## 2. Description of Related Art

**[0006]** Cystinuria is a hereditary disorder caused by mutations in the *SLC3A1* and *SLC7A9* genes encoding the kidney proximal tubule's cystine and dibasic amino acid transporter that leads to abnormal excretion of cystine (the disulfide form of the amino acid cysteine) and the formation of cystine crystals/stones in the urinary tract. There are few therapeutics available to patients suffering from the hereditary disorder cystinuria wherein a defective kidney transporter is unable to re-uptake cystine during renal filtration. Cystine, the disulfide form of the amino acid L-cysteine, is highly insoluble and in cystinuria patients reaches high concentrations in the urinary tract resulting in the formation of cystine crystals and stones. Existing therapies that reduce circulating cystine levels partially prevent urinary tract stone formation but have significant adverse effects that limit their use. Therapies are needed to reduce and prevent cystine stone formation in the kidney and bladder.

## SUMMARY

**[0007]** In one aspect, the present disclosure refers to an isolated, modified primate cystathionine-γ-lyase (CGL) enzyme comprising the following substitutions relative to a native human CGL amino acid sequence (SEQ ID NO: 1), wherein the modified enzyme has both cystinase and cysteinase activity, said substitutions being: alanine at position 193, glycine at position 311, valine at position 339, and serine at position 353. The present disclosure describes the engineering of primate cystathionine-gamma-lyase ("CGL") enzymes such that both L-cystine and L-cysteine (referred to herein as "L-cyst(e)ine") can be efficiently degraded from serum, and providing the modified CGL enzymes in a formulation suitable for human therapy. To develop an enzyme displaying low $K_M$ and high catalytic activity, $k_{cat}$, as compared to the native enzyme, the native enzyme was engineered by modifying selected amino acids, which modifications result in an enzyme having dramatically improved enzymatic properties. As such, modified CGL enzymes, as described herein, overcome a major deficiency in the art by providing novel enzymes that comprise human or primate polypeptide sequences having improved L-cyst(e)ine-degrading catalytic activity. As this enzyme is comprised of a human sequence, it is not likely to induce adverse immunological responses. As such, these modified enzymes may be suitable for human therapy and have low immunogenicity.

**[0008]** Methods are disclosed of utilizing an engineered human cystathionine-gamma-lyase (CGL) enzyme that efficiently converts cystine to cysteine-persulfide, which subsequently decays to free cysteine and $H_2S$, such that it is a suitable therapy for treating cystinuria patients by preventing cystine accumulation and formation of stones in the kidney and urinary tract. In addition, the engineered human CGL enzyme can convert cysteine to pyruvate, ammonia, and hydrogen sulfide, in effect reducing the amount of cysteine that can oxidize to form cystine. As cystine is a non-essential amino acid, which is normally

**WHAT IS CLAIMED IS:**

1. An isolated, modified primate cystathionine-γ-lyase (CGL) enzyme comprising ~~at least~~ the following substitutions relative to a native human CGL amino acid sequence (SEQ ID NO: 1), wherein the modified enzyme has both cystinase and cysteinase activity, said substitutions being ~~selected from the group consisting of~~:

~~(a)~~ alanine at position 193, glycine at position 311, valine at position 339, and serine at position 353.~~;~~

~~(b) proline at position 200, glycine at position 311, valine at position 339, and serine at position 353;~~

~~(c) alanine at position 193, proline at position 200, glycine at position 311, valine at position 339, and serine at position 353;~~

~~(d) glutamic acid at position 55, threonine at position 59, methionine at position 91, lysine at position 234, aspartic acid at position 336, and valine at position 339;~~

~~(e) glutamic acid at position 55, threonine at position 59, serine at position 91, lysine at position 234, aspartic acid at position 336, and valine at position 339;~~

~~(f) serine at position 189, glycine at position 193, glycine at position 311, valine at position 339, and serine at position 353;~~

~~(g) arginine at position 163, glycine at position 311, valine at position 339, and serine at position 353;~~

~~(h) tryptophan at position 51, glutamic acid at position 55, threonine at position 59, aspartic acid at position 336, and valine at position 339;~~

~~(i) glutamic acid at position 55, alanine at position 193, glycine at position 311, aspartic acid at position 336, valine at position 339, and serine at position 353;~~

~~(j) histidine at position 200, glycine at position 311, valine at position 339, and serine at position 353;~~

~~(k) glycine at position 311, valine at position 339, and serine at position 353;~~

~~(l) isoleucine at position 59, valine at position 339, and serine at position 353;~~

