

RNA (mRNA) transcripts, forming complex gene regulatory networks. Target recognition for miRNA is based on partial complementarity between the miRNA and the 3' untranslated region (3'UTR) of each target mRNA, although targeting is also observed in open reading frames (ORFs) at lower frequency. While miRNAs are naturally occurring as small RNAs repressors for gene expression, artificial short interfering RNAs (siRNAs) have been developed as powerful tools for experimental gene repression. In contrast to the partial complementarity observed with miRNA target interactions, siRNAs are designed to be perfectly complementary to a single mRNA target, enabling the repression of individual genes and are well established and can be designed and generated on demand.

[011] In order to enable a tailored gene regulation of multiple specific target lipases self-designed and non-naturally occurring artificial miRNAs (amiRNA) need to be designed. Such artificially designed miRNAs to target multiple desired mRNAs are rare and not established. *De novo* design of miRNAs is difficult and has not been shown for targets such as hydrolases (e.g., lipases and/or carboxylesterases). Moreover, while some natural miRNAs have been identified that improve e.g., productivity, no natural miRNAs seem to have been described or identified that would target and downregulate an enzyme class such as hydrolases. Thus, there is a need to target multiple hydrolases simultaneously involved in PS degradation and/or associated with immunogenicity (including lipases and/or carboxylesterases).

SUMMARY

[012] Based on micro-conserved regions in the mRNA sequence of two sets of target HCPs, we provide a proof-of-concept for a simultaneous multi-lipase knockdown in CHO cells using single artificial miRNAs (amiRNAs). The designed amiRNAs were not only able to reduce PS degradation but lay the foundation to expand this tool to other areas of cell line phenotype engineering.

[013] The aim of the present disclosure is to provide a cell line engineering approach for simultaneously targeting multiple hydrolases acting on ester bonds, such as lipases, in mammalian cells to reduce hydrolase activity, particularly PS degrading activity and/or immunogenic activity of contaminating hydrolases in compositions comprising a recombinant protein of interest, such as mAbs, produced in said mammalian cells. Therefore, highly conserved transcripts of hydrolases acting on ester bonds, particularly lipases, were targeted by *de novo* designed artificial microRNAs (amiRNA). These amiRNAs simultaneously knock-down multiple hydrolases, such as lipases, via perfect and/or imperfect mRNA target binding and can be used for stable or transient expression of amiRNAs targeting multiple hydrolases (e.g., lipases) along with the transgene of interest. Methods of reducing hydrolase expression in a mammalian cells using said amiRNA(s) resulting in reduced hydrolytic activity and thus polysorbate degradation in a product produced in the mammalian cells relates to all unit operations of the manufacturing process including the production process using cell cultures (vial thaw until harvest process, including HCCF), downstream intermediates and composition comprising said intermediates, as well as more final compositions, such as bulk drug substance (DS) and formulated drug product (DP). Particularly, a proof-of-concept is provided that artificial miRNAs

comprising designed artificial miRNA guide strand sequences targeting multiple hydrolases (e.g., lipases or carboxylesterases) can be *de novo* designed and are effective in reducing and/or preventing PS degrading activity in the harvested cell culture fluid of a mammalian cell used for expression of a protein of interest (due to the presence of contaminating HCPs with hydrolase activity acting on ester bonds). Because these artificial miRNAs can target multiple targets simultaneously and can further be used as a mixture of different artificial miRNAs, this approach allows tackling contaminating PS degradation in compositions comprising a protein of interest already in the upstream process and without knowing the exact hydrolase responsible for the observed degradation.

[014] In one aspect the disclosure relates to an artificial microRNA (miRNA) comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds. The two or more hydrolases are endogenous hydrolases of a mammalian cell, preferably of a human or a rodent cell, more preferably of a CHO cell. The artificial miRNA guide strand sequence may have perfect or imperfect complementarity to target sequences of at least two mRNAs encoding the two or more mammalian hydrolases. Preferably, the artificial miRNA guide strand sequence has imperfect complementarity to all or all but one target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases. The target sequences are located in the 3' untranslated regions (3'UTR) and/or in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases. In certain examples, the artificial miRNA targeting two or more mammalian hydrolases reduces expression and/or activity of said two or more hydrolases in a mammalian cell, preferably a rodent or human cell, more preferably a CHO cell.

