CVs) of 20 mM Na-Phosphate, 1.5 M Ammonium Sulfate, pH 7.0 at a flow rate of 4 CVs/min. The conditioned load was then applied to the anion exchange unit. The unit was washed by applying 20 CVs equilibration buffer followed by a second wash of 20 CVs of wash buffer 2 (20 mM Na-phosphate, 0.5 M magnesium sulfate, pH 7.0) which was found to remove RNA impurities. Plasmid DNA was then eluted by applying a gradient from wash buffer 2 to elution buffer (20 mM Na-Phosphate, 1.0 M Magnesium Sulfate, pH 7.0) in 25 CVs. Finally, the chromatography unit was stripped by applying 30 CVs of 4 M NaCl.

[0102] A representative chromatography profile of the anion exchange unit (Sartobind Q) is shown in **Figure 4** with a zoom-in on the elution gradient. Two pools were established: Pool 1 comprising fractions 1C2 to 2B4 (light grey box) and Pool 2 comprising fractions 1C4 to 2B2 (dark grey box).

b) Assessment of residual impurities

[0103] Fractions from the AEX chromatography step with Sartobind Q were analyzed by agarose gel electrophoresis (cf. **Table 3** below for the loading scheme of the agarose gel) and the results are shown in **Figure 5**.

Table 3: Loading scheme of fractions from the anion exchange chromatography step (Sartobind Q) as analyzed by agarose gel electrophoresis.

Well no.	Sample
1	1 kb ladder
2	Load
3	Load FT

Well no.	Sample
6	Fr. 1C3
7	Fr. 1C4
8	Fr. 1C5

Well no.	Sample
11	Fr. 2A4
12	Fr. 2B1
13	Fr. 2B3

4	Wash 2	9	Fr. 2A1	14	Strip
5	Fr. 1A2	10	Fr. 2A2	15	1 kb ladder

[0104] High amounts were loaded on the agarose gel to visualize impurities. Figure 5 shows that RNA was largely removed during the second wash step (see, e.g., lane 4; washing buffer: 50 mM Tris-base/TAPS ([tris(hydroxymethyl)methylamino]propanesulfonic acid), 0.5 M magnesium sulfate, pH 7.0). For the gradient elution under the chosen conditions, it was observed that oc pDNA had a lower retention time than SC pDNA (see again Figure 5).

[0105] Fractions from the AEX chromatography step (Sartobind Q) were also analyzed by HPLC. AEX-HPLC traces from the Sartobind Q elution pool 1 (cf. **Figure 4**; dashed line) and a standard (solid line) are shown in **Figure 6** and the results are summarized in **Table 4** below. To measure residual RNA within the load and elution pool 1, the pDNA was transferred to water by performing a tangential flow filtration step (Pellicon 3 cassette, 30 kDa, C screen, Ultracel membrane, Cat#: P3C030C00, Merck Millipore). Since pool 2 was a subset of pool 1, residual RNA was not measured for pool 2.

Table 4: Analytical results for the AEX unit operation.

Sample	**UV Absorption**		**AEX HPLC**	**RNA**
Product description	A260 (AU)	DNA titer (µg/mL)	Sum OC and L-Form (% impurities)	Residual RNA (µg/mL)
Load	1.2	60	n/a	59.1
Pool 1	6.0	302	4	< 1.6
Pool 2	8.5	425	3	< LoQ

[0106] The results summarized in **Table 4** above demonstrate that RNA was removed quantitatively. The oc pDNA and linear pDNA was reduced to low levels of 4% and 3% for Pool 1 and Pool 2, respectively. Taken together, the gradient elution allowed separation of RNA, as well as open-circular plasmid DNA (oc pDNA) from super-coiled plasmid DNA (sc pDNA). SDS-PAGE analysis (**Figure 7**) confirmed that proteinaceous impurities were likewise removed quantitatively by the AEX chromatography step (cf. Table 5 for the loading scheme of the SDS-PAGE gel).

Table 5: Loading scheme of samples as analyzed by SDS-PAGE.

Lane	**Sample**	**Lane**	**Sample**
1	Ladder	6	Blank
2	Blank	7	Load 1:2 dilution
3	Load non-diluted	8	Elution pool non-diluted
4	Blank	9	Elution 1:1 dilution
5	Load 1:1 dilution	10	Elution 1:2 dilution

Example 3: Improved primary recovery process and pDNA pUC19 purification using Sartobind Q and magnesium sulfate for the elution with a Tris buffer system

[0107] E. coli cells harboring the plasmid pUC19 were grown and lysed as described in more detail below. All buffers used throughout the process were filtered through 0.2 µm filters. A schematic representation of the primary recovery process including the AEX chromatography step is depicted in **Figure 8**.

