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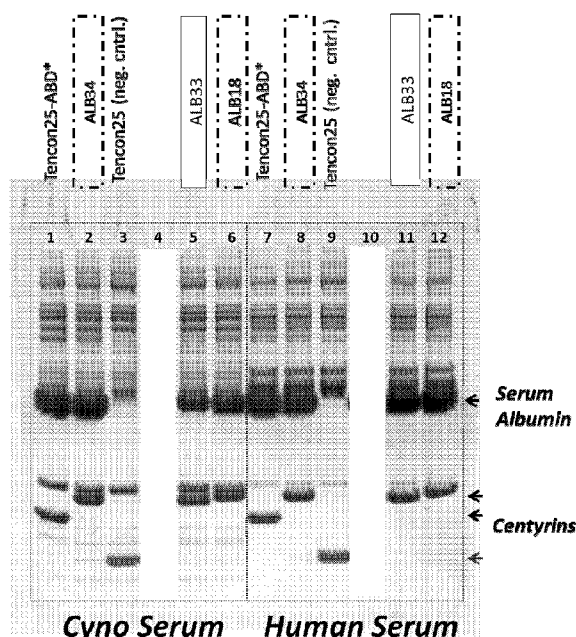
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(54) Title: SERUM ALBUMIN-BINDING FIBRONECTIN TYPE III DOMAINS

Figure 1



(57) Abstract: Fibronectin type III domains (FN3) that specifically bind to serum albumin, related polynucleotides capable of encoding serum albumin-specific FN3 domains, cells expressing the FN3 domains, as well as associated vectors, detectably labeled FN3 domains and FN3 domains fused to a heterologous moiety are useful in extending the half-life of molecules in diagnostic and therapeutic applications.

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SERUM ALBUMIN-BINDING FIBRONECTIN TYPE III DOMAINS

Sequence Listing

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 31, 2017, is named JBI5089WOPCT.txt and is 49,228 bytes in size.

Technical Field

The present invention relates to fibronectin type III (FN3) domains that specifically bind serum albumin. Such FN3 domains may be used, for example, for extending the *in vivo* serum half-life of drugs or proteins conjugated thereto. Methods for the production of such molecules and pharmaceutical compositions comprising them are also provided.

Background

Rapid elimination of biotherapeutic molecules from the blood stream can contribute to limited clinical effectiveness of these molecules or result in more frequent dosing for the patient. One method of elimination that commonly occurs is renal clearance due to glomerular filtration. This route of elimination is most associated with smaller biotherapeutics, as the rates of kidney filtration are greatly reduced for molecules with a molecular weight of greater 50,000 daltons (Kontermann, Curr Opin Biotechnol 2011). Several approved biotherapeutic drugs contain active portions that on their own fall below the filtration limit and are thus cleared quickly. To overcome this limitation, a number of technologies have been introduced to effectively increase the size of the therapeutic molecule to reduce kidney filtration.

Modification of therapeutic proteins to add Human Serum Albumin (HSA) has been shown to be an effective way to increase the hydrodynamic radius of a protein and reduce glomerular filtration. HSA exhibits a long half-life *in vivo* due to FcRn recycling and with a concentration of approximately 40 g/L, HSA is the most abundant protein found in the blood. FcRn recycling leads to a long half-life of approximately 19 days in humans. Additionally, biodistribution studies suggest that albumin may distribute within the body to areas important for targeting disease, such as inflamed joints or tumors

