therapeutic and non-healthcare applications. Notably, DNA is used as the template to manufacture large quantities of RNA, particularly mRNA, and producing DNA on a commercial scale is therefore critical to the mass-production of RNA vaccines, for example. Thus, the requirement for clean, efficient DNA manufacture on an industrial scale is currently growing exponentially. The DNA produced using the present invention may be used as a template for manufacturing SARS-Cov-2 mRNA vaccines and the like.

Summary

The present ~~invention~~ disclosure relates to a process for cell-free production or synthesis of DNA. The process may allow for enhanced production of DNA compared to current methodologies, i.e. an increased or greater yield, a more efficient process or the ability to perform enzymatic DNA synthesis in an environment with fewer additional components than thought possible under current methodologies. This significantly increases productivity whilst reducing the cost of synthesising DNA, particularly on a large scale.

In order to achieve a high yield at an industrial scale it is necessary to utilise high concentrations of the "building blocks" of DNA, the nucleotides (notably dNTPs). Changing the parameters of the reaction conditions alone will not increase the yields from an enzymatic reaction.

Given the provision of nucleotides to the enzymatic reaction is as a salt, increasing the quantity of nucleotides results in a significant increase in the ionic strength of the reaction mixture. The ionic strength is a function of the concentration of all ions present. Such is an important consideration as the enzymes catalysing the DNA synthesis reaction are proteins, and an increased ionic strength can result in protein unfolding and thus inactivation of enzyme activity.

Further, it can be considered that the presence of a salt can also influence the pH of the reaction mixture. Depending on the acid-base properties of the component ions, a salt can dissolve in water to provide a neutral solution (strong acid/strong base), a basic solution (weak acid/strong base) or acidic solution (strong acid/weak base). Thus, by increasing the concentration of a nucleotide salt, or any other salt to the reaction mixture (magnesium chloride as an example), this may also influence the pH and further limit the pH stabilising performance of any buffering agent present. Thus, the addition of a higher concentration of nucleotide salt, for example, may lead to sub-optimal pH control, which impacts on the enzymatic DNA synthesis notably in terms of lowering DNA yields or adversely affecting the fidelity of DNA synthesis. Therefore, there are numerous considerations to be made in relation to the "scaling-up" of enzymatic DNA manufacture, and the present inventors have devised ways to increase the yield without adversely affecting the DNA synthesis reaction.

In general, the present ~~invention~~disclosure relates to enzymatic DNA synthesis using a nucleotidyltransferase, such as a polymerase enzyme or other DNA synthesising enzyme, any of which can optionally be engineered to give it particular properties.

The present ~~invention~~disclosure may relate to DNA synthesis from a nucleic acid template or DNA synthesis *de novo*, without a template, depending on the nucleotidyltransferase used.

The present ~~invention~~disclosure may relate to isothermal methods of synthesising DNA that do not require temperature to be cycled via heating and cooling during amplification, but may allow for the use of heat to initially denature the template, if present. The ~~invention~~disclosure preferably relates to the use of polymerase enzymes that are capable of replicating a nucleic acid template via strand-displacement replication, independently or with the help of other enzymes.

The processes of the ~~invention~~disclosure involve the use of nucleotides in the forms of complexes with associated ions, which may also be referred to herein as counter-ions. The nucleotide complex is generally present in solution, and therefore the associated counter-ion may or may not be dispersed in solution. Due to the nature of their preparation, the counter-ions may effectively be "shared" between the nucleotides, such that there is a ratio of counter-ion to nucleotide which is not a whole number. In solution with the nucleotide ionic species are divalent counter-ions and monovalent counter-ions, such that the partial or complete charge balance for each nucleotide is contributed by a mixture of monovalent and divalent cations. The use of such complexes of a nucleotide ionic species and a mixture of cation "counter-ion" species has not hitherto been seen, and is therefore a unique proposition. The provision can be such that the complex is electrically neutral, or may have a net negative charge. The nucleotide complexes may be supplied in solution, or may be dispersed in solution by adding solid nucleotide complexes to the nucleotidyltransferase in solution.