~~(m) methionine at position 91 and valine at position 339; and~~

~~(n) valine at position 339 and serine at position 353.~~

~~2. The isolated, modified CGL enzyme of claim 1, wherein the modified CGL enzyme is a modified *Pongo abelii* CGL enzyme.~~

~~3. The isolated, modified CGL enzyme of claim 2, wherein the modified *Pongo abelii* CGL enzyme comprises substitutions selected from the group consisting of:~~

~~(a) P193A, T311G, E339V, and V353S;~~

~~(b) A200P, T311G, E339V, and V353S;~~

~~(c) P193A, A200P, T311G, E339V, and V353S;~~

~~(d) H55E, V59T, L91M, N234K, T336D, and E339V;~~

~~(e) H55E, V59T, L91S, N234K, T336D, and E339V;~~

~~(f) T189S, P193G, T311G, E339V, and V353S;~~

~~(g) T163R, T311G, E339V, and V353S;~~

~~(h) A51W, H55E, V59T, T336D, and E339V;~~

~~(i) H55E, P193A, T311G, T336D, E339V, and V353S;~~

~~(j) A200H, T311G, E339V, and V353S;~~

~~(k) T311G, E339V, and V353S;~~

~~(l) V59I, E339V, and V353S;~~

~~(m) L91M and E339V; and~~

~~(n) E339V and V353S.~~

2~~4~~. The isolated, modified CGL enzyme of claim 1, wherein the modified CGL enzyme is a modified human CGL enzyme~~, a modified *Pan troglodytes* CGL enzyme, or a modified *Pan paniscus* CGL enzyme~~.

~~5. The isolated, modified CGL enzyme of claim 4, wherein the modified human CGL enzyme, the modified *Pan troglodytes* CGL enzyme, or the modified *Pan paniscus* CGL enzyme comprises substitutions selected from the group consisting of:~~

~~(a) P193A, T311G, E339V, and I353S;~~

~~(b) A200P, T311G, E339V, and I353S;~~

~~(c) P193A, A200P, T311G, E339V, and I353S;~~

~~(d) H55E, E59T, L91M, N234K, T336D, and E339V;~~

~~(e) H55E, E59T, L91S, N234K, T336D, and E339V;~~

~~(f) T189S, P193G, T311G, E339V, and I353S;~~

~~(g) T163R, T311G, E339V, and I353S;~~

~~(h) A51W, H55E, E59T, T336D, and E339V;~~

~~(i) H55E, P193A, T311G, T336D, E339V, and I353S;~~

~~(j) A200H, T311G, E339V, and I353S;~~

~~(k) T311G, E339V, and I353S;~~

~~(l) E59I, E339V, and I353S;~~

~~(m) L91M and E339V; and~~

~~(n) E339V and I353S.~~

~~6. The isolated, modified CGL enzyme of claim 1, wherein the modified CGL enzyme is a modified *Macaca fascicularis* CGL enzyme.~~

~~7. The isolated, modified CGL enzyme of claim 6, wherein the modified *Macaca fascicularis* CGL enzyme comprises substitutions selected from the group consisting of:~~

~~(a) P193A, T311G, E339V, and I353S;~~

~~(b) A200P, T311G, E339V, and I353S;~~

~~(c) P193A, A200P, T311G, E339V, and I353S;~~

~~(d) H55E, E59T, L91M, N234K, T336D, and E339V;~~

~~(e) H55E, E59T, L91S, N234K, T336D, and E339V;~~

~~(f) T189S, P193G, T311G, E339V, and I353S;~~

~~(g) V163R, T311G, E339V, and I353S;~~

~~(h) A51W, H55E, E59T, T336D, and E339V;~~

~~(i) H55E, P193A, T311G, T336D, E339V, and I353S;~~

~~(j) A200H, T311G, E339V, and I353S;~~

~~(k) T311G, E339V, and I353S;~~

~~(l) E59I, E339V, and I353S;~~

~~(m) L91M and E339V; and~~

~~(n) E339V and I353S.~~

3. The isolated, modified CGL enzyme of claim 1 or 2, wherein the modified CGL enzyme is at least 95% identical to SEQ ID NO: 1.

4. The isolated, modified CGL enzyme of any one of claims 1-3, wherein the modified CGL has the sequence of SEQ ID NO: 6.

5. The isolated, modified CGL enzyme of any one of claims 1-4, wherein the modified CGL enzyme has a $k_{cat}/K_M$ for L-cystine and/or L-cysteine that is greater than the $k_{cat}/K_M$ of human cystathionine-γ-lyase.