[0108] For the primary recovery, a 76.4 g pellet of E. coli harboring the plasmid pUC19 was suspended with 764 mL suspension buffer (50 mM Tris, 10 mM EDTA) for 1 hour. Addition of 764 mL lysis buffer (1 % SDS, 0.2 M NaOH) started lysis of the cells. The lysis process was stopped after 4 minutes by adding 198 mL neutralization buffer (3 M Potassium Acetate, 2 M Acetic acid, chilled at 5 °C). It was found that 0.26 volumes are sufficient for neutralization.

[0109] 1800 mL of the neutralized solution was conditioned in a tulip-shaped tank by adding 1800 mL of conditioning buffer (200 mM Tris-base/TAPS, 3 M Ammonium Sulfate pH 7.0) to increase the density of the solution. The tulip-shaped tank facilitates removal of insoluble particles and thus improves the filtration behavior of the conditioned solution when applied to a depth filter (Clarisolve; Cat#: CS40MS01L3; 40 µm, Merck Millipore). The pH value of the filtrate was adjusted to 7.0 by adding 194 mL 1 M NaOH.

[0110] The chromatography unit was equilibrated with 20 column volumes (CVs) of 50 mM Tris-base/TAPS, 1.5 M ammonium sulfate, pH 7.0 at a flow rate of 4 CVs/min. The depth filtrate was then applied to the AEX unit (Sartobind Q; Cat# 96IEXQ42EUC11—A; 3 mL bed volume; Sartorius). Binding of plasmid DNA was performed at a concentration of 1.5 M ammonium sulfate. The load flow-through showed high absorbance values indicating that proteins and a significant amount of RNA species did not bind to the AEX material under these loading conditions. The unit was then washed by applying 20 CVs equilibration buffer followed by a second wash with 20 CVs of wash buffer (50 mM Tris-Base/TAPS, 0.5 M magnesium sulfate, pH 7.0) to remove RNA impurities. Plasmid DNA was eluted by applying a gradient from wash to elution buffer (50 mM Tris-Base/TAPS, 1.0 M magnesium sulfate, pH 7.0) in 25 CVs. The gradient elution allowed separation of mainly two species, wherein the first peak contained oc pDNA and the second peak contained sc pDNA. The chromatography unit was finally stripped by applying 30 CVs of 4 M NaCl. A representative chromatography profile of the anion exchange unit (Sartobind Q) is shown in **Figure 9**.

Claims

1. A method for isolating or purifying a plasmid DNA (pDNA) of interest from a mixture, the method comprising:

contacting the mixture with an anion exchange material in the presence of a solution comprising a kosmotropic salt at a concentration that allows selective binding of the pDNA of interest to the anion exchange material;
wherein the kosmotropic salt is selected from the group consisting of: ammonium sulfate, ammonium citrate, ammonium phosphate, potassium phosphate, sodium citrate, sodium phosphate, and mixtures thereof;

wherein the concentration of the kosmotropic salt is greater than 0.5 M; and

eluting the pDNA of interest with an eluent comprising a chaotropic salt at a concentration that provides an eluant containing the purified pDNA;
optionally wherein
(i) the fraction(s) comprising said pDNA in the eluant is/are collected and/or
(ii) said pDNA of interest is isolated from any one or all of the collected fractions; and/or
(iii) the method is conducted in the absence of organic solvents, detergents, glycols, hexamine cobalt, spermidine, and/or polyvinylpyrrolidone.

2. The method of claim 1, wherein
(i) the plasmid DNA is super-coiled plasmid DNA (sc pDNA); and/or
(ii) the plasmid DNA comprises mammalian DNA, bacterial DNA, non-coding DNA, or viral DNA; optionally wherein the plasmid DNA comprises DNA capable of expressing a polypeptide of interest; and/or
(iii) the mixture comprises super-coiled plasmid DNA, open-circular plasmid DNA, linear plasmid DNA, genomic DNA, RNA, lipopolysaccharides, endotoxins, proteins, or any combination thereof.

3. The method of claim 1 or claim 2, wherein the purity of the purified and/or isolated plasmid DNA is at least 90%, or at least 95%, or at least 98%.

4. The method of any one of claims 1 to 3, wherein
(i) the concentration of the kosmotropic salt is 0.8 M to 4.0 M; and/or
(ii) the concentration of the chaotropic salt that provides an eluant containing the purified pDNA is 0.25 M to 4.0 M; and/or
(iii) the elution step comprises a gradient elution by varying the concentration of the chaotropic salt in the eluent; optionally wherein the concentration of the chaotropic salt in the eluent is increased.