The nucleotide complexes of the present ~~invention~~disclosure are in solution, and comprise a nucleotide (also described here as a nucleotide ion or ionic species) associated with at least two different positive counter-ions (cations). It is preferred that one of these counter-ions is a monovalent cation, i.e. it has a single positive charge due to the loss of one electron. It is preferred that one of these counter-ions is a divalent cation, i.e. it has a double positive charge due to the loss of two electrons. In order to increase the yield and/or efficiency of the DNA synthesis, the nucleotides are provided as a complex with a mixed counter-ion provision of monovalent and divalent cations.

It will be understood that as a nucleotide has four negative charges, it is usual to provide four positive charges, generally through 4 monovalent cations to maintain electrical neutrality, and most commercial nucleotide salts are obtained on this basis. Commercially available salts are generally limited to lithium or sodium salts. However, in the processes of the present ~~invention~~disclosure,

when the nucleotide complex is in solution, it is possible that less than 4 positive charges are supplied by the monovalent or divalent cations used as the counter-ions. Without wishing to be bound by theory, the inventors postulate that the remaining charges may be provided by entities such as the hydronium ion, in order to reach electrical neutrality, if required.

Accordingly there is provided a cell-free process for the enzymatic synthesis of DNA comprising the use of nucleotide complexes in solution, wherein said complex in solution comprises a nucleotide and between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide.

It will be appreciated that the term nucleotide as used here may also be read as a nucleotide ion or nucleotide ionic species; it is the nucleotide entity without any counter-ion present.

Alternatively worded, there is provided:

A cell-free process for the enzymatic synthesis of DNA comprising obtaining a nucleotide complex in solution, wherein said complex is a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide, and adding a nucleotidyltransferase.

A cell-free process for the enzymatic synthesis of DNA comprising obtaining a nucleotide complex in solution, wherein said complex is a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide, in combination with a nucleotidyltransferase.

A cell-free process for the enzymatic synthesis of DNA using a nucleotidyltransferase comprising combining said enzyme and a nucleotide complex in solution, wherein said complex is a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide

Also provided is a novel nucleotide complex in solution comprising a nucleotide and between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide.

It will be appreciated that the term nucleotide as used here may also be read as a nucleotide ion or nucleotide ionic species; it is the nucleotide entity without any counter-ion present.

It will be appreciated that in solution the ions forming the complex may or may not dissociate.

It is preferred that the enzymatic DNA synthesis is for the manufacturing of DNA on a larger scale, i.e. for therapeutic or prophylactic use (stated as grams per litre reaction mixture), rather than lab-scale amplification (scale of ng to mg per litre). It is in this scaling-up of laboratory scale amplification that the present inventors have found that it is not as simple as providing more substrate and other components and finding that the yield follows suit. Thus, the process

comprises the use of nucleotide complexes in general at a concentration equal or greater than 30mM, said concentration being determined when the nucleotide complexes are combined with the nucleotidyltransferase. The admixing of the nucleotide complexes and the enzyme results in the formation of a reaction mixture. The concentration is determined in the reaction mixture in which the process is performed. Thus, the concentration of the nucleotide complexes is determined in the reaction mixture at the time that the nucleotide complexes are added. The concentration is therefore the initial concentration or concentration at the initiation of the process.

Accordingly there is provided a cell-free process for the enzymatic DNA synthesis comprising the use of nucleotide complexes at a concentration of at least 30mM in solution, wherein said complexes each comprise a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations.

Accordingly there is provided a cell-free process for the enzymatic synthesis of DNA comprising obtaining a nucleotide complex in solution at a concentration of at least 30mM, said complex comprising a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide, and adding a nucleotidyltransferase. The enzymatic DNA synthesis may involve any enzyme capable of synthesising DNA, notably a nucleotidyltransferase, the definition of which herein includes all enzymes capable of transferring a nucleotide to the end of a nascent polynucleotide chain, either on the basis of a template or *de novo*. The nucleotidyltransferase may include a polymerase or a modified polymerase, such as a DNA polymerase or an RNA polymerase. The polymerase may be from any of the known families of DNA polymerase, including families A, B, C, D, X, Y and RT. An example of a DNA polymerase from family X is terminal deoxynucleotidyl transferase.

The nucleotidyltransferase may be present in solution, and the nucleotide complexes can be added as a solid preparation to the enzyme in solution, for example as a lyophilised powder.

The enzymatic DNA synthesis may occur *de novo* without the use of a template.