[015] The artificial miRNA guide strand sequence has about 19 to 25 nucleotides, preferably 20 to 24, more preferably 21 to 23 nucleotides. Further, the artificial miRNA guide strand sequence hybridizes to the target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases independently, without the formation of a bulge loop and/or with formation of a maximum of two bulge loops, preferably without the formation of a bulge loop. Preferably, the artificial miRNA guide strand sequence has at least 55% sequence complementarity with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases; and/or wherein the miRNA guide strand sequence has at least 13 Watson-Crick base pairings with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases. Examples of suitable miRNA guide strand sequences are artificial miRNA guide strand sequence having the nucleotide sequence of SEQ ID NO: 1 (LP 8mer), SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 3 (LH 8mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical). The artificial miRNA may be a pri-miRNA, a pre-miRNA, a double stranded mature miRNA (comprising the passenger strand and the guide strand) or a single stranded miRNA guide strand. The artificial miRNA may also be a single or double stranded miRNA mimic.

[016] In certain examples, one of the two or more mammalian hydrolases is lipoprotein lipase (LPL). The target sequence(s) in the mRNA encoding LPL is preferably within nucleotides 560-590,

700-745, 930-960 or 1330-1360 of SEQ ID NOs: 6 or 7, preferably within nucleotides 560-590, 705-745, or 930-960 of SEQ ID NOs: 6 or 7. In additional or alternative examples, the two or more mammalian hydrolases are selected from the group consisting of lipoprotein lipase (LPL), phospholipase B-like 2 (PLBL2), phospholipase A1 (Pla1a), inactive pancreatic lipase-related protein 1 (PNLIPRP1), inactive pancreatic lipase-related protein 2 (PNLIPRP2), pancreatic triacylglycerol lipase precursor (PNLIP), lipase member I precursor (LipI), lipase member H precursor (LipH), lipase member G precursor (LipG), lipase member C precursor (LipC) and isoforms thereof.

[017] In certain examples, the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergo base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9) complementary to the target sequence(s) and having 0, 1, 2 or 3 mismatches with the target sequence(s), preferably 0, 1 or 2 mismatches, and (ii) a GC-content of 55-70% wherein all or all but one or all but two guanine (G) or cytosine (C) are complementary to the target sequence(s); and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 1, 2 or 3 mismatches with the target sequence(s), preferably wherein the mismatches are not consecutive mismatches, and (ii) a GC-content of $\leq 50\%$ in the last 6 nucleotides, wherein 1 or 2 of the complementary nucleotides are G or C. Examples of such artificial miRNA guide strand sequences are artificial miRNA guide strand sequences having the nucleotide sequence of SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).

[018] In other examples, the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s), and (ii) a GC-content of 55-75%, wherein at least 4 of the consecutive nucleotides are complementary to the target sequence(s) are G or C; and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s), preferably wherein the last nucleotide is complementary to the target sequence(s) and/or the penultimate nucleotide is a mismatch, and (ii) a GC-content of 30-50% in the last 6 nucleotides, preferably wherein at least 2 of the complementary nucleotides are G or C. Examples of such artificial miRNA guide strand sequences are artificial miRNA guide strand sequence having the nucleotide sequence of SEQ ID NO: 1 (LP 8mer) or SEQ ID NO: 2 (LP 6mer).

[019] In another aspect, the disclosure provides a mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA according to the disclosure. The mammalian expression vector may further comprise at least one gene of interest encoding a recombinant protein of interest. In certain examples, the mammalian expression vector comprises a polynucleotide sequence encoding two or more of the artificial miRNAs according to the disclosure, wherein the artificial miRNAs are identical or different.

[020] In yet another aspect, the disclosure relates to a mammalian cell comprising the artificial miRNAs according to the disclosure or the mammalian expression vector according to the disclosure. The mammalian cell may be a human or a rodent cell, preferably a rodent cell, more preferably a CHO cell. In a preferred example, the mammalian cell further expresses a recombinant protein of interest.

[021] In yet another aspect, the disclosure relates to a use of the artificial miRNA according to the disclosure in a mammalian cell for preventing expression of at least two endogenous hydrolases in the mammalian cell and/or preventing hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell.

[022] Provided is a method for manufacturing a protein of interest comprising the steps of: (a) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into a mammalian cell; (b) introducing an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds into the mammalian cell, wherein the artificial miRNA is introduced before, simultaneously or after step (a) into the mammalian cell, wherein the artificial miRNA is introduced as RNA or as a mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA; (c) cultivating said mammalian cell under conditions that allow expression of the recombinant protein of interest; and (d) harvesting the recombinant protein of interest in the harvested cell culture fluid. The method according to the disclosure may further comprise the following steps: (e) purifying the recombinant protein of interest; and (f) formulating the purified recombinant protein of interest into a composition. The harvested cell culture fluid has reduced hydrolase activity and/or reduced PS degrading activity. Preferably the harvested cell culture fluid has reduced hydrolase activity and/or reduced PS degrading activity compared to harvested cell culture fluid comprising the recombinant protein of interest produced in a mammalian cell not comprising or encoding the artificial miRNA. In a preferred example, the composition comprising the purified recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity.

