Summary

In one aspect, the present disclosure refers to a cell-free process for the enzymatic synthesis of DNA comprising the use of nucleotide salts, wherein said salts comprise a monovalent cation with an ionic radius that is greater than that of the sodium ion.

In another aspect, the present disclosure refers to a cell-free process for the enzymatic synthesis of DNA comprising the use of nucleotide salts , wherein said nucleotide salts are present at a concentration of at least 10mM and are either:

(a) a nucleotide salt comprising a monovalent cation whose ionic radius is greater than that of the sodium ion, or

(b) two or more nucleotide salts, each salt comprising different monovalent cations, wherein at least one of the cations has an ionic radius greater than that of the sodium ion.

In yet another aspect, the present disclosure refers to the use of nucleotide salts comprising caesium cations in the enzymatic cell-free synthesis of DNA.

In a further aspect, the present disclosure refers to a method of amplifying a DNA template using a DNA polymerase wherein maintaining a ratio of divalent cations to nucleotides in the reaction mixture of 0.5:1 or below is required, comprising the use of nucleotide salts comprising caesium ions.

In another aspect, the present disclosure refers to the use of nucleotide salts comprising rubidium cations in the enzymatic cell-free synthesis of DNA.

In yet another aspect, the present disclosure refers to a cell-free method of amplifying a DNA template, said method comprising contacting said template and a DNA polymerase with nucleotides in the form of a salt in an amount equal to or greater than 40mM, preferably greater than 60mM or optionally greater than 80mM wherein said salt comprises ammonium ions.

In a further aspect, the present disclosure refers to an enzymatic DNA synthesis which is performed under conditions of reduced concentrations of divalent cations, preferably magnesium, comprising the use of nucleotides in the form of salts comprising a monovalent cation with an ionic radius greater than that of the sodium ion.

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

The present disclosure relates generally to isothermal methods of amplifying 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 DNA template. The present disclosure preferably relates to the use of polymerase enzymes that are capable of replicating a DNA template via strand-displacement replication, independently or with the help of other enzymes.

The processes of the present disclosure involve the use of nucleotides in the forms of salts. The salts include a positive counter-ion (cation). It is preferred that this counter-ion is a monovalent cation, i.e. it has a single positive charge due to the loss of one electron. In order to increase the yield and/or efficiency of the DNA synthesis, the monovalent cations may not be exclusively sodium or lithium ions or a mixture thereof, but at least a portion of the cations will have an ionic radius greater than that of the sodium ion. The presence of a proportion of sodium or lithium in the salts or in the process in general may be tolerated, but it is preferred that the salt comprises monovalent cations with an ionic radius greater than that of the sodium ion. It will be understood that as a nucleotide has four negative charges, in general four monovalent cations will be present in the salt to maintain electrical neutrality.

Accordingly there is provided a cell-free process for the enzymatic synthesis of DNA comprising the use of nucleotides supplied as salts, wherein said salts comprise a monovalent cation with an ionic radius that is greater than that of the sodium ion.

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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, rather than lab-scale amplification. 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. For nucleotide salts with sodium and lithium, these were found to be inhibitory to the DNA synthesis enzymes at higher concentrations. The present inventors have found alternative ways around this inhibition. The present disclosure allows for the reaction set-up to be altered, and therefore for the use of nucleotide salts at a concentration equal to or greater than 10mM. Thus, the process comprises the use of nucleotide salts at a concentration equal or greater than 10mM, said concentration being determined when the nucleotides are added. The concentration is determined in the reaction mixture in which the process is performed. Thus, the concentration of the nucleotides is determined in the reaction mixture at the time that the nucleotides 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 nucleotides supplied as salts at a concentration of at least 10mM, wherein said salts comprise a monovalent cation with an ionic radius that is greater than that of the sodium ion.

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Any nucleotide salt as described here may have up to four monovalent cations present to maintain electrical neutrality.

Further, it is possible to use mixtures of nucleotide salts in the enzymatic DNA synthesis or cell-free process.

Accordingly there is provided a cell-free process for the enzymatic DNA synthesis comprising the use of nucleotides supplied as salts, wherein said nucleotides are either:

(a) in the form of a salt with a single monovalent cation whose ionic radius is greater than that of the sodium ion, or

(b) in the form of salts with 2 or more different monovalent cations, wherein at least one of the cations has an ionic radius greater than that of the sodium ion.

The nucleotides in this example may be provided at a concentration of greater than 10mM.

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initial concentration of nucleotide (or nucleotide salt). 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 salts, unless further nucleotides are supplied to replenish the concentration. Further, since the nucleotide/nucleotide salts will be used or consumed by the process, i.e. the DNA synthesis reaction, the concentration of the nucleotide/nucleotide salts will fall as the process progresses. In certain examples, further nucleotides/nucleotide salts 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 salts include monovalent cations with an ionic radius greater than that of the sodium ion, the requirement for a divalent cation in the synthesis is reduced. 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 nucleotide salts. This is because magnesium is required at the active site of some polymerase 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. However, under certain conditions, the present inventors have developed a method wherein the requirement for magnesium or other divalent cations is much reduced. This is important, since reducing the components included in the DNA synthesis notably reduces costs, but furthermore a higher concentration of magnesium is related to a decreased fidelity in DNA synthesis.