(Wunder, Muller-Ladner, Stelzer, Funk, Neumann, Stehle, Pap, Sinn, Gay and Fiehn, J Immunol 170: 4793-4801 2003). As such, the serum half-life of a number of proteins has been increased by producing them as either C-terminal or N-terminal fusions to HSA. Successful fusions include interferon alpha (Flisiak and Flisiak, Expert Opin Biol Ther 10: 1509-1515 2010), human growth hormone (Osborn, Sekut, Corcoran, Poortman, Sturm, Chen, Mather, Lin and Parry, Eur J Pharmacol 456: 149-158 2002), tumor necrosis factor (Muller, Schneider, Pfizenmaier and Wajant, Biochem Biophys Res Commun 396: 793-799 2010), coagulation factor IX (Metzner, Weimer, Kronthaler, Lang and Schulte, Thromb Haemost 102: 634-644 2009), coagulation factor VIIa (Schulte, Thromb Res 122 Suppl 4: S14-19 2008), insulin (Duttaroy, Kanakaraj, Osborn, Schneider, Pickeral, Chen, Zhang, Kaithamana, Singh, Schulingkamp, Crossan, Bock, Kaufman, Reavey, Carey-Barber, Krishnan, Garcia, Murphy, Siskind, McLean, Cheng, Ruben, Birse and Blondel, Diabetes 54: 251-258 2005), urokinase (Breton, Pezzi, Molinari, Bonomini, Lansen, Gonzalez De Buitrago and Prieto, Eur J Biochem 231: 563-569 1995), hirudin (Sheffield, Smith, Syed and Bhakta, Blood Coagul Fibrinolysis 12: 433-443 2001), and bispecific antibody fragments (Muller, Karle, Meissburger, Hofig, Stork and Kontermann, J Biol Chem 282: 12650-12660 2007) among others. HSA fusion proteins may have long serum half-lives, however large scale production of such fusion proteins is limited to eukaryotic expression systems. Additionally, the large size of HSA may lead to a loss in activity of the therapeutic due to steric hindrance.

An alternative method to take advantage of the long serum half-life of albumin is to produce therapeutic proteins as fusions to peptides or protein domains that bind to serum albumin in blood. These molecules can be selected to bind to albumin with various affinities, allowing the fusion protein to bind to and release from albumin with defined rates and residence times *in vivo*. For example, phage display was used to select a series of cysteine-constrained peptides that bind to albumins of different species. Expression of a Fab antibody fragment as a fusion to this peptide significantly increased the serum half-life of the Fab in mice and rabbits (Dennis, Zhang, Meng, Kadkhodayan, Kirchhofer, Combs and Damico, J Biol Chem 277: 35035-35043 2002) (US 20040253247A1). Subsequent studies have shown that fusion of this peptide to an antibody fragment leads to better peak tumor accumulation and more homogeneous tumor distribution compared to Fab and mAb molecules targeting the same antigen (Dennis, Jin, Dugger, Yang, McFarland, Ogasawara, Williams, Cole, Ross and Schwall,

Cancer Res 67: 254-261 2007) (US20050287153A1). A number of antibody fragments that bind specifically to albumin have been selected in a similar manner. A camelid V_{HH} antibody fragment (Nanobody) that binds to HSA was fused to another Nanobody that binds to TNF-alpha. The serum half-life of this molecule was extended from 54 minutes to 2.2 days in mice and showed accumulation in inflamed joints and efficacy in a mouse model of rheumatoid arthritis (Coppieters, Dreier, Silence, de Haard, Lauwereys, Casteels, Beirnaert, Jonckheere, Van de Wiele, Staelens, Hostens, Revets, Remaut, Elewaut and Rottiers, Arthritis Rheum 54: 1856-1866 2006). The same albumin binding Nanobody was fused to anti-EGFR Nanobodies, resulting in increased half-life and better tumor accumulation in xenograft models (Tijink, Laeremans, Budde, Stigter-van Walsum, Dreier, de Haard, Leemans and van Dongen, Mol Cancer Ther 7: 2288-2297 2008). Similarly, anti-albumin domain antibodies (dAbs) have been generated that bind to albumin. Fusion of these dAbs to the interleukin-1 receptor increased the half-life from 2 minutes to 4.3 hours in rats (Holt, Basran, Jones, Chorlton, Jespers, Brewis and Tomlinson, Protein Eng Des Sel 21: 283-288 2008). This technology has also been applied to fusions with interferon alpha 2b (Walker, Dunlevy, Rycroft, Topley, Holt, Herbert, Davies, Cook, Holmes, Jespers and Herring, Protein Eng Des Sel 23: 271-278 2010). In a similar manner, naturally occurring albumin binding domains from bacteria with affinity for albumin have been used for in vivo half-life extension (Anderson et al. 2011, Orlova et al., 2013, Malm et al., 2014). Jacobs et al. recently described a consensus designed albumin binding domain and demonstrated the potential for tuning of serum exposure profile based on affinity for albumin (Jacobs, Gibbs, Conk, Yi, Maguire, Kane, O'Neil, Protein Eng Des Sel 10:385-93, 2015). Despite their utility as research tools, the utility of such bacterially derived domains for therapeutic applications may be limited by their potential for immunogenicity and drug clearance by the resulting antibodies.

Thus, there is a need for serum albumin-binding molecules that can increase the serum half-life of therapeutics, that can possess desirable biophysical properties (e.g., substantially monomeric, well-folded, etc), that can be manufactured in a cost effective manner, and that are small enough to permit tissue penetration.

