

Eylea[®] (Regeneron, Tarrytown, NY) for the treatment of various ocular conditions, including wet type AMD, and is formulated for intravitreal administration. The fusion protein is also marketed as Zaltrap[®] (ziv-aflibercept) (Regeneron, Tarrytown, NY) for the treatment of certain types of cancer and is formulated for intravenous administration.

An attribute of a protein can have an important role in at least the quality of a protein product. Accordingly, provided herein are methods of characterizing and modifying the attributes of aflibercept and compositions comprising aflibercept with attributes, and related advantages.

SUMMARY

The present disclosure provides compositions of aflibercept, including compositions comprising a mixture of aflibercept species. Also provided herein are aflibercept species. In one aspect, the present disclosure provides a Y92L clipped species of aflibercept comprising SEQ ID NO: 3. In another aspect, the present disclosure provides a mixture of aflibercept species, wherein the amount of Y92L clipped species in the composition is less than 5.0%, as determined by rCE-SDS. In yet another aspect, the present disclosure provides a method of producing a composition comprising a mixture of aflibercept species comprising: subjecting a cell culture fluid comprising a mixture of aflibercept species to a purification process comprising an anion exchange chromatography (AEX) step followed by a hydrophobic interaction chromatography (HIC) step. In a further aspect, the present disclosure provides a composition comprising a mixture of aflibercept species manufactured by the method as described herein.

In one example, a species of aflibercept has one or more different attributes than another species of aflibercept. In some examples, the difference is the presence or absence of an attribute. In another example, the difference is the level or amount of an attribute. Also, provided herein are methods of characterizing one or more attributes of aflibercept as well as methods of modifying one or more attributes of aflibercept, purifying aflibercept, and producing compositions of aflibercept.

One example of the present disclosure is an aflibercept species that is a Y92L clipped species of aflibercept. The Y92L clipped species can comprise SEQ ID NO: 3.

Another example of the present disclosure is a composition comprising a mixture of aflibercept species, including the Y92L clipped species. In one example, the amount of Y92L clipped species in the composition is less than 5.0%, as determined by reduced capillary electrophoresis sodium dodecyl sulfate (rCE-SDS). In one example, the amount of Y92L clipped species in the composition is less than 0.8%, as determined by rCE-SDS. In some examples, the amount of Y92L clipped species in the composition is less than 3.0%, between 1.0% and 5.0% or between 1.0% and 3.0%, about 1.1%, about 3.0% or about 4.7% of the composition, as determined by rCE-SDS. In one example, the amount of Y92L clipped species in the composition is about 0.4%, as determined by rCE-SDS.

In another example of the present disclosure, the composition comprising a mixture of aflibercept species comprises N68 occupied species of aflibercept. In one example, at least 30% of the aflibercept species is occupied at position N68, as determined by rCE-SDS. In some examples, at least 50%, between 50-60%, about 39%, about 53%, about 54%, or about 55% of the aflibercept species is occupied at position N68. In some examples, the composition also comprises Y92L clipped species, such as between 1% and 3%, less than 0.8%, about 1.1% or about 0.4% of the composition.

The present disclosure also provides a composition comprising a mixture of aflibercept species, wherein the total sialic acid content of aflibercept species is between 6.0 and 10.0 mol/mol protein, as determined by LC-MS based peptide mapping. In one example, the total sialic acid content is about 6.8, about 8.5 or about 9.5 mol/mol protein. In some examples, the total sialic acid content is about 9.5 mol/mol protein, and wherein between 54-55% of the aflibercept species is occupied at position N68, and wherein the amount of Y92L clipped species in the mixture is about 1.1%.

Another example of the present disclosure is a composition comprising a mixture of aflibercept species, wherein the aflibercept species comprise less than 13% afucosylation in the Fc domain. The percentage of afucosylation can be determined by any method known in the art, such as LC-MS based peptide mapping. In one example, the aflibercept species comprise between 1.0-12% afucosylation in the Fc domain. In another example, the aflibercept species comprise less than 12%, 11%, 10%, 9%, 8%, 7%, 6%, or 5% afucosylation in the Fc domain. In one example, the aflibercept species comprise less than 10% afucosylation in the Fc domain. In another example, the aflibercept species comprise less than 5% afucosylation in the Fc domain. In another

example, the aflibercept species comprise about 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5% or 4% afucosylation in the Fc domain. In yet another example, the aflibercept species comprise about 4% afucosylation in the Fc domain.

Another example of the present disclosure is a composition comprising a mixture of aflibercept species, wherein the aflibercept species comprise between 0.1-2.0 (mol/mol protein) of sialic acid at N68; between 1.0-25.0% O-glycosylation at T33; between 70%-99% N-glycosylation at N36; between 20%-75% of N-glycosylation at N68; between 0.1-1.0% high mannose in the Fc domain; between 26% galactosylation in the Fc domain; about 1% sialylation in the Fc domain; or any combination thereof.