The divalent cations may comprise one or more metals selected from the list consisting of: Mg^{2+}, Be^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+} or Zn^{2+}, preferably Mg^{2+} or Mn^{2+}. The ratio between the metal cations and the nucleotide salts may be about 1:1 in the reaction mixture. Ratios lower than 1:1 are desirable and are preferable in DNA synthesis since ratios higher than 1:1 can lead to some infidelity in DNA synthesis. The divalent cations may be provided to the enzymatic DNA synthesis in the form of any suitable salt.

The present disclosure therefore also relates to an enzymatic DNA synthesis which is performed under conditions of reduced divalent cations comprising the use of nucleotide salts with a monovalent cation or cations with an ionic radius greater than that of the sodium ion. Reduced in this context is in comparison to an identical reaction where lithium or sodium ions are present in the nucleotide salt.

The inventors have surprisingly found that the use of nucleotide salts with alternative counter-ions such as ammonium and caesium ions in the processes of the present disclosure reduces the requirement for buffering agents to be included in the enzymatic DNA synthesis. This again is

advantageous, since it reduces the cost of the synthesis reaction and could be beneficial for DNA synthesis for therapeutic use.

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. The required components may include the DNA synthesising enzyme , i.e. a polymerase, the nucleotide salts, and a divalent cation (as a salt), with optional additional components required depending on the conditions of the reaction, selected from a template, a denaturing agent, a pyrophosphatase, or one or more primers. These components may form the reaction mixture.

Thus, the provision to the process, i.e. the reaction mixture, of at least a proportion of nucleotides as salts with a monovalent positive counter-ion (cation) with an ionic radius greater than that of the sodium ion 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 alone, for example as solely lithium or sodium salts, or a mixture of these two ions. The provision of different nucleotide salts to those 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 to lower the requirement for a divalent cation co-factor for the reaction mixture, most notably magnesium.

In one example, a template directs the enzymatic DNA synthesis in the processes. This template may be a DNA template. 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 to enter between double stranded template, 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 the 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 example of the present disclosure may be: The template may be circular. 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. 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 per litre of reaction mixture, notably 16g/l or more, preferably up to 30g/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 salts) may be described as the percentage of nucleotides or salts thereof supplied to the reaction mixture which are successfully incorporated into a product over the course of the reaction.

The cell-free process requires at least one nucleotide. One or more further nucleotides may then be added. The nucleotides or further nucleotides 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 salts thereof. Each individual nucleotide salt may comprise up to four monovalent cations to maintain electrical neutrality. The nucleotide salts

Accordingly, the use of nucleotide salts comprising any one of caesium, ammonium, ammonium derivatives or rubidium cations in the enzymatic cell-free synthesis of DNA forms part of the present disclosure. Thus, use of nucleotide salts comprising these ions forms part of the present disclosure.

The enzymatic cell-free synthesis of DNA with such ions can be carried out in the presence of low levels of divalent cation, less than about 1:1, preferably at a ratio of 0.2:1 to 0.8:1 divalent cation to nucleotide, preferably 0.2:1 to 0.5:1. The ions are the counter-ion in a nucleotide salt.

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 very small amount of cations provided by the presence of a chemical used to denature the template, such as sodium, potassium or ammonium hydroxide. The ions are the counter-ion in a nucleotide salt.

Should a low level of magnesium ions be desirable in the enzymatic DNA synthesis, the inventors have found that a reliable nucleotide for such a synthesis is a nucleotide salt with caesium ions.

Accordingly, the present disclosure provides an enzymatic cell-free synthesis of DNA wherein maintaining a ratio of divalent ions to nucleotides of 0.5:1 or below is required, comprising the use of nucleotide salts comprising caesium ions.

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:

Figure 1A to 1E are plots showing results obtained with experiments using varied starting concentrations of nucleotide salts with different counter-ions and different initial/starting concentrations of magnesium ions (as $MgCl_2$) in a DNA synthesis reaction. Each plot shows the obtained raw DNA yield (g/l) versus the theoretical DNA yield (g/l) corresponding to the total initial/starting nucleotide salt concentration (mM). On all of the plots the dashed line shows 80% efficiency of conversion of the nucleotide salts into DNA, the solid line shows 100% efficiency of conversion. Figure 1A depicts the DNA synthesis results obtained using lithium-dNTPs; Figure 1B the results achieved with sodium-dNTPs, Figure 1C the results obtained with potassium-dNTPs Figure 1D the results achieved with ammonium-dNTPs, and Figure 1E, the results when using caesium-dNTPs;