

[52] In one aspect, the invention relates to an RNA produced by a method of the invention.

[53] In one aspect, the invention relates to a composition comprising RNA produced by the method of the invention.

[54] In one aspect, the invention relates to a use of the RNA produced by the method of the invention or use of the composition comprising RNA produced by the method of the invention, in the manufacture of a medicament for treating a subject, wherein (i) the RNA or composition comprising RNA are to be obtained, and (ii) the medicament is to be administered to the subject.

[55] In one aspect, the invention relates to a method of producing an RNA by in vitro transcription, wherein the method comprises restricting concentration of UTP or functional analogs thereof during the in vitro transcription reaction.

[56] In one aspect, the invention relates to an in vitro transcription reaction comprising an RNA template comprising a promoter that directs transcription of a template to generate a transcript, optionally with a polyA sequence element; an RNA polymerase that acts on the promoter; and adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof, wherein the starting concentration of UTP or functional analog thereof, is lower than the concentration of CTP and/or ATP or functional analogs thereof.

[57] In one aspect, a use of the RNA produced by a method of the invention or use of the composition comprising RNA produced by a method of the invention, in the manufacture of a medicament for treating a subject.

[58] In one embodiment, the invention relates to a method for reducing the amount of double-stranded RNA resulting from in vitro transcription of RNA, the method comprising in vitro transcribing RNA from a template under transcription conditions in which the amount or concentration of UTP or a functional analog thereof is a limiting amount or concentration for the transcribing of the RNA in comparison to the amount or concentration of one or more of ATP, CTP and/or GTP or a respective analog thereof.

[59] In one embodiment, the invention relates to a method for reducing the amount of double-stranded RNA in a composition comprising RNA resulting from in vitro transcription of RNA, the method comprising in vitro transcribing RNA from a template under transcription conditions in which the amount or concentration of UTP or a functional analog thereof is a limiting amount or concentration for the transcribing of the RNA in comparison to the

amount or concentration of one or more of ATP, CTP and/or GTP or a respective analog thereof.

[60] In one embodiment, the invention relates to a method for reducing the immunogenicity of a composition comprising RNA resulting from *in vitro* transcription of RNA, the method comprising *in vitro* transcribing RNA from a template under transcription conditions in which the amount or concentration of UTP or a functional analog thereof is a limiting amount or concentration for the transcribing of the RNA in comparison to the amount or concentration of one or more of ATP, CTP and/or GTP or a respective analog thereof.

[61] In an embodiment, the double-stranded RNA is the result of at least two distinct RNA molecules annealing to each other, i.e., the result of inter-molecular binding. In an embodiment, the double-stranded RNA is the result of intra-molecular binding, i.e., parts of an RNA molecule annealing to itself, e.g., in cases where the transcript backfolds on itself.

Brief Description of the Drawing

[62] **Figure 1** demonstrates exemplary results for dsRNA content generated by an IVT reaction transcribing unmodified RNA with step-wise addition of NTP. RNA was *in vitro* transcribed with reduced starting concentration (limited) of indicated NTP. G – GTP, A-ATP, U-UTP, A/U-ATP+UTP. The limited NTP were fed step-wise to the IVT reaction until the final concentration NTP was reached for all NTPs. All RNA were co transcriptionally capped using CC413 cap analog. As a control the limitation of GTP was used. **A.** RNA yield was unaffected compared to control by the type of NTP that was fed over the course of the reaction. **B.** RNA integrity was unaffected compared to control by the type of NTP that was fed over the course of the reaction. **C.** dsRNA content was increased compared to control when ATP was fed and reduced when UTP was fed. Feeding both ATP and UTP abrogated each other's effect, resulting in a dsRNA content comparable to that of control (GTP fed). **D.** Capping efficiency was reduced compared to control when GTP was not fed.

[63] **Figure 2** demonstrates exemplary results for dsRNA content generated by an IVT reaction of unmodified RNA with step-wise addition of UTP or GTP and UTP, which rescues capping efficiency. RNA was *in vitro* transcribed with reduced starting concentration (limited) of indicated NTP. G – GTP, U-UTP, G/U-GTP+UTP. The limited NTP were step wise fed to the IVT reaction until the final concentration NTP was reached for all NTPs. As a control the limitation of GTP was used. All RNA were co

Claims

We claim:

