As the successes of the viral vector approaches in clinical trials begin to build towards regulatory approval and commercialisation, attention has focused on the emerging bottleneck in mass production of good manufacturing practice (GMP) grade vector material (Van der Loo JCM, Wright JF., 2016, *Human Molecular Genetics*, 25(R1):R42–R52). A similar bottleneck exists in the production of GMP grade recombinant proteins.

A way to overcome this challenge is to find new ways to maximise transfection efficiency during production of viral vector or recombinant protein. Thus, there is a need in the art to provide alternative and improved transfection methods for use in the production of e.g. viral vectors and other biological agents, which help to address the known issues associated with the mass production of GMP grade material, such as GMP grade vector material.

SUMMARY

The present inventors have shown that polymer/nucleic acid complexes are only stable for a short period of time in the presence of salt before there is a negative effect on transfection efficiency. This limits the use of cationic polymers in mass production of biological agents because the optimum incubation period is too short to be practically possible at large scale GMP production.

The present disclosure provides an improved method for cell transfection. In this regard, the present inventors have surprisingly found that polymer/nucleic acid complexes prepared by mixing the nucleic acid and cationic polymer in a substantially salt-free aqueous solution (e.g. water), followed by the addition of salt to induce polymer/nucleic acid complex maturation, elongates the time for optimum polymer/nucleic acid complex maturation and provides high transfection efficiency. Thus, an optimum concentration of salt can be used to elongate the time for optimal polymer/nucleic acid complex maturation (i.e. the incubation time). The present inventors have further surprisingly found that the growth of the polymer/nucleic acid complexes can be curtailed and the complexes stabilised by dilution of the solution of polymer/nucleic acid complexes (thereby diluting the salt) to further extend the incubation time. Hence, the method described herein is advantageous as it provides the ability to control the initiation and/or termination of polymer/nucleic acid complex maturation. This in turn is highly advantageous for the mass production of GMP grade material, as it removes time constraints as an issue for the transfection process, i.e. improves manufacturing process flexibility.

The present inventors have surprisingly found that the ability to control the initiation and/or termination of polymer/nucleic acid complex maturation, combined with the reduced cell toxicity, is advantageous as it provides the ability to scale the preparation of the

polymer/nucleic acid complexes with the cell number during transfection. This in turn is highly advantageous for the mass production of GMP grade material, as it enables the use of higher cell densities (e.g. cell densities achieved using perfusion culture) for the transfection process, i.e. improves manufacturing process capacity and yield.

The present disclosure relates to such process for preparing a solution of polymer/nucleic acid complexes and use of the solution for transfection of cells, for example in the production of lentiviral vector.

In one example, the disclosure provides an *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;
b) adding a salt to the mixture produced in step a) to form a salt solution;
c) optionally incubating the salt solution produced in step b); and/or
d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c),

wherein said salt is not a valproic acid salt, isobutyric acid salt or isovaleric acid salt.

In a further example, the disclosure provides an *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;
b) adding a salt to the mixture produced in step a) to form a salt solution;
c) optionally incubating the salt solution produced in step b); and/or
d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c),

wherein said salt is added only prior to contacting the solution of polymer/nucleic acid complexes with a cell.

In a further example, the disclosure provides an *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;
b) adding a salt to the mixture produced in step a) to form a salt solution;

c) optionally incubating the salt solution produced in step b); and/or

d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c),

wherein the final salt concentration in step b) is between about 10 mM to about 100 mM.

In a further example, the disclosure provides an *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;

b) adding a salt to the mixture produced in step a) to form a salt solution;

c) optionally incubating the salt solution produced in step b); and/or

d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c),

wherein said salt has a degree of dissociation of at least 0.95 in the substantially salt free aqueous solution**.**

In a further aspect, the disclosure provides an *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;

b) adding a salt to the mixture produced in step a) to form a salt solution;

c) optionally incubating the salt solution produced in step b); and/or

d) diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c).

In a further example, the disclosure provides an *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;

b) adding a salt to the mixture produced in step a) to form a salt solution;

c) incubating the salt solution produced in step b); and/or

d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c).

In a further aspect, the disclosure provides a solution of polymer/nucleic acid complexes obtained or obtainable by the methods of the disclosure.

In a further aspect, the disclosure provides a solution comprising polymer/nucleic acid complexes and a salt, wherein the salt concentration is between about 10 mM to about 100 mM.

In a further aspect, the disclosure provides the use of a solution of polymer/nucleic acid complexes of the disclosure for the transfection of the nucleic acid into cells.In a further example, the disclosure provides the use of a solution of polymer/nucleic acid complexes of the disclosure in a method for the production of a retroviral vector.

In some examples, the nucleic acid is selected from DNA, RNA, oligonucleotide molecule, and mixtures thereof.