5. The method of any one of claims 2 to 4, wherein the elution step includes fractionation of super-coiled plasmid DNA from other plasmid DNA forms such as linear plasmid DNA and open-circular plasmid DNA, and, optionally, other nucleic acid molecules.

6. The method of any one of claims 1 to 5, wherein
(i) the kosmotropic salt is ammonium sulfate; optionally wherein the concentration of ammonium sulfate is 0.5 M to 2.0M; and/or
(ii) wherein the chaotropic salt is selected from the group consisting of: ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, guanidinium hydrochloride, and mixtures thereof, optionally wherein the chaotropic salt is magnesium sulfate; further optionally wherein the concentration of magnesium sulfate is 0.5M to 1.0M; and/or
(iii) the solution comprising the kosmotropic salt has a pH in the range of 5.0 to 10.0, or of 5.0 to 8.0, or 6.0 to 8.0, or pH 7.

7. The method of any one of claims 1 to 6, wherein the anion exchange material is selected from an anion exchange membrane, an ion exchange resin, a three-dimensional microporous hydrogel structure, a packed bed of superporous beads, macroporous beads, a monolith, agarose beads, cross-linked agarose, silica beads, large pore gels, methacrylate-based beads, polystyrene-based beads, cellulose-based beads, dextran-based beads, bisacrylamide-based beads, polyvinylether-based beads, ceramic-based beads, or polymer-based beads;
optionally wherein the anion exchange material is
(i) an anion exchange membrane; optionally wherein the anion exchange membrane has an average pore size of 3.0μm to 5.0μm; preferably wherein the average pore size is 3.0μm; or
(ii) an ion exchange resin, optionally wherein the ion exchange resin has an average particle diameter from 30 μm to 300μm.

8. The method of any one of claims 1 to 7, wherein the method further comprises separating solid components from the mixture comprising the pDNA of interest prior to contacting the mixture with an anion exchange material in the presence of the solution comprising the salt at a concentration that allows selective binding of the pDNA to the anion exchange material,
optionally wherein said removal of solid components is achieved by filtration or phase separation.

9. The method of claim 8, wherein the removal of solid components is achieved via two-phase separation,
optionally wherein
(i) a buffer is added to increase the density of the mixture to be greater than 1.1

kg/L, optionally wherein the buffer comprises a kosmotropic salt, preferably wherein the kosmotropic salt is ammonium sulfate; and/or

(ii) wherein the lower phase of the two-phase mixture having a higher density than the top phase is subjected to a depth filtration.

10. The method of any one of claims 1 to 9, wherein the method further comprises the step of

(i) precipitating the pDNA from the eluant; and/or

(ii) filtering the eluant by tangential-flow filtration to isolate the pDNA; optionally wherein the average pore size of the filtration membrane used in the tangential-flow filtration step is ≤ 0.2 µm; and/or

(iii) lyophilization of the purified pDNA of interest, optionally wherein the lyophilization is carried out in the presence of a carbohydrate; and/or

(iv) collecting a flow-through following the step of contacting the mixture with an anion exchange material in the presence of the solution comprising the salt at a concentration that allows selective binding of the pDNA to the anion exchange material; optionally wherein the flow-through comprises RNA, genomic DNA, proteins, cellular fractions, or combinations thereof, preferably wherein the flow-through comprises RNA; and/or

(v) washing the anion exchange material with a washing buffer solution prior to the elution of the pDNA of interest; optionally wherein the washing buffer solution comprises a chaotropic salt at a concentration lower than the concentration required for the elution of the pDNA of interest, wherein the chaotropic salt is preferably selected from the group consisting of: ammonium chloride, potassium chloride, sodium chloride, magnesium sulfate, magnesium chloride, magnesium nitrate, and mixtures thereof.

11. The method of any one of claims 1 to 10, wherein the method further comprises a further purification step following the step of eluting and optionally isolating the pDNA; optionally wherein the second purification step comprises use of a thiophilic aromatic adsorption chromatography medium having a selectivity that allows super-coiled plasmid DNA to be separated from open circular and/or linear DNA.

12. The method of any one of claims 1 to 11, wherein the method further comprises the step of

(i) using the pDNA to express a polypeptide of interest; or

(ii) using the pDNA to produce and harvest a polypeptide of interest; optionally wherein the method further comprises the step of formulating the polypeptide of interest into a pharmaceutical composition.

13. The method of any one of claims 2 to 11, wherein the method further comprises the step of using the purified plasmid DNA for the production of RNA.

14. The method of any one of claims 1 to 13, wherein the method includes the steps of cell harvesting and washing, cell lysis, neutralization, and flocculate removal prior to contacting the resulting mixture comprising the pDNA of interest with the anion exchange material.

15. The method of any one of claims 1 to 14, wherein the method does not comprise a $CaCl_2$ precipitation step to remove RNA.