SUMMARY

The present invention comprises serum albumin-binding fibronectin type III (FN3) domains. Also described are related polynucleotides capable of encoding the provided FN3 domains, cells expressing the provided FN3 domains, as well as associated vectors. In addition, methods of using the provided FN3 domains are described. For example, given the extended serum half-life of albumin, albumin-binding peptides make ideal fusion partners for creation of therapeutic proteins with long serum half-lives.

In some embodiments, the present invention comprises an isolated FN3 domain, wherein the FN3 domain binds to domain I or III of human serum albumin, and wherein the serum half-life of the FN3 domain is at least 10-fold higher than the serum half-life of that of Tencon sequence of SEQ ID NO: 67. In other embodiments, the albumin-specific FN3 domains bind human serum albumin and cynomolgus monkey serum albumin. In yet other embodiments, the albumin-specific FN3 domain is based on Tencon sequence of SEQ ID NO: 1. In further embodiments, the albumin-specific FN3 domain is based on Tencon27 sequence of SEQ ID NO: 4. In some embodiments, the albumin-specific FN3 domain is based on the SEQ ID NO: 1 or the SEQ ID NO: 4, optionally having substitutions at residues positions 11, 14, 17, 37, 46, 73, and/or 86. In some embodiments, the albumin-specific FN3 domain is isolated from the library comprising the sequence of SEQ ID NOs: 2, 3, 5, 6, 7 or 8.

In some embodiments, the albumin-specific FN3 domain comprises a modified amino acid sequence in the C, CD, F and FG loops relative to Tencon sequence of SEQ ID NO: 1. In some embodiments, the albumin-specific FN3 domain comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 51-53. In some embodiments, the albumin-specific FN3 domain has a serum half-life of at least about 25 hours in cynomolgus monkey.

In addition to the described albumin-specific FN3 domains, also provided are polynucleotide sequences capable of encoding the described antibodies and antigen-binding fragments. Vectors comprising the described polynucleotides are also provided, as are cells expressing the albumin-specific FN3 domains herein. Also described are cells capable of expressing the disclosed vectors. These cells may be mammalian cells (such as 293F cells, CHO cells), insect cells (such as Sf7 cells), yeast cells, plant cells, or

bacteria cells (such as *E. coli*). A process for the production of the described FN3 domains is also provided.

The present invention also comprises methods of fusing or otherwise associating the described albumin-specific FN3 domains to various molecules to extend the half-lives of such molecules. For example, the extended serum half-life of serum albumin makes it an ideal fusion partner for creation of therapeutic proteins with long serum half-lives. As such, the albumin-specific FN3 domains have utility in the extending of half-lives of therapeutic drugs.

In one embodiment, a pharmaceutical composition comprising the albumin-specific FN3 domain is administered in order to improve the *in vivo* half-life of the therapeutic partner. Such half-life extension can be assayed by a variety of methods known in the art, including, for example, by monitoring the pharmacokinetics of the albumin-binding FN3 domain. Specific assays for these methods are provided in the EXAMPLES.

Within the scope of the invention are kits including the disclosed albumin-specific (binding) FN3 domains. The kits may be used to carry out the methods of using the albumin-specific FN3 domains provided herein, or other methods known to those skilled in the art. In some embodiments the described kits may include the FN3 domains described herein and reagents for use in detecting the presence of human serum albumin in a biological sample. The described kits may include one or more of the FN3 domains described herein and a vessel for containing the FN3 domains when not in use, instructions for use of the FN3 domains affixed to a solid support, and/or detectably labeled forms of the FN3 domains, as described herein.

Brief Description of Drawings

Figure 1 shows the indirect pull-down of endogenous albumin from cynomolgus monkey and human serum using albumin-binding FN3 domains. FN3 domains that bind to domain 1 of albumin are boxed with dotted lines; FN3 domains that bind to domain 3 of albumin are boxed with solid lines. All constructs are prepared as bispecific genetic fusions with one null FN3 domain (TC25) and one albumin-binding FN3 domain. *ABD refers to albumin binding domain (ref. J.T. Andersen, R. Pehrson, V. Tolmachev, M.B. Daba, L. Abrahmsen, C. Ekblad, *J. Biol. Chem.* 286:5234-5241 2011).