6. The isolated, modified CGL enzyme of any one of claims 1-5, wherein the modified CGL enzyme has a $k_{cat}/K_M$ for L-cystine that is greater than 10,000 $s^{-1}M^{-1}$ and/or wherein the modified CGL enzyme has a $k_{cat}/K_M$ for L-cysteine that is greater than 1,000 $s^{-1}M^{-1}$.

7~~8~~. The isolated, modified CGL enzyme of any one of claims 1-6~~7~~, further comprising a heterologous peptide segment or a polysaccharide.

8~~9~~. The isolated, modified CGL enzyme of claim 7~~8~~, wherein the heterologous peptide segment is an XTEN peptide, an IgG Fc, an albumin, or an albumin binding peptide.

9~~10~~. The isolated, modified CGL enzyme of claim 7~~8~~, wherein the polysaccharide comprises polysialic acid polymers.

10~~11~~. The isolated, modified CGL enzyme of any one of claims 1-9~~10~~, wherein the enzyme is coupled to polyethylene glycol (PEG).

11~~12~~. The isolated, modified CGL enzyme of claim 10~~11~~, wherein the enzyme is coupled to the PEG via one or more lysine residues.

12~~13~~. A nucleic acid comprising a nucleotide sequence encoding the enzyme of any one of claims 1-6~~7~~.

13~~14~~. The nucleic acid of claim 12~~13~~, wherein the nucleic acid is codon optimized for expression in bacteria, fungus, insects, or mammals.

14~~15~~. The nucleic acid of claim 13~~14~~, wherein the bacteria are *E. coli*.

15~~16~~. The nucleic acid of claim 14~~15~~, wherein the nucleic acid comprises a sequence according to ~~one of~~ SEQ ID NOs: ~~81-95~~96.

16~~17~~. An expression vector comprising the nucleic acid of any one of claims 12~~13~~-15~~16~~.

17~~18~~. A host cell comprising the nucleic acid of any one of claims 12~~13~~-15~~16~~.

18~~19~~. The host cell of claim 17~~8~~, wherein the host cell is a bacterial cell, a fungal cell, an insect cell, or a mammalian cell.

19~~20~~. A therapeutic formulation comprising an enzyme of any one of claims 1-10~~12~~, or the nucleic acid of any one of claims 12~~13~~-15~~16~~, in a pharmaceutically acceptable carrier.

2021. Use of a formulation of claim 1920 in the manufacture of a medicament for treating a subject having or at risk of developing cystinuria.

2122. The use of claim 2021, wherein the subject is maintained on a L-cystine and/or L-cysteine restricted diet.

2223. The use of claim 2021, wherein the subject is maintained on a methionine-restricted diet.

2324. The use of claim 2021, wherein the subject is maintained on a normal diet.

2425. The use of claim 2021, wherein the subject is a human patient.

2526. The use of claim 2021, wherein the formulation is to be administered intravenously, intraarterially, intraperitoneally, intramuscularly, intravascularly, subcutaneously, by injection, by infusion, by continuous infusion, or via a catheter.

2627. The use of claim 2021, wherein the subject has previously been treated for cystinuria and the enzyme is to be administered to prevent the recurrence of cystinuria.

2728. The use of claim 2021, further comprising at least a second cystinuria therapy to be administered to the subject.

2829. The use of claim 2728, wherein the second cystinuria therapy is a surgical therapy or a shock wave therapy.

2930. The use of claim 2728, wherein the second cystinuria therapy comprises an agent or drug selected from the group consisting of: bicarbonate, tris-hydroxymethylene-aminomethane (tromethamine-E), acetylcysteine, D-penicillamine, α-mercaptopropionylglycine, and captopril.

3031. The use of claim 2021, wherein the medicament reduces the level of cysteine and/or cystine in the subject's plasma and/or serum.

3132. The use of claim 2021, wherein the medicament reduces the level of cysteine and/or cystine in the subject's urine.

3233. The use of claim 2021, wherein the medicament prevents the formation of cystine stones in the subject's urinary tract.

3334. The use of claim 2021, wherein the medicament prevents the formation of cystine stones in the subject's kidneys.

3435. The use of claim 2021, wherein the medicament reduces the number of cystine stones in the subject's kidneys.

3536. Use of an isolated or modified CGL enzyme of any of claims 1 to 1112 or a composition comprising said modified CGL enzyme in the manufacture of a medicament for therapeutic application to a cystinuria patient.