In some examples, the nucleic acid is DNA, preferably plasmid DNA.

In some examples, the cationic polymer is a polymer-based transfection reagent.

In some examples, the cationic polymer is selected from polyethylenimine (PEI), a dendrimer, DEAE-dextran, polypropyleneimine (PPI), chitosan [poly-(β-1/4)-2-amino-2-deoxy-D-glucopyranose], poly-L-lysine (PLL), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone) (PCL), and a derivative thereof.

In some examples, the cationic polymer is PEI or a derivative thereof, preferably selected from linear PEI, branched PEI, PEGylated PEI, JetPEI, PEIPro®, PEI MAX and PTG1+.

In some examples, the salt is selected from a sodium salt, a magnesium salt, a potassium salt, a calcium salt, a phosphate salt and a mixture thereof.

In some examples, the salt is phosphate buffered saline (PBS).

In some examples, the salt is PBS and PBS is added in step b) to a final concentration of between about 0.1X PBS to about 1.0X PBS, preferably wherein the PBS is added to a final concentration of between about 0.1X PBS to about 0.3X PBS.

In some examples, the salt is added in step b) to a final concentration of between about 10 mM to about 100 mM, preferably wherein the salt is added in step b) to a final concentration of between about 20 mM to about 100 mM.

In some examples, step a) comprises mixing a plurality of nucleic acid molecules and a plurality of cationic polymer molecules in a substantially salt free aqueous solution.

In some examples, the method comprises the step of incubating the salt solution produced in step b).

In some examples, the salt solution is incubated for about 1 minute to up to about 2 hours, preferably wherein the salt solution is incubated for about 20 minutes to about 90 minutes.

In some examples, the salt solution is incubated for about 60 minutes.

In some examples, the method comprises the step of diluting the salt solution produced in step b) or the salt solution following incubation of step c).

In some examples, the salt is PBS and the salt solution is diluted to a final concentration of between about 0.05X PBS to about 0.15X PBS, preferably wherein the salt solution is diluted to a final concentration of about 0.1X PBS.

In some examples, the nucleic acid encodes a retroviral vector component.

In some examples, the retroviral vector is a replication defective retroviral vector.

In some examples, the retroviral vector is a lentiviral vector, preferably wherein the lentiviral vector is HIV-1, HIV-2, SIV, FIV, BIV, EIAV, CAEV or Visna.

In some examples, the vector component is selected from:

a) the RNA genome of the lentiviral vector;
b) env or a functional substitute thereof;
c) gag-pol or a functional substitute thereof; and/or
d) rev or functional substitute thereof.

In some examples, the method further comprises the steps of:

e) optionally culturing a mammalian cell;
f) transfecting the mammalian cell using the solution of polymer/nucleic acid complexes obtained by the method according to any one of the preceding claims;
g) optionally introducing a nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes into the mammalian cell;
h) optionally selecting for a mammalian cell which has the nucleic acid(s) integrated within its genome;

i) optionally culturing the mammalian cell under conditions in which the nucleic acid(s) is (are) expressed;
j) optionally culturing the mammalian cell under conditions in which the retroviral vector is produced; and
k) optionally isolating the retroviral vector.

In some examples, the method comprises the step of culturing a mammalian cell.

In some examples culturing is by perfusion culture.

In some examples, the step of culturing a mammalian cell in a perfusion culture is performed for about 10 hours to about 96 hours.

In some examples, the method further comprises the step of inoculating a cell culture vessel (e.g. bioreactor) with a mammalian cell prior to the step of culturing a mammalian cell.

In some examples, the method further comprises the step of performing a rapid media exchange after the step of inoculating the cell and prior to the step of transfecting the cell.

Suitably, the step of culturing a mammalian cell comprises at least one rapid media exchange.

In some examples, the method comprises the step of culturing the mammalian cell under conditions in which the nucleic acid(s) is expressed.

In some examples, the method comprises the step of culturing the mammalian cell under conditions in which the retroviral vector is produced.

In some examples, the method comprises the step of isolating the retroviral vector.

In some examples, the mammalian cells are HEK293T cells.

In some examples, the mammalian cells are suspension-adapted mammalian cells.

In some examples, the transfection, introduction and/or culturing step(s) is (are) performed in suspension in a serum-free medium.

In some examples, the transfection is a transient transfection.

In some examples, the transfection, introduction and/or culturing step(s) is (are) carried out in a volume of at least 50 L, preferably wherein the transfection, introduction and culturing steps are carried out in a volume of at least 50 L.

In some examples, the nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes is introduced into the mammalian cell by transfection or by electroporation.
In some examples, the nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes encodes any viral vector components selected from:

a) the RNA genome of the lentiviral vector;
b) env or a functional substitute thereof;
c) gag-pol or a functional substitute thereof; and/or
d) rev or functional substitute thereof;

that are not encoded by the nucleic acid of the solution of polymer/nucleic acid complexes.