Figure 2 shows the pharmacokinetic profile of albumin-binding FN3 domains in cynomolgous monkeys following a single IV dose of 1mg/kg (H9) or 2mg/kg (ALB18, or ALB33).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Definitions

Various terms relating to aspects of the description are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definitions provided herein.

As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “a cell” includes a combination of two or more cells, and the like.

The term “about” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of up to $\pm 10\%$ from the specified value, as such variations are appropriate to perform the disclosed methods. Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

“Isolated” means a biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins that have been “isolated” thus include nucleic acids and proteins purified by standard purification methods. “Isolated” nucleic acids, peptides and proteins can be part of a composition and still be isolated if such composition is not part of the native environment of the nucleic acid, peptide, or protein. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids. An “isolated” FN3 domain, as used herein, is intended to refer to an FN3 domain which is substantially free of other FN3 domains having different antigenic specificities (for instance, an isolated FN3 domain that specifically binds to human serum albumin is substantially free of FN3 domains that specifically bind antigens other than human serum albumin). An isolated FN3 domain that specifically binds to an epitope, isoform or variant of human serum albumin may, however, have cross-reactivity to other related antigens, for instance from other species (such as serum albumin species homologs).

The term “fibronectin type III (FN3) domain” (FN3 domain) as used herein refers to a domain occurring frequently in proteins including fibronectins, tenascin, intracellular cytoskeletal proteins, cytokine receptors and prokaryotic enzymes (Bork and Doolittle, *Proc Nat Acad Sci USA* 89:8990-8994, 1992; Meinke *et al.*, *J Bacteriol* 175:1910-1918, 1993; Watanabe *et al.*, *J Biol Chem* 265:15659-15665, 1990). Exemplary FN3 domains are the 15 different FN3 domains present in human tenascin C, the 15 different FN3 domains present in human fibronectin (FN), and non-natural synthetic FN3 domains as described for example in U.S. Pat. No. 8,278,419. Individual FN3 domains are referred to by domain number and protein name, e.g., the 3rd FN3 domain of tenascin (TN3), or the 10th FN3 domain of fibronectin (FN10).

The term “specifically binds” or “specific binding” as used herein refers to the ability of the FN3 domain of the invention to bind to a predetermined antigen with a dissociation constant (K_D) of about 1×10^{-6} M or less, for example about 1×10^{-7} M or less, about 1×10^{-8} M or less, about 1×10^{-9} M or less, about 1×10^{-10} M or less, about 1×10^{-11} M or less, about 1×10^{-12} M or less, or about 1×10^{-13} M or less. Typically the described FN3 domain binds to a predetermined antigen (i.e. human serum albumin) with a K_D that is at least ten fold less than its K_D for a nonspecific antigen (for example casein) as measured by surface plasmon resonance using for example a Proteon Instrument (BioRad). The described FN3 domains that specifically bind to human serum albumin may, however, have cross-reactivity to other related antigens, for example to the same predetermined antigen from other species (homologs), such as *Macaca Fascicularis* (cynomolgous monkey, cyno) or *Pan troglodytes* (chimpanzee).

By “serum half-life”, as used herein can generally be defined as the time taken for the serum concentration of the amino acid sequence, compound, or polypeptide to be reduced by 50%, *in vivo*, for example due to degradation of the sequence or compound and/or clearance or sequestration of the sequence or compound by natural mechanisms. The *in vivo* half-life of an amino acid sequence, compound or polypeptide of the invention can be determined in any manner known per se, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art, and may for example generally involve the steps of suitably administering to a warm-blooded animal (i.e. to a human or to another suitable mammal, such as a mouse, rabbit, rat, pig, dog or a primate, for example monkeys from the genus *Macaca* (such as, and in particular, cynomolgous monkeys (*Macaca fascicularis*) and/or rhesus monkeys (*Macaca mulatta*)) and baboon (*Papio ursinus*)) a suitable dose of the amino acid sequence, compound or polypeptide of the disclosure; collecting blood samples or other samples from said animal; determining the level or concentration of the amino acid sequence, compound or polypeptide of the invention in said blood sample; and calculating, from (a plot of) the data thus obtained, the time until the level or concentration of the amino acid sequence, compound or polypeptide of the invention has been reduced by 50% compared to the initial level upon dosing. Reference is for example made to the Experimental Part below, as well as to the standard handbooks, such as Kenneth, A et al: Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists and Peters et al, Pharmacokinete

analysis: A Practical Approach (1996). Reference is also made to "Pharmacokinetics", M Gibaldi & D Perron, published by Marcel Dekker, 2nd Rev. edition (1982).