The aflibercept species can further comprise less than 13% afucosylation in the Fc domain, such as between 1.0-12% afucosylation in the Fc domain, or about 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5% or 4% afucosylation in the Fc domain, such as less than 5% afucosylation in the Fc domain. In one example, the aflibercept species comprise about 4% afucosylation in the Fc domain.

The aflibercept species in a mixture can also comprise about 0.7 (mol/mol protein) of sialic acid at N68; about 4% O-glycosylation at T33; about 95% N-glycosylation at N36; about 26% of N-glycosylation at N68; about 1% high mannose in the Fc domain; about 26% galactosylation in the Fc domain; about 1% sialylation in the Fc domain; less than 13% afucosylation in the Fc domain, such as between 1.0-12% afucosylation in the Fc domain, or about 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5% or 4% afucosylation in the Fc domain; or any combination thereof. In one example, the aflibercept species in a mixture comprises about 0.7 (mol/mol protein) of sialic acid at N68; about 4% O-glycosylation at T33; about 95% N-glycosylation at N36; about 26% of N-glycosylation at N68; about 1% high mannose in the Fc domain; about 6% afucosylation in the Fc domain; about 26% galactosylation in the Fc domain; about 1% sialylation in the Fc domain; or any combination thereof.

The aflibercept species in a mixture can also comprise about 0.7 (mol/mol protein) of sialic acid at N68; about 4% O-glycosylation at T33; about 95% N-glycosylation at N36; about 26% of N-glycosylation at N68; about 1% high mannose in the Fc domain; about 26% galactosylation in the Fc domain; about 1% sialylation in the Fc domain; less than 13% afucosylation in the Fc domain, such as between 1.0-12% afucosylation in the Fc domain, or about 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5% or 4% afucosylation in the Fc domain; less than 0.8% of Y92L clipped species, such as about 0.4%; or any combination thereof.

The aflibercept species in a mixture can also comprise about 0.8 (mol/mol protein) of sialic acid at N68; about 8.3% O-glycosylation at T33; about 90.9% N-glycosylation at N36; about 51.9% of N-glycosylation at N68; about 0.4% high mannose in the Fc domain; about 24.4% galactosylation in the Fc domain; about 3.8% sialylation in the Fc domain; less than 13% afucosylation in the Fc domain, such as between 1.0-12% afucosylation in the Fc domain, or about 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5% or 4% afucosylation in the Fc domain; or any combination thereof. In one example, the aflibercept species in a mixture comprises about 0.8 (mol/mol protein) of sialic acid at N68; about 8.3% O-glycosylation at T33; about 90.9% N-glycosylation at N36; about 51.9% of N-glycosylation at N68; about 0.4% high mannose in the Fc domain; about 4% afucosylation in the Fc domain; about 24.4% galactosylation in the Fc domain; about 3.8% sialylation in the Fc domain; or any combination thereof.

The aflibercept species in a mixture can also comprise about 0.8 (mol/mol protein) of sialic acid at N68; about 8.3% O-glycosylation at T33; about 90.9% N-glycosylation at N36; about 51.9% of N-glycosylation at N68; about 0.4% high mannose in the Fc domain; about 24.4% galactosylation in the Fc domain; about 3.8% sialylation in the Fc domain; less than 13% afucosylation in the Fc domain, such as between 1.0-12% afucosylation in the Fc domain, or about 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5% or 4% afucosylation in the Fc domain; less than 0.8% of Y92L clipped species, such as about 0.4%; or any combination thereof. In one example, the aflibercept species in a mixture comprises about 0.8 (mol/mol protein) of sialic acid at N68; about 8.3% O-glycosylation at T33; about 90.9% N-glycosylation at N36; about 51.9% of N-glycosylation at N68; about 0.4% high mannose in the Fc domain; about 4% afucosylation in the Fc domain; about 24.4% galactosylation in the Fc domain; about 3.8% sialylation in the Fc domain; about 0.4% of Y92L clipped species; or any combination thereof. In one example, the aflibercept species in a mixture comprises about 0.8 (mol/mol protein) of sialic acid at N68; about 8.3% O-glycosylation at T33; about 90.9% N-glycosylation at N36; about 51.9% of N-glycosylation at N68; about 0.4% high mannose in the Fc domain; about 4% afucosylation in the Fc domain; about 24.4% galactosylation in the Fc domain; about 3.8% sialylation in the Fc domain; and about 0.4% of Y92L clipped species.

The present disclosure also provides a method of increasing the binding of a composition comprising a mixture of aflibercept species to placental growth factor (PlGF) and/or VEGF-A comprising reducing the amount of Y92L clipped species in

the mixture or by reducing the N68 occupancy of aflibercept. The PlGF can be PlGF-1 or PlGF-2. In one example, the method of reducing the amount of Y92L clipped species in the mixture comprises purifying aflibercept with an anion exchange chromatography step followed by a hydrophobic interaction chromatography step.