The enzymatic DNA synthesis may involve a template, such as a nucleic acid template, including a DNA template.

The enzymatic DNA synthesis may take place in a reaction mixture, comprising the components described here.

Alternatively written, there is provided a cell-free process for synthesising DNA in solution comprising contacting a template with at least one nucleotidyltransferase in the presence of one or more nucleotide complexes , wherein said nucleotide complexes comprise a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per

nucleotide. Optionally the concentration of said nucleotide complexes is at least 30mM, preferably 40mM.

Alternatively put, the nucleotide complexes include a mixture of divalent and monovalent cations, along with the nucleotide itself. Accordingly there is provided a cell-free process for synthesising DNA comprising contacting a template with at least one nucleotidyltransferase in the presence of one or more nucleotide complexes to form a reaction mixture, wherein said nucleotide complexes are present at a concentration of at least 30mM and comprise a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations.

It is preferred that when the concentration of the nucleotide or nucleotide complex is referred to, this is the concentration of nucleotide (or complex thereof) when the process begins, i.e. the starting or initial concentration of nucleotide (or nucleotide complex). Thus, it is the concentration after addition to the reaction mixture. It will be appreciated that addition of other components can be made during the process; such additions may dilute the concentration of the nucleotides/nucleotide complexes, unless further nucleotides/nucleotide complexes are supplied to replenish the concentration. Further, since the nucleotide/nucleotide complexes will be used or consumed by the process, i.e. the DNA synthesis reaction, the concentration of the nucleotide/nucleotide complexes will fall as the process progresses. In certain ~~embodiments~~examples, further nucleotides/nucleotide complexes may be added as the process progresses in order to replenish substrates for the enzymatic reaction.

The inventors have surprisingly found that if the nucleotide complexes include a mixture of monovalent and divalent cations that the yield can be further improved. This further improvement is compared to a conventional nucleotide salt with 4 monovalent cations. Comparative Examples are included here. This effect is most notable for higher concentrations of nucleotide complex, for example above 30mM. The inventors have also noted that the association of magnesium or manganese cations with the nucleotide complex means that no additional magnesium or manganese is required in the reaction mixture in order to synthesize DNA. This is beneficial in reducing components and therefore cost.

Convention dictates, for example, that magnesium (a divalent cation) is present in DNA synthesis reactions at a minimum ratio of at least 1:1 with the nucleotides. This is because magnesium may be required at the active site of some nucleotidyltransferase enzymes; it may form a complex with the nucleotide prior to incorporation and further may form its own salt with the phosphate ionic species released during DNA synthesis. The benefit of including magnesium or manganese in the nucleotide complex is that this reduces or removes the requirement for additional magnesium or manganese. It assists to maintain an approximate 1:1 ratio (or below 1:1 ratio) of

magnesium or manganese to nucleotide, which the inventors have previously identified as desirable in large scale DNA synthesis reactions. This is important, since reducing the components included in the DNA synthesis notably reduces costs, but furthermore a higher concentration (greater than 1:1) of magnesium is related to a decreased fidelity in DNA synthesis.

The divalent cations associated with the nucleotide in the complex may comprise one or more metals selected from the list consisting of: magnesium (Mg^{2+}), beryllium (Be^{2+}), calcium (Ca^{2+}), strontium (Sr^{2+}), manganese (Mn^{2+})or zinc (Zn^{2+}), preferably Mg^{2+} or Mn^{2+}. The ratio between the divalent metal cations and the nucleotide (nucleotide ion or nucleotide ionic species) may be about 1:1 in solution, but is preferably between 0.2:1 and 2:1, optionally 0.5:1 to 1.5:1. Ratios lower than 1:1 are desirable and are preferable in DNA synthesis since ratios higher than 1:1 may lead to some infidelity in DNA synthesis. The provision of the divalent cations in relation to the nucleotide complex may therefore reduce or remove the need to add additional divalent cations to the reaction mixture. However, should further be required, these divalent cations may be provided to the enzymatic DNA synthesis in the form of any suitable salt.