1. A method of producing an RNA comprising transcribing RNA from a DNA template using a reaction mix which comprises adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof, wherein the starting concentration of UTP, or a functional analog thereof, is lower than the starting concentration of CTP and/or ATP, or a functional analog thereof, wherein the method comprises supplementing the reaction mix during the course of the transcription reaction with a composition which comprises UTP, or a functional analog thereof, and is substantially free of CTP or ATP, or a functional analog thereof.
2. A method of producing an RNA comprising transcribing RNA from a DNA template using a reaction mix which comprises adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof, wherein the starting concentration of CTP, or a functional analog thereof, is equal to the starting concentration of ATP, or a functional analog thereof, and wherein the starting concentration of UTP, or a functional analog thereof, is lower than the starting concentration of CTP or ATP, or a functional analog thereof, wherein the method comprises supplementing the reaction mix with UTP, or a functional analog thereof, during the course of the transcription reaction.
3. A method of producing a composition comprising RNA having a reduced double- stranded (ds) RNA content, wherein the method comprises transcribing RNA from a DNA template using a reaction mix which comprises adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof, wherein the starting concentration of UTP, or a functional analog thereof, is lower than the starting concentration of CTP and/or ATP, or a functional analog thereof, wherein the method comprises supplementing the reaction mix during the course of the transcription reaction with a composition which comprises UTP, or a functional analog thereof, and is substantially free of CTP or ATP, or a functional analog thereof.

4. A method of producing a composition comprising RNA having a reduced double- stranded (ds) RNA content, wherein the method comprises transcribing RNA from a DNA template using a reaction mix which comprises adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof, wherein the starting concentration of CTP, or a functional analog thereof, is equal to the starting concentration of ATP, or a functional analog thereof, and wherein the starting concentration of UTP, or a functional analog thereof, is lower than the starting concentration of CTP or ATP, or a functional analog thereof, wherein the method comprises supplementing the reaction mix with UTP, or a functional analog thereof, during the course of the transcription reaction.

5. The method of claim 3 or 4, wherein:

a) the double-stranded (ds) RNA content and/or the immunogenicity of the composition comprising RNA is reduced compared to the dsRNA content and/or immunogenicity of a composition comprising RNA transcribed from the same DNA template using equimolar amounts of adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof.

6. The method of any one of claims 1 to 5, wherein uridine triphosphate (UTP), or a functional analog thereof, is present in a starting concentration that limits the rate of transcription.

7. The method of any one of claims 1 to 6, wherein the ratio of the starting concentration of uridine triphosphate (UTP), or a functional analog thereof, to the starting concentration of cytidine triphosphate (CTP) or adenosine triphosphate (ATP), or a functional analog thereof, is between about 1:1.5 and about 1:15.

8. The method of any one of claims 1 to 7, wherein the reaction mix is supplemented with uridine triphosphate (UTP), or a functional analog thereof:
a) when the concentration of UTP, or a functional analog thereof, nears depletion; and/or
b) at least once during the course of the transcription reaction; and/or

c) continuously or periodically with uridine triphosphate (UTP), or a functional analog thereof, during the course of the transcription reaction.

9. The method of any one of claims 1 to 8, wherein supplementing the reaction mix with uridine triphosphate (UTP), or a functional analog thereof, maintains or restores the initial ratio of the concentration of UTP, or a functional analog thereof, to the concentration of cytidine triphosphate (CTP) or adenosine triphosphate (ATP), or a functional analog thereof.

10. The method of any one of claims 1 to 9, wherein the reaction mix is supplemented with uridine triphosphate (UTP), or a functional analog thereof, until the end of the transcription reaction.

11. The method of any one of claims 1 to 10 wherein the starting concentration of guanosine triphosphate (GTP), or a functional analog thereof, is lower than the starting concentration of cytidine triphosphate (CTP) or adenosine triphosphate (ATP), or a functional analog thereof, optionally wherein the reaction mix is supplemented with guanosine triphosphate (GTP), or a functional analog thereof, during the course of the transcription reaction.

12. The method of claim 11, wherein guanosine triphosphate (GTP), or a functional analog thereof, is present in a starting concentration that limits the rate of transcription.

13. The method of claim 11 or 12, wherein the ratio of the starting concentration of guanosine triphosphate (GTP), or a functional analog thereof, to the starting concentration of cytidine triphosphate (CTP) or adenosine triphosphate (ATP), or a functional analog thereof, is between about 1:1.5 and about 1:15.

14. The method of claim 11, wherein the reaction mix is supplemented with guanosine triphosphate (GTP), or a functional analog thereof:

a) when the concentration of GTP, or a functional analog thereof, nears depletion; and/or

- b) at least once during the course of the transcription reaction; and/or
- c) continuously or periodically with guanosine triphosphate (GTP), or a functional analog thereof, during the course of the transcription reaction.