As will also be clear to the skilled person (see for example pages 6 and 7 of WO 04/003019 and in the further references cited therein), the half-life can be expressed using parameters such as the $t_{1/2}$ -alpha, $t_{1/2}$ -beta and the area under the curve (AUC). In the present specification, a "half-life" refers to a decrease in any one of these parameters, such as any two of these parameters, or essentially all three of these parameters.

The term "pharmacokinetics" or "pharmacokinetic" is used according to its art accepted meaning and refers to the study of the action of drugs in the body, for example the effect and duration of drug action, the rate at they are absorbed, distributed, metabolized, and eliminated by the body etc.

The term "substituting" or "substituted" or "mutating" or "mutated" as used herein refers to altering, deleting or inserting one or more amino acids or nucleotides in a polypeptide or polynucleotide sequence to generate a variant of that sequence.

The term "randomizing" or "randomized" or "diversified" or "diversifying" as used herein refers to making at least one substitution, insertion or deletion in a polynucleotide or polypeptide sequence.

"Variant" as used herein refers to a polypeptide or a polynucleotide that differs from a reference polypeptide or a reference polynucleotide by one or more modifications for example, substitutions, insertions or deletions.

The term "library" refers to a collection of variants. The library may be composed of polypeptide or polynucleotide variants.

"Tencon" as used herein refers to the synthetic fibronectin type III (FN3) domain having the sequence shown in SEQ ID NO: 1 and described in U.S. Pat. Publ. No. US2010/0216708.

"Polynucleotide," synonymously referred to as "nucleic acid molecule," "nucleotides" or "nucleic acids," refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition,

“polynucleotide” refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. “Modified” bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, “polynucleotide” embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. “Polynucleotide” also embraces relatively short nucleic acid chains, often referred to as oligonucleotides.

A “vector” is a replicon, such as plasmid, phage, cosmid, or virus in which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

As used herein, the term "host cell" can be any type of cell, e.g., a primary cell, a cell in culture, or a cell from a cell line. In specific embodiments, the term "host cell" refers to a cell transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule, e.g., due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome. The terms “expression” and “production” are used synonymously herein, and refer to the biosynthesis of a gene product. These terms encompass the transcription of a gene into RNA. These terms also encompass translation of RNA into one or more polypeptides, and further encompass all naturally occurring post-transcriptional and post-translational modifications. The expression or production of an antibody or antigen-binding fragment thereof may be within the cytoplasm of the cell, or into the extracellular milieu such as the growth medium of a cell culture. The meaning of “substantially the same” can differ depending on the context in which the term is used. Because of the natural sequence variation likely to exist among heavy and light chains and the genes encoding them, one would expect to find some level of variation within the amino acid sequences or the genes encoding the antibodies or antigen-binding fragments described herein, with little or no impact on their unique binding properties (e.g., specificity and affinity). Such an expectation is due in part to the degeneracy of the genetic code, as well as to the evolutionary success of conservative

amino acid sequence variations, which do not appreciably alter the nature of the encoded protein.

Overview of the disclosed FN3 domains

Tencon (SEQ ID NO: 1) is a non-naturally occurring fibronectin type III (FN3) domain designed from a consensus sequence of fifteen FN3 domains from human tenascin-C (Jacobs *et al.*, Protein Engineering, Design, and Selection, 25:107-117, 2012; U.S. Pat. Publ. No. 2010/0216708). The crystal structure of Tencon shows six surface-exposed loops that connect seven beta-strands as is characteristic to the FN3 domains, the beta-strands referred to as A, B, C, D, E, F, and G, and the loops referred to as AB, BC, CD, DE, EF, and FG loops (Bork and Doolittle, Proc Natl Acad Sci USA 89:8990-8992, 1992; U.S. Pat. No. 6,673,901). These loops, or selected residues within each loop, may be randomized in order to construct libraries of fibronectin type III (FN3) domains that may be used to select novel molecules that bind serum albumin. Table 1 shows positions and sequences of each loop and beta-strand in Tencon (SEQ ID NO: 1).