Further, the method developed here by the present inventors can be performed in a large range of conditions with respect to the other components present. These conditions range from a conventional level of buffering to the provision of no further buffering agents, effectively performing the reaction with the required components in water. Increasing the concentration of a buffering agent may increase buffering capacity to directly improve pH control but chemical buffers can also chelate a range of metal ions including magnesium ions and may adversely interfere with the balance of mono- and divalent cations required for optimal DNA yields. Using concentrations of buffering agents at the lowest possible levels while balancing other essential reaction components to maintain pH within an acceptable band for optimal DNA production may, therefore, be desirable. Those skilled in the art will appreciate that some of the counter-ions proposed here may have their own buffering ability, or entities released or produced during DNA synthesis (for example pyrophosphates and phosphates) may also help buffer against excessive pH change.

The required components, regardless of buffering agent provision, may include the DNA synthesising enzyme (nucleotidyltransferase), such as a polymerase, the nucleotide complexes, with optional additional components required depending on the conditions of the reaction, selected from divalent metal cations provided as a salt, a template, a denaturing agent, a pyrophosphatase, or one or more primers/primase. These components may form the reaction mixture. Thus, in its most basic form, the reaction mixture is simply the nucleotide complexes plus a nucleotidyltransferase. It will be understood that it is desirable that the reaction mixture does not contain superfluous ionic species, since such entities may undesirably affect the DNA synthesis. Other than the ions present in

the nucleotide complex, other ions may be present in minimal amounts, such as in denaturing agents (for example sodium or potassium hydroxide) or in buffering agents. It may be necessary to further supplement magnesium or manganese salts, depending on the nucleotide complex selected and enzyme involved in the reaction. It is preferred that the concentration of "additional ions" in the reaction mixture, such as at the start of the reaction, may be kept to a minimal level, for example less than 50mM, less than 40mM, less than 30 mM, less than 20mM, or less than 10mM. Such additional ions are ions other than those supplied with the nucleotide complex or generated therefrom during the course of the reaction.

Thus, the provision to the process, i.e. the reaction mixture, of nucleotides as complexes with a mixed counter ion provision is advantageous, since this surprisingly allows for improved DNA yield and/or an improved efficiency of conversion of the nucleotides into DNA. These improvements may be compared to an analogous reaction mixture where all the nucleotides are supplied as conventional salts with the requisite monovalent cation alone. The provision of novel nucleotide complexes instead of nucleotide salts conventionally used has some further surprising advantages, such as the ability to lower the concentration of buffering agents in the reaction mixture, in some instances to zero, and/or to lower, decrease or remove entirely the requirement for the additional provision of a divalent cation co-factor for the reaction mixture, most notably magnesium, which is usually added as a salt. Given that this salt may no longer be required, the present ~~invention~~disclosure has the effect of reducing the ionic strength of the reaction mixture, since if the divalent cation salt is not added, there is no provision to the reaction mixture of the associated anion (e.g. $MgCl_2$ – therefore the addition of two chloride ions is avoided). The ionic strength of a solution is a measure of the concentration of ions in that solution. Ionic compounds, when dissolved in water, dissociate into ions. As used herein, the unit of measurement is molar (mol/L). The inventors postulate that a reduction in the ionic strength of the reaction mixture may be beneficial to the process. It is possible that the monovalent cations provided with the nucleotide entity are inhibitory to the process in high concentrations. Alternatively or additionally, in a standard process, magnesium or manganese is supplied as a salt, and high concentrations of the associated anion from this salt may also be inhibitory to the process, for example the chloride ion. Furthermore, should it be desirable to manipulate the DNA produced using the ~~invention~~disclosure, the inventors have seen that enzymes introduced into the reaction mixture, such as enzymes that cleave and ligate target sequences (DNA processing enzymes), prefer the conditions of lower ionic strength, since these are generally added at the end of the DNA synthesis reaction, when the ionic strength may have increased significantly with conventional nucleotide and divalent salts. Thus, the product from such a DNA synthesis reaction

may be suitable for further processing enzymatically (such as use as a template to produce RNA using RNA polymerase).

