

poly(A)polymerases, nor does it disclose the use of the immobilized poly(A)polymerase for producing polynucleotidylated ribonucleic acid molecules.

5 M. Prakash (2010, *Enzyme Biotechnology*, Discovery Publishing Pvt. Ltd) describe the binding of anti-poly(A) polymerase antibodies with hepatoma poly(A) polymerase dripped on DBM-paper. Again, the neither a bacterial PNP/PAP was used nor was the enzyme used for production of stable and homogeneous poly(N/A) RNA molecules.

10 The patent application US 2012/0214171 A1 discloses immobilization of RNA polymerases which are neither structurally nor regarding their biological activity comparable to the claimed immobilized PNP/PAPs. Immobilization is achieved using oligohistidine tags on functionalized solid supports. The document does neither suggest immobilization of RNA polymerases via covalent binding nor as
15 regards benefits of using RNA polymerases of specific biological origin. The document does also neither mention nor suggest immobilization of poly(A/N)polymerases or any benefit of using such enzymes.

Thus, the state of the art PNPs and polynucleotidylation reaction methods do neither
20 straight-forwardly nor cost-effectively produce homogeneous poly(N/A)RNA molecules.

These and other problems are solved by the claimed subject matter, in particular by the employment of an immobilized poly(N/A) polymerase.

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SUMMARY

As solution to the above discussed problems, disclosed herein is a poly(N)polymerase (PNP) immobilized onto a solid support, a method of producing the PNP and uses thereof. Further disclosed are an enzyme reactor and a kit
30 comprising the PNP.

In one aspect, the present invention refers to a poly(N)polymerase characterized in that the poly(N)polymerase is a bacterial poly(N)polymerase and immobilized onto a solid support by covalent binding, wherein the poly(N)polymerase stays active when
5 immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase. In another aspect, the present invention refers to use of a poly(N)polymerase being immobilized onto a solid support by covalent binding for producing polynucleotidylated ribonucleic acid (poly(N)RNA)
10 molecules, preferably polyadenylated ribonucleic acid (poly(A)RNA) molecules, wherein the poly(N)polymerase stays active when immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase. In yet another aspect, the present invention refers to an enzyme reactor comprising a
15 poly(N)polymerase being immobilized onto a solid support or comprising a poly(N)polymerase as disclosed herein or comprising a poly(N)polymerase obtainable by the method disclosed herein. In a further aspect, the present invention refers to a kit comprising a poly(N)polymerase characterized in that the poly(N)polymerase is immobilized onto a solid support by covalent binding, wherein
20 the poly(N)polymerase stays active when immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase, a poly(N)polymerase reaction buffer, and nucleoside monophosphates, optionally a nucleotide mixture, further optionally an RNA polymerase, and optionally an RNA
25 in vitro transcription buffer.

The immobilization of the PNP onto a solid support has a number of advantages over classical methods wherein the PNP is free in solution together with the other components of the polynucleotidyl reaction, such as RNA molecules, nucleotides,
30 salts, buffer components etc.

First a PNP which is immobilized onto a solid support may be used repeatedly and

for different RNA molecules to be polynucleotidylated which makes the polynucleotidylation reaction much more time-effective (quicker separation), cost-effective and more ecologic since less chemicals and other materials are needed for provision of PNP and its separation from the polynucleotidylated RNA.

- 5 Immobilization enhanced the stability of the enzyme PNP (e.g. poly(A)polymerase (PAP)) since aggregation and denaturation of the protein are reduced. The reaction can be performed in batch or even fed-batch mode.

Second the immobilization of PNPs facilitates purification of the polynucleotidylated RNA (poly(N) RNA). In fact, the simple removal of the reaction mixture provides for separation of the PNP from the other reaction components and separation steps such as heat denaturation, extraction and precipitation may be avoided. This also reduces impurities (e.g., denatured PNP proteins or fragments) in the produced poly(N) RNA solution.

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The immobilization is an important aspect when talking about poly(N) RNA molecules being homogeneous with respect to the length of the poly(N) tail since the length of the poly(N) tail is significantly determined by the molar ratio between RNA and nucleotide provided in the polymerization reaction. Therefore, PNP can be provided in an amount which does not limit the reaction and homogenous poly(N) tails are produced.