Library designed based on Tencon sequence may thus have randomized FG loop, or randomized BC and FG loops, such as libraries TCL1 or TCL2 as described below. The Tencon BC loop is 7 amino acids long, thus 1, 2, 3, 4, 5, 6 or 7 amino acids may be randomized in the library diversified at the BC loop and designed based on Tencon sequence. The Tencon FG loop is 7 amino acids long, thus 1, 2, 3, 4, 5, 6 or 7 amino acids may be randomized in the library diversified at the FG loop and designed based on Tencon sequence. Further diversity at loops in the Tencon libraries may be achieved by insertion and/or deletions of residues at loops. For example, the FG and/or BC loops may be extended by 1-22 amino acids, or decreased by 1-3 amino acids. The FG loop in Tencon is 7 amino acids long, whereas the corresponding loop in antibody heavy chains ranges from 4-28 residues. To provide maximum diversity, the FG loop may be diversified in sequence as well as in length to correspond to the antibody CDR3 length range of 4-28 residues. For example, the FG loop can further be diversified in length by extending the loop by additional 1, 2, 3, 4 or 5 amino acids.

Library designed based on Tencon sequence may also have randomized alternative surfaces that form on a side of the FN3 domain and comprise two or more beta strands, and at least one loop. One such alternative surface is formed by amino

acids in the C and the F beta-strands and the CD and the FG loops (a C-CD-F-FG surface). A library design based on Tencon alternative C-CD-F-FG surface is described in U.S. Pat. Publ. No. US2013/0226834. Library designed based on Tencon sequence also includes libraries designed based on Tencon variants, such as Tencon variants having substitutions at residues positions 11, 14, 17, 37, 46, 73, or 86 (residue numbering corresponding to SEQ ID NO: 1), and which variants display improved thermal stability. Exemplary Tencon variants are described in US Pat. Publ. No. 2011/0274623, and include Tencon27 (SEQ ID NO: 4) having substitutions E11R, L17A, N46V and E86I when compared to Tencon of SEQ ID NO: 1.

Table 1.

FN3 domain	Tencon (SEQ ID NO: 1)
A strand	1-12
AB loop	13-16
B strand	17-21
BC loop	22-28
C strand	29-37
CD loop	38-43
D strand	44-50
DE loop	51-54
E strand	55-59
EF loop	60-64
F strand	65-74
FG loop	75-81
G strand	82-89

Tencon and other FN3 sequence based libraries may be randomized at chosen residue positions using a random or defined set of amino acids. For example, variants in the library having random substitutions may be generated using NNK codons, which encode all 20 naturally occurring amino acids. In other diversification schemes, DVK codons may be used to encode amino acids Ala, Trp, Tyr, Lys, Thr, Asn, Lys, Ser, Arg,

Asp, Glu, Gly, and Cys. Alternatively, NNS codons may be used to give rise to all 20 amino acid residues and simultaneously reducing the frequency of stop codons. Libraries of FN3 domains with biased amino acid distribution at positions to be diversified may be synthesized for example using Slonomics® technology (<http://www.sloning.com>). This technology uses a library of pre-made double stranded triplets that act as universal building blocks sufficient for thousands of gene synthesis processes. The triplet library represents all possible sequence combinations necessary to build any desired DNA molecule. The codon designations are according to the well known IUB code.

The FN3 domains specifically binding human human serum albumin described herein may be isolated by producing the FN3 library such as the Tencon library using *cis* display to ligate DNA fragments encoding the scaffold proteins to a DNA fragment encoding RepA to generate a pool of protein-DNA complexes formed after *in vitro* translation wherein each protein is stably associated with the DNA that encodes it (U.S. Pat. No. 7,842,476; Odegrip *et al.*, Proc Natl Acad Sci U S A 101, 2806-2810, 2004), and assaying the library for specific binding to human serum albumin by any method known in the art and described in the Example. Exemplary well known methods which can be used are ELISA, sandwich immunoassays, and competitive and non-competitive assays (see, e.g., Ausubel *et al.*, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York). The identified FN3 domains specifically binding human serum albumin are further characterized according to the desired characteristics.

The FN3 domains specifically binding human serum albumin described herein may be generated using any FN3 domain as a template to generate a library and screening the library for molecules specifically binding human serum albumin using methods provided within. Exemplary FN3 domains that may be used are the 3rd FN3 domain of tenascin C (TN3) (SEQ ID NO: 68), Fibcon (SEQ ID NO: 69146), and the 10th FN3 domain of fibronectin (FN10) (SEQ ID NO: 70). Standard cloning and expression techniques are used to clone the libraries into a vector or synthesize double stranded cDNA cassettes of the library, to express, or to translate the libraries *in vitro*. For example ribosome display (