15. The method of any one of claims 11 to 14, wherein supplementing the reaction mix with guanosine triphosphate (GTP), or a functional analog thereof, maintains or restores the initial ratio of the concentration of GTP, or a functional analog thereof, to the concentration of cytidine triphosphate (CTP) or adenosine triphosphate (ATP), or a functional analog thereof.

16. The method of any one of claims 11 to 15, wherein the reaction mix is supplemented with guanosine triphosphate (GTP), or a functional analog thereof, until the end of the transcription reaction.

17. The method of any one of claims 1 to 16, wherein the method does not comprise supplementing the transcription mix with cytidine triphosphate (CTP) and/or adenosine triphosphate (ATP), or a functional analog thereof, during the course of the transcription reaction.

18. The method of any one of claims 1 to 17, wherein the reaction mix comprises a start nucleotide corresponding to the first nucleotide in the RNA molecule, optionally wherein the start nucleotide is a nucleoside monophosphate, a nucleoside diphosphate, a nucleoside triphosphate or a dinucleoside triphosphate.

19. The method of claim 18, wherein the start nucleotide is a 5' cap or a 5' cap analog, optionally wherein the 5' cap analog is selected from the group consisting of G[5']ppp[5']G, m⁷G[5']ppp[5']G, m₃^{2,2,7}G[5']ppp[5']G, m₂^{7,3'-O}G[5']ppp[5']G (3'-ARCA), m₂^{7,2'-O}GpppG (2'-ARCA), m₂^{7,2'-O}GppspG D1 (β-S-ARCA D1), m₂^{7,2'-O}GppspG D2 (β-S-ARCA D2) and m₂^{7,3'-O}Gppp(m^{2'-O})ApG (CC413).

20. The method of claim 19, wherein the 5' cap or 5' cap analog in the reaction mix is present in excess compared to guanosine triphosphate (GTP), or a functional analog thereof, optionally wherein the ratio of the starting concentration of 5' cap or 5' cap analog to the starting concentration of guanosine triphosphate (GTP), or a functional analog thereof, is between about 2:1 and about 20:1, and optionally wherein the ratio of the starting concentration of 5' cap or 5' cap analog to the starting concentration of guanosine triphosphate (GTP), or a functional analog thereof, is about 4:1.

21. The method of any one of claims 1 to 20, wherein the reaction mix further comprises an RNA polymerase, a buffer and at least one monovalent or divalent cation, optionally wherein the cation is Li^+ , Na^+ , K^+ , NH_4^+ , tris(hydroxymethyl)aminomethane cation, Mg^{2+} , Ba^{2+} or Mn^{2+} , and/or optionally wherein the RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase.

22. The method of any one of claims 1 to 21, wherein:

- a) the functional analog of uridine triphosphate (UTP) is selected from the group consisting of Pseudo-UTP, N1- Methylpseudo-UTP, 2-Thio-UTP and 4-Thio-UTP; and/or
- b) the functional analog of guanosine triphosphate (GTP) is selected from the group consisting of 7-Deaza-GTP, N1-Methyl-GTP and O6-Methyl-GTP; and/or
- c) the DNA template encodes one or more of a 5' untranslated region (UTR), a 3' UTR, an open reading frame and a poly(A)-tail; and/or
- d) the RNA comprises one or more of a 5' untranslated region (UTR), a 3' UTR, an open reading frame and a poly(A)-tail, optionally wherein the RNA encodes at least one peptide or protein; and/or
- e) the RNA is mRNA; and/or
- f) the pH value of the reaction mix is kept substantially constant during the course of the transcription reaction; and/or
- g) the progress of the transcription reaction is monitored in real time; and/or
- h) the method is performed using a bioreactor.

23. An RNA produced by the method of any one of claims 1 to 22.
24. A composition comprising RNA produced by the method of any one of claims 3 to 22.
25. Use of the RNA produced by the method of any one of claims 1 to 22 or use of the composition comprising RNA produced by the method of any one of claims 3 to 22, in the manufacture of a medicament for treating a subject, wherein
- (i) the RNA or composition comprising RNA are to be obtained, and
 - (ii) the medicament is to be administered to the subject.
26. Use of the RNA of claim 23 or use of the composition comprising RNA of claim 24, in the manufacture of a medicament for treating a subject.
27. A method of producing an RNA by *in vitro* transcription, the method comprising:
restricting concentration of UTP or functional analogs thereof during the *in vitro* transcription reaction.
28. An *in vitro* transcription reaction comprising:
an RNA template comprising a promoter that directs transcription of a template to generate a transcript with a polyA sequence element;
an RNA polymerase that acts on the promoter; and
adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP), or functional analogs thereof, wherein the starting concentration of UTP or functional analog thereof, is lower than the concentration of CTP and/or ATP or functional analogs thereof.