In a further example, the disclosure provides a method for producing a retroviral vector comprising the steps of:

a) optionally culturing a mammalian cell;
b) transfecting a mammalian cell using solution of polymer/nucleic acid complexes of the disclosure or a solution of polymer/nucleic acid complexes of the disclosure;
c) optionally introducing at least one nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes into the mammalian cell;
d) optionally selecting for a mammalian cell which has the nucleic acid sequences encoding the viral vector components integrated within its genome; and
e) culturing the mammalian cell under conditions in which the retroviral vector is produced.

In some examples culturing is by perfusion culture
In some examples, the step of culturing the cell in a perfusion culture is performed for about 10 hours to about 96 hours.
In some examples, the method further comprises the step of isolating the retroviral vector.

In some examples, the retroviral vector is a replication defective retroviral vector.

In some examples, the retroviral vector is a lentiviral vector, preferably wherein the lentiviral vector is HIV-1, HIV-2, SIV, FIV, BIV, EIAV, CAEV or Visna.

In some examples, the mammalian cell is a HEK293T cell.

In some examples, the mammalian cell is a suspension-adapted cell.

In some examples, the method is performed in suspension in a serum-free medium.

In some examples, step a), step b), step c) and/or step e) is carried out in a volume of at least 50 L, preferably wherein step a), step b), step c) and step e) are carried out in a volume of at least 50 L.

In some examples, the nucleic acid is introduced into the cell by transfection or by electroporation in step c).

In some examples, the transfection is a transient transfection.

In some examples, the at least one nucleic acid optionally introduced into the mammalian cell in step b) encodes any lentiviral vector components selected from the group consisting of:

(i) the RNA genome of the lentiviral vector;

(ii) env or a functional substitute thereof;

(iii) gag-pol or a functional substitute thereof; and/or

(iv) rev or functional substitute thereof;

that are not encoded by the nucleic acid of the solution of polymer/nucleic acid complexes.

In a further aspect, the disclosure provides a method for transfecting a mammalian cell comprising the steps of:

a) culturing a mammalian cell in a perfusion culture; and

b) transfecting the mammalian cell using a solution of polymer/nucleic acid complexes as described herein.

In some examples, the step of culturing a mammalian cell in a perfusion culture is performed for about 10 hours to about 96 hours.

Suitably, the mammalian cell is a mammalian cell as described herein.

In some examples, the method is performed in suspension in a serum-free medium.

In some examples, step a) and/or step b) is carried out in a volume of at least 50 L, preferably wherein step a) and step b) are carried out in a volume of at least 50 L.

In some examples, the transfection is a transient transfection.

In a further aspect, the present disclosure provides a use of a cationic polymer in an *in vitro* method for controlling the initiation and/or termination of the maturation of polymer/nucleic acid complexes, the method comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;

b) adding a salt to the mixture produced in step a) to form a salt solution;

c) optionally incubating the salt solution produced in step b); and

d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c).

CLAIMS

1. An *in vitro* method for producing a solution of polymer/nucleic acid complexes comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;

b) adding a salt to the mixture produced in step a) to form a salt solution;

c) optionally incubating the salt solution produced in step b); and

d) diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c).

2. The method according to claim 1, wherein:

i. the nucleic acid is selected from DNA, RNA, oligonucleotide molecule, and mixtures thereof;
ii. the cationic polymer is a polymer-based transfection reagent;
iii. the cationic polymer is selected from polyethylenimine (PEI), a dendrimer, DEAE-dextran, polypropyleneimine (PPI), chitosan [poly-(β-1/4)-2-amino-2-deoxy-D-glucopyranose], poly-L-lysine (PLL), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone) (PCL), and a derivative thereof;
iv. the cationic polymer is PEI or a derivative thereof;
v. the cationic polymer is PEI or a derivative thereof selected from linear PEI, branched PEI, PEGylated PEI, JetPEI, PEIPro®, PEI MAX and PTG1+;
vi. the salt is selected from a sodium salt, a magnesium salt, a potassium salt, a calcium salt, a phosphate salt and a mixture thereof; and/or
vii. the salt is phosphate buffered saline (PBS).

3. The method according to claim 1 or claim 2, wherein the salt is PBS and PBS is added in step b) to a final concentration of:

i. between 0.1X PBS to 1.0X PBS; or
ii. between 0.1X PBS to 0.3X PBS.

4. The method according to any one of claims 1 to 3, wherein the salt is added in step b) to a final concentration of between 10 mM to 100 mM or between 20 mM to 100 mM.