[022a] In another aspect, provided is a method for manufacturing a protein of interest comprising the steps of: (a) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into a mammalian cell; (b) introducing an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds into the mammalian cell, wherein the artificial miRNA is introduced before, simultaneously or after step (a) into the mammalian cell, wherein the artificial miRNA is introduced as RNA or as a mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA; (c) cultivating said mammalian cell under conditions that allow expression of the recombinant protein of interest; and (d) harvesting the recombinant protein of interest in the harvested cell culture fluid; and optionally further comprises the following steps: (e) purifying the recombinant protein of interest; and (f) formulating the purified recombinant protein of interest into a composition.

[023] In certain examples, the artificial miRNA is the artificial miRNA according to the disclosure and/or the mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA is the mammalian expression vector according to the disclosure. The two or more mammalian hydrolases are endogenous hydrolases of the mammalian cell, preferably the mammalian cell is a human or a rodent cell, more preferably a CHO cell.

[024] The disclosure further encompasses a composition comprising a recombinant protein of interest, wherein the recombinant protein of interest is obtainable by the method of the disclosure and the composition preferably further comprises PS (such as PS20 or PS80), more preferably at a concentration of 0.1 g/L or more. For instance, in such a composition comprising PS (PS20 or PS80) preferably less than about 10% of PS is degraded when the composition is stored at about 2°C to about 8°C for at least six months. The composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity, preferably the composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity compared to a composition comprising the recombinant protein of interest produced in a mammalian cell without introducing or comprising the artificial miRNA.

DESCRIPTION OF THE FIGURES

[025] **Figure 1: Protein sequence alignments of LPL with its eight homologs (Lipase 1a – 1h) and LPL with PLBL2.** Lipase amino acid sequences (LPL, Lipase 1a – 1h and PLBL2) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type “Global alignment with free end gaps” of the Geneious Prime® Software. (A) Amino acids 140 – 320 of the LPL with its eight homologs (Lipase 1a - 1h) alignment. (B) Amino acids 240 – 450 of the LPL with PLBL2 alignment. Consensus: Black regions = sequences conserved between all lipases; grey = sequences partially conserved between lipases; light grey = less conserved sequences between lipases. Identity: The bars indicate the degree of homology between lipases. Lipase 1 = LPL; Lipase 2 = PLBL2; Lipase 1a = Pla1a; Lipase 1b = PNLIPRP1; Lipase 1c = PNLIPRP2; Lipase 1d = PNLIP; Lipase 1e = LipI; Lipase 1f = LipH; Lipase 1g = LipG; Lipase 1h = LipC.

[026] **Figure 2: Transcript sequence alignments of LPL with its eight homologs (Lipase 1a - 1h) and LPL with PLBL2 for identification of amiRNA binding sites.** Lipase transcript sequences (LPL, Lipase 1a – 1h and PLBL2) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type “Global alignment with free end gaps” of the Geneious Prime® Software. (A) Transcript sequences 480 – 1020 of the LPL with its eight homologs (Lipase 1a - 1h) alignment. Designed amiRNAs (grey arrows): 9 = LH 9mer; NC = LH Non-canonical; 8 = LH 8mer. (B) Transcript sequences 620 – 1630 of the LPL with PLBL2 alignment. Designed amiRNAs (grey arrows): 6 = LP 6mer; 8 = LP 8mer. Consensus: Black regions = sequences conserved between all lipases; grey = sequences partially conserved between lipases; light grey = less conserved sequences between lipases. Identity: The bars indicate the degree of homology between lipases. Lipase 1 = LPL; Lipase 2 = PLBL2; Lipase 1a = Pla1a; Lipase 1b = PNLIPRP1; Lipase 1c = PNLIPRP2; Lipase 1d = PNLIP; Lipase 1e = LipI; Lipase 1f = LipH; Lipase 1g = LipG; Lipase 1h = LipC.

[027] **Figure 3: Binding of *de novo* designed amiRNAs against LPL and its eight homologs (Lipase 1a-1h).** Lipase transcript sequences (LPL, Lipase 1a – 1h) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type “Global alignment with free end gaps” of the Geneious Prime® Software resulting in the consensus sequence numbering