5 Another example of the present disclosure is a method of producing a composition comprising a mixture of aflibercept species. The method can comprise subjecting a cell culture fluid comprising a mixture of aflibercept species to a purification process comprising an anion exchange chromatography (AEX) step followed by a hydrophobic interaction chromatography (HIC) step, wherein a lower
10 amount of Y92L clipped species is produced as compared to a purification process comprising an AEX step followed by a cation exchange chromatography (CEX) step. In some examples, the purification process does not include a CEX step.

 Another method of producing a composition comprising a mixture of aflibercept species comprises harvesting cells producing aflibercept, subjecting the harvested cell
15 culture fluid to a Protein A column, and performing anion exchange chromatography (AEX) followed by cation exchange chromatography (CEX) or hydrophobic interaction chromatography (HIC). In some examples, AEX is followed by HIC, and optionally, the method does not include CEX. In some examples, AEX is followed by CEX. In one example, the cells are Chinese Hamster Ovary (CHO) cells, which can be grown
20 by perfusion or fed-batch. The cells can be harvested by acid precipitation, centrifugation, microfiltration, depth filtration or any combination thereof. The method can also further comprise one or more viral inactivation and/or viral filtration step(s), or ultrafiltration/diafiltration (UF/DF) step. In some examples, the method does not comprise performing CEX prior to performing AEX, HIC, and/or size exclusion
25 chromatography (SEC).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts attributes of aflibercept mapped onto its structure.

Figure 2 is a graph of the relative VEGF-A binding (%) as a function of the percentage of Y92L clipped species of aflibercept.

30 **Figure 3** is a graph of the relative PlGF-1 binding (%) as a function of the percentage of N68 occupancy of aflibercept.

Figure 4 depicts 2 versions of a process to produce aflibercept. In the first process, AEX chromatography is followed by CEX. In the second process, AEX chromatography is followed by HIC.

Figure 5 depicts the reduced capillary electrophoresis-sodium dodecyl sulfate (rCE-SDS) results of aflibercept from hydrophobic interaction chromatography (HIC) fractions (F1-F5), along with the aflibercept species represented by each peak.

5 **Figure 6** depicts the reduced capillary electrophoresis-sodium dodecyl sulfate (rCE-SDS) results of aflibercept manufactured with a process using anion exchange followed by cation exchange (DS) and aflibercept manufactured with a process using anion exchange followed by hydrophobic interaction chromatography.

Figure 7 depicts the percentage of Y92L clipped species of aflibercept grown in various culture conditions.

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CLAIMS

What is claimed is:

1. A Y92L clipped species of aflibercept comprising SEQ ID NO: 3.
2. A composition comprising a mixture of aflibercept species, wherein the amount of Y92L clipped species in the composition is less than 5.0%, as determined by rCE-SDS
3. The composition of claim 2, wherein the amount of Y92L clipped species is less than 2 %, less than 1 % or less than 0.8 %.
4. The composition of claim 2, wherein the amount of Y92L clipped species is about 1.1 % or about 0.4 %.
5. A method of producing a composition comprising a mixture of aflibercept species comprising:

subjecting a cell culture fluid comprising a mixture of aflibercept species to a purification process comprising an anion exchange chromatography (AEX) step followed by a hydrophobic interaction chromatography (HIC) step.
6. The method of claim 5, wherein the purification process does not include a CEX step.
7. The method of any one of claims 5-6, comprising:
 - a) harvesting cells producing aflibercept;
 - b) subjecting the harvested cell culture fluid to a Protein A column; and
 - c) performing anion exchange chromatography (AEX) followed by hydrophobic interaction chromatography (HIC).
8. The method of any one of claims 5 and 7, wherein AEX is followed by CEX.
9. The method of any one of claims 5-8, wherein the cells are Chinese Hamster Ovary (CHO) cells.
10. The method of any one of claims 5-9, wherein the cells are grown by perfusion or fed-batch.
11. The method of any one of claims 5-10, wherein the cells are harvested by acid precipitation, centrifugation, depth filtration or any combination thereof.
12. The method of any one of claims 5-11, further comprising one or more viral inactivation and/or viral filtration step(s).

13. The method of any one of claims 5-12, further comprising a ultrafiltration/diafiltration (UF/DF) step.
14. The method of any one of claims 5-13, wherein the method does not comprise performing size exclusion chromatography (SEC).
15. The method of any one of claims 5, 7-14, wherein a lower amount of Y92L clipped species is produced as compared to a purification process comprising an AEX step followed by a cation exchange chromatography (CEX) step.
16. The method of any one of claims 5-15, wherein the amount of Y92L clipped species is less than 2 %, less than 1 % or less than 0.8 %.
17. The method of claim 5-16, wherein the amount of Y92L clipped species is about 1.1 % or about 0.4 %.
18. A composition comprising a mixture of aflibercept species manufactured by the method of claim 5.