In one ~~aspect~~example, a template directs the enzymatic DNA synthesis in the processes. This template may be any nucleic acid template, for example a DNA or RNA template. The template may be a natural nucleic acid, an artificial nucleic acid or a combination of the two. The amplification of the template is preferably via strand-displacement. The amplification of the template is preferably isothermal, i.e. there is no requirement to cycle between low and high temperatures to progress the amplification. In this scenario, heat may be used at the start to denature the template, if required, or the template may be denatured by chemical means. However, once the template has been denatured, if appropriate, to allow any primers or indeed primase enzyme to enter between double stranded templates, the temperature may be maintained at a range of temperatures that do not affect the denaturation of the template and product. Isothermal temperature conditions require that the reaction is not heated to a point where the template and products denature (compared to PCR which requires cycles of heat to denature the template and product). Generally such reactions are performed at a constant temperature, depending on the preference of the enzyme itself. The temperature may be any suitable temperature for the enzyme.

The cell-free process preferably involves amplification of the template via strand displacement replication. This synthesis releases a single stranded DNA, which may in turn be copied into double stranded-DNA, using a polymerase. The term strand displacement describes the ability to displace downstream DNA encountered during synthesis, wherein the polymerase opens the double-stranded DNA in order to extend the nascent single strand. DNA polymerases with varying degrees of strand displacement activity are available commercially. Alternatively, strand displacement can be achieved by supplying a DNA polymerase and a separate helicase. Replicative helicases may open the duplex DNA and facilitate the advancement of the leading-strand polymerase.

Independently, optional features of any ~~aspect~~ example of the ~~invention~~disclosure may be: The template may be circular. The DNA may be synthesized by amplification of a template, optionally by strand displacement replication. The strand displacement amplification of said DNA template may be carried out by rolling circle amplification (RCA). The polymerase may be Phi29, or a variant thereof. The amplification of DNA may be isothermal amplification, i.e. at a constant temperature. A primer or primase may be used to initiate amplification. A nickase may be used to generate a primer “in situ” on a double stranded circular template. The one or more primers may be random primers. A pair or set of primers may be used. The synthesised DNA may comprise concatamers comprising

tandem units of DNA sequence amplified from the DNA template. The DNA template may be a closed linear DNA; preferably the DNA template is incubated under denaturing conditions to form a closed circular single stranded DNA.

The quantity of DNA that may be synthesised is equal to or higher than 3g/ litre of reaction mixture, notably 16g/l or more, preferably up to 25g/l and beyond.

The amount of DNA that may be synthesised may exceed 60% of the calculated maximum yield for the reaction mixture. Preferably, the amount of DNA that may be synthesised may exceed 80% of the calculated maximum yield. The calculated maximum yield is based upon the theoretical yield should all nucleotides be incorporated into a product, and this can be calculated by those skilled in the art.

The efficiency of DNA synthesis from the nucleotides (or nucleotide complexes) may be described as the percentage of nucleotides or complexes thereof supplied to the reaction mixture which are successfully incorporated into a product over the course of the reaction.

The speed of the DNA synthesis may also be improved by the present ~~invention~~disclosure, reducing the time taken to make a significant DNA yield.

The cell-free process requires at least one nucleotide complex. One or more further nucleotide complexes may then be added. Any suitable number of phosphate groups may be present, as required. However, it is preferred that the nucleotides/nucleotide complexes or further nucleotides/nucleotide complexes are deoxyribonucleoside triphosphates (dNTPs) or a derivative or modified version thereof. The nucleotides or further nucleotides are one or more of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and derivatives thereof. The nucleotides or further nucleotides are provided as complexes thereof. Each individual nucleotide complex may, but is not required to, be charged balanced with various cations providing 4 positive charges to maintain electrical neutrality. The nucleotide complexes used in the process may include one or more monovalent cations, i.e. one or more species of monovalent cation and one or more divalent cations, i.e. one or more species of divalent cation. It will be appreciated that these may dissociate in solution, and because of this, the number of cations associated with each nucleotide complex does not need to be a whole number, since in solution the ions may dissociate. The nucleotide complex may be considered to be a salt if the charges are entirely balanced.

In general, the inventors have mixed two different nucleotide complexes (one with solely divalent cation(s) and the other with solely monovalent cations) together in order to prepare the nucleotide complexes used in the method of the ~~invention~~disclosure. The nucleotide complex may each independently comprise a complex where not all of the negative charges are balanced. This has

several advantages. Nucleotides complexed with divalent cations have a low solubility, and therefore are not in routine use for any application. Particular issues are seen with nucleotides complexed with magnesium ions; they are not in solution and are unusable in their current form. However, when mixed with nucleotide complexes associated with a monovalent cation or cations, the mixture is soluble and forms a solution. Therefore, this provides an elegant way of utilising a previously desired but unworkable nucleotide complex.

The monovalent ion may be a sole species of ion, or a mixture of different species of ion. The divalent ion may be a sole species of ion or a mixture of different species of ion.

0.2 to 2.5 monovalent cations are generally present in or associated with the nucleotide complex according to the present ~~invention~~disclosure. This range includes 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4 and 2.5 monovalent ions per nucleotide complex. It is possible that the ions are shared between the nucleotide ions. It is, however, possible that the level of monovalent cation could be reduced to minimal levels or even complete absence, by the alternative use of buffering agents to ensure solubility of the nucleotide complexes associated with divalent cations. This would have further beneficial effects, since the level of monovalent cation associated with the nucleotide complex could be taken lower than 0.2:1 and may even reach 0.1:1 or zero. Suitable buffering agents for this ~~embodiment~~ example include those that are not capable or complexing the metal cations present in the reaction mixture, since protons are released when a buffer forms complexes with metal ions. For example, buffers HEPES and HEPPS both have negligible metal ion affinity, or BES, which does not interact with magnesium.

It is preferred that the concentration of nucleotides or complexes thereof in the process, i.e. in the reaction mixture, may be more than 30mM and up to at least 300mM. Such concentrations are important in the production of higher yields of DNA, which in the case of the two concentrations given can be as high as 9.75 g/l to 97.5g/l. It is preferred that the concentration of nucleotide or complexes thereof stated is at the start of the process, i.e. is the starting or initial concentration of nucleotides or complexes thereof in the reaction mixture, which also includes the enzyme necessary for DNA synthesis. Subsequent addition of further components may reduce this concentration, and their use by the DNA synthesis enzyme will also reduce the concentration from the starting concentration. Those skilled in the art will be aware of how to calculate the concentration of nucleotides/nucleotide complexes as the process is prepared, based upon the volume of the other components and the stock nucleotide complex solution/powder used.

It should be noted that in the art of DNA synthesis or amplification, the term “nucleotide” is used when the author means “nucleotide salt”, since the provision and utilisation in DNA synthesis of nucleotides without any form of counter-ion is not currently possible. The process may be a batch

process or a continuous flow process. The batch may be a closed batch (i.e. all of the reaction components are provided at the start of the DNA synthesis) or further components can be supplied to the reaction as required during the process, such as described in WO2016/034849, incorporated herein by reference. Should further additions be required, this will dilute the concentration of the nucleotide or nucleotide complexes, unless further nucleotide complexes are added to replenish or increase the concentration.

The present inventors have found that each of the different counter-ions may add a particular characteristic to the enzymatic DNA synthesis reaction. For example the use of ammonium ions in nucleotide complexes may result in the use of some higher concentrations of nucleotides.

The enzymatic cell-free synthesis of DNA with such ions can be carried out in minimal buffering agents, in which no additional salts which have been shown to enhance DNA synthesis or assist in primer binding, or detergents, are added. This minimal buffer may comprise an agent (a buffering agent) to stabilise the pH. The minimal buffering agent may contain a small amount of cations provided by the presence of a chemical used to denature the template, such as sodium, potassium or ammonium hydroxide. In the Examples, 5mM sodium or potassium hydroxide was used as a denaturant, but this concentration may be altered to suit the conditions of the reaction, within the skill of those used to performing DNA denaturation, and amounts of sodium, potassium or ammonium hydroxide could be provided from 2.5mM, up to 5mM, up to 10mM, 15mM, 20mM or 25mM or more may be employed dependent upon the nature of the template. Therefore, the reaction mixture may contain a small or minimal amount of cations and anions which were not originally associated with the nucleotide complex.

Thus, in one aspect, the present disclosure provides a cell-free process for the enzymatic synthesis of DNA in solution comprising obtaining a nucleotide complex, where said nucleotide complex comprises a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide and adding a nucleotidyltransferase.

Further advantages are described below.

Brief description of the Drawings

The present invention will be described further below with reference to exemplary embodiments and the accompanying drawings, in which:

Figures 1A to 1B are plots showing results obtained with experiments using varied starting concentrations of nucleotide salts for either sodium alone (dNTP:$4Na^+$)(continuous line) or a mixed sodium/magnesium dNTP (dNTP:$2Na^+$/Mg^{2+})(dotted line). The plot depicts the reaction yields in g/l (A) and the reaction efficiency as a % (B) for each type of dNTP plotted vs the start dNTP concentration used. Figure 1C shows DNA produced after 1 week of RCA, with more viscous DNA solutions corresponding to higher yields remaining attached to the base of the Eppendorf tube when inverted.

Claims

1. A cell-free process for the enzymatic synthesis of DNA in solution comprising obtaining a nucleotide complex, where said nucleotide complex comprises a nucleotide associated with between 0.2 and 2 divalent cations and between 0.2 and 2.5 monovalent cations per nucleotide and adding a nucleotidyltransferase.

2. A cell-free process according to claim 1 wherein the nucleotide complex is a salt.

3. A cell-free process according to any one of claims 1 or 2 wherein said nucleotide complex is electrically neutral.

4. A cell-free process according to claim 3 wherein in order to obtain electrical neutrality the complex associates with one or more hydrogen or hydronium ions.

5. A cell-free process according to any preceding claim wherein the complex is associated with between about 0.5 and 1.5 divalent cations, preferably 1 divalent cation per nucleotide entity.

6. A cell-free process according to any preceding claim wherein the complex is associated with between about 0.2 and 2 monovalent cations.

7. A cell-free process according to any preceding claim wherein said divalent cations are independently selected from: magnesium (Mg^{2+}), beryllium (Be^{2+}), calcium (Ca^{2+}), strontium (Sr^{2+}), manganese (Mn^{2+}) or zinc (Zn^{2+}), preferably Mg^{2+} or Mn^{2+}.

8. A cell-free process according to any preceding claim wherein said monovalent cations are independently selected from: an alkali metal, a transition metal, or a polyatomic ion.

9. A cell-free process according to claim 8 wherein said monovalent cations may be independently selected from a polyatomic ion such as an oxonium ion, preferably ammonium or a derivative thereof.

10. A cell-free process according to claim 8 wherein said monovalent cations may be an alkali metal, independently selected from : lithium (Li^{+}), sodium (Na^{+}), potassium (K^{+}), rubidium (Rb^{+}), caesium (Cs^{+}) or francium (Fr^{+}).

11. A cell-free process according to any preceding claim, wherein said nucleotide complex in solution is obtained by mixing nucleotides complexed with divalent cations and a solution of nucleotides complexed with monovalent cations , preferably wherein the divalent and monovalent cations are present at a ratio of less than 4:1 cation: nucleotide, optionally 3.5:1, 3:1 or 2.5:1 or below.

12. A cell-free process according to claim 11 wherein said nucleotide complex with divalent cations is poorly soluble until mixing with nucleotides complexed with monovalent cations.

13. A cell-free process according to claims 11 and 12 wherein the nucleotide complex is soluble.

~~14.~~ A cell-free process according to any preceding claim wherein said nucleotide complex is obtained at a concentration of at least 30mM, or~~.~~

~~15.~~14. ~~A cell-free process according to any preceding claim wherein said nucleotide complex is obtained at a concentration of~~ at least 40mM.

~~16.~~15. A cell-free process according to any preceding claim wherein said nucleotide complex and nucleotidyltransferase form a reaction mixture.

~~17.~~16. A cell-free process according to claim 16 wherein further components are added to the reaction mixture, including but not limited to any one or more of the following:

a) template nucleic acid;

b) primer;

c) primase;

d) denaturing agent, such as sodium or ammonium hydroxide;

e) buffering agents; including buffering salts;

f) pyrophosphatase; and/or

g) magnesium or manganese salts

~~18.~~17. A cell-free process according to claim 17 wherein magnesium or manganese salts are added to the reaction mixture as a co-factor for the nucleotidyltransferase, such that the total ratio of the magnesium and/or manganese to nucleotide does not exceed 2:1.

~~19.~~18. A cell-free process according to any preceding claim wherein said nucleotidyltransferase is a DNA polymerase, preferably a strand-displacing DNA polymerase.

~~20.~~19. A cell-free process according to claim 19 wherein said nucleotidyltransferase is capable of isothermal DNA synthesis.

~~21.~~20. A cell- free process according to any one of claims 1-18 wherein said nucleotidyltransferase does not require a template.