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Claims

1. A poly(N)polymerase characterized in that the poly(N)polymerase is a bacterial poly(N)polymerase and immobilized onto a solid support by covalent binding, wherein the poly(N)polymerase stays active when immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase.
2. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase is a poly(A)polymerase.
3. The poly(N)polymerase according to claim 1, wherein, the poly(N)polymerase is immobilized by covalent binding to a thiol-activated solid support, haloacetyl functionalized solid support, epoxy-functionalized solid support, pyridyl disulfide-functionalized solid support, maleimide-activated solid support or a mixture thereof, preferably the poly(N)polymerase is immobilized by covalent binding to a thiol-activated solid support, haloacetyl functionalized solid support, pyridyl disulfide-functionalized solid support, maleimide-activated solid support or a mixture thereof.
4. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase is immobilized via a thiol group of at least one cysteine residue.
5. The poly(N)polymerase according to claim 1, wherein the covalent binding is a disulfide bridge, thioester bond or a thioether bond, preferably the covalent binding is a disulfide bridge or a thioether bond.
6. The poly(N)polymerase according to claim 1, wherein the solid support comprises a member selected from the group consisting of sepharose, thiopropyl-sepharose, sephadex, agarose, silica, magnetic beads, methacrylate beads, and nanoparticles, preferably the solid support comprises a member selected from the group consisting of sepharose, thiopropyl-sepharose, sephadex, agarose, silica, magnetic beads, and nanoparticles.
7. The poly(N)polymerase according to claim 1, wherein the solid support comprises a reactive group selected from the group consisting of thiol, haloacetyl, pyridyl disulfide, epoxy, maleimide and mixtures thereof.

8. The poly(N)polymerase according to claim 1, wherein the solid support is selected from the group consisting of activated thiol sepharose, thiopropyl-sepharose, thiol-activated sephadex, thiol-activated agarose, silica-based thiol-activated matrix, silica-based thiol-activated magnetic beads, pyridyl disulfide-functionalized nanoparticles, maleimide-
5 activated agarose and mixtures thereof.
9. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase is derived from *Escherichia coli*, *Streptomyces coelicolor*, *Meiothermus silvanus*, *Bacillus subtilis*, *Thermus aquaticus*, *Shigella flexneri*, *Shigella dysenteriae*, *Citrobacter koseri*,
10 *Salmonella bongori*, *Salmonella enterica*, *Trabulsiella guamensis*, *Kluyvera ascorbata*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus gallinarum*, *Grimontia indica*, or *Salinivibrio costicola*.
10. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase
15 comprises an amino acid sequence being at least 90% identical to an amino acid sequence as depicted in any one of SEQ ID NOs: 1 to 22, 24 to 155, or 203, preferably comprises an amino acid sequence being at least 90% identical to any one of SEQ ID NOs: 1, 2, 3, 16 to 22, 24 to 155, or 203, more preferably at least 90% identical to any one of SEQ ID NOs: 17 to 22, 32 to 83, 85-111, 113-139, 141-145, 146-148, 150-152, 154-155, or 203, even more
20 preferably at least 90% identical to any one of SEQ ID NOs: 18, 58-83, 85-111, 113-139, or 203, and most preferably at least 90% identical to SEQ ID NO: 113.
11. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase comprises at least one newly introduced cysteine residues compared to a native
25 poly(N)polymerase, preferably the poly(N)polymerase comprises an amino acid sequence as depicted in any one of SEQ ID NOs: 17-22, 32-155, or 203.
12. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase comprises only one cysteine residue or is mutated to comprise only one cysteine residue,
30 preferably the poly(N)polymerase comprises an amino acid sequence as depicted in any one of SEQ ID NOs: 2, 16-22, 24-83, 85-111, 113-139, 141-144, 146-148, 150-152, 154-155, or 203.
13. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase
35 comprises a linker element as depicted in SEQ ID Nos: 156-180.

14. The poly(N)polymerase according to claim 1, wherein the poly(N)polymerase comprises a purification tag as depicted in SEQ ID NOs: 181-201.

15. A method for producing a poly(N)polymerase characterized in that the
5 poly(N)polymerase is a bacterial poly(N)polymerase and immobilized onto a solid support by covalent binding, comprising a step of contacting the poly(N)polymerase with a solid support under conditions suitable for immobilizing the poly(N)polymerase to the solid support by covalent binding, wherein the poly(N)polymerase stays active when immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group
10 consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase.

16. Use of a poly(N)polymerase being immobilized onto a solid support by covalent binding for producing polynucleotidylated ribonucleic acid (poly(N)RNA) molecules,
15 preferably polyadenylated ribonucleic acid (poly(A)RNA) molecules, wherein the poly(N)polymerase stays active when immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase.

20 17. An enzyme reactor comprising a poly(N)polymerase being immobilized onto a solid support or comprising a poly(N)polymerase according to any one of claims 1 to 14 or comprising a poly(N)polymerase obtainable by the method according to claim 15.

18 A kit comprising a poly(N)polymerase characterized in that the poly(N)polymerase
25 is immobilized onto a solid support by covalent binding, wherein the poly(N)polymerase stays active when immobilized onto a solid support and wherein the poly(N)polymerase is selected from the group consisting of poly(A)polymerase, poly(U)polymerase, poly(G)polymerase and poly(C)polymerase, a poly(N)polymerase reaction buffer, and nucleoside monophosphates, optionally a nucleotide mixture, further optionally an RNA
30 polymerase, and optionally an RNA *in vitro* transcription buffer.