5. The method according to any one of the preceding claims, wherein step a) comprises mixing a plurality of nucleic acid molecules and a plurality of cationic polymer molecules in a substantially salt free aqueous solution.

6. The method according to any one of the preceding claims, wherein the method comprises the step of incubating the salt solution produced in step b).

7. The method according to claim 6, wherein:

i. the salt solution is incubated for 1 minute to up to 2 hours;
ii. the salt solution is incubated for 20 minutes to 90 minutes; or
iii. the salt solution is incubated for about 60 minutes.

8. The method according to any one of the preceding claims, wherein the salt is PBS and the salt solution is diluted to a final concentration of between 0.05X PBS to 0.15X PBS.

9. The method according to any one of the preceding claims, wherein the nucleic acid encodes a retroviral vector component.

10. The method according to claim 9, wherein the retroviral vector is a replication defective retroviral vector.

11. The method according to claim 9 or claim 10, wherein the retroviral vector is a lentiviral vector.

12. The method according to claim 11, wherein the vector component is selected from:

a) the RNA genome of the lentiviral vector;
b) env or a functional substitute thereof;
c) gag-pol or a functional substitute thereof; and/or
d) rev or functional substitute thereof.

13. The method according to any one of the preceding claims, wherein the method further comprises the steps of:

e) culturing a mammalian cell in a perfusion culture;
f) transfecting a mammalian cell using the solution of polymer/nucleic acid complexes obtained by the method according to any one of the preceding claims;
g) introducing a nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes into the mammalian cell;
h) selecting for a mammalian cell which has the nucleic acid(s) integrated within its genome;
i) culturing the mammalian cell under conditions in which the nucleic acid(s) is (are) expressed;

j) culturing the mammalian cell under conditions in which the retroviral vector is produced; and/or
k) isolating the retroviral vector.

14. The method according to claim 13, wherein:

i. the step of culturing a mammalian cell in a perfusion culture is performed for 10 hours to 96 hours;
ii. the mammalian cell is a HEK293T cell;
iii. the mammalian cell is a suspension-adapted mammalian cell;
iv. the transfection, introduction and/or culturing step(s) is (are) performed in suspension in a serum-free medium;
v. the transfection is a transient transfection;
vi. the transfection, introduction and/or culturing step(s) is (are) carried out in a volume of at least 50 L;
vii. the nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes is introduced into the mammalian cell by transfection or by electroporation; and/or
viii. the nucleic acid that is different to the nucleic acid of the solution of polymer/nucleic acid complexes encodes any viral vector components selected from:

i) the RNA genome of the lentiviral vector;
ii) env or a functional substitute thereof;
iii) gag-pol or a functional substitute thereof; and/or
iv) rev or functional substitute thereof;

that are not encoded by the nucleic acid of the solution of polymer/nucleic acid complexes.

15. A solution of polymer/nucleic acid complexes obtained or obtainable by the method according to any one of claims 1-14.

16. The solution of polymer/nucleic acid complexes according to claim 15, wherein the solution is obtained or obtainable by the method according to any one of claims 9 to 12.

17. A solution comprising polymer/nucleic acid complexes and a salt, wherein the salt concentration is between 10 mM to 100 mM or the salt concentration is less than 90 mM or the salt is PBS and the concentration of PBS is less than 0.3X PBS.

18. The solution of polymer/nucleic acid complexes according to claim 17, wherein the nucleic acid encodes a component for producing a retroviral vector.

19. Use of a solution of polymer/nucleic acid complexes according to any one of claims 15 to 18 for the transfection of the nucleic acid into cells.

20. A method for transfecting a mammalian cell comprising the steps of:

a) culturing a mammalian cell in a perfusion culture; and

b) transfecting the mammalian cell using solution of polymer/nucleic acid complexes as defined in 9 to 12 or a solution of polymer/nucleic acid complexes according to claim 16 or claim 18.

21. The method according to claim 20, wherein:

i. the step of culturing a mammalian cell in a perfusion culture is performed for 10 hours to 96 hours;.
ii. the mammalian cell is a HEK293T cell;
iii. the mammalian cell is a suspension-adapted cell;
iv. the method is performed in suspension in a serum-free medium;
v. step a) and/or step b) is carried out in a volume of at least 50 L; and/or
vi. the transfection is a transient transfection.

22. Use of a cationic polymer in an *in vitro* method for controlling the initiation and/or termination of the maturation of polymer/nucleic acid complexes, the method comprising the steps of:

a) mixing a nucleic acid and a cationic polymer in a substantially salt free aqueous solution;

b) adding a salt to the mixture produced in step a) to form a salt solution;

c) optionally incubating the salt solution produced in step b); and

d) optionally diluting the salt solution produced in step b) or diluting the salt solution following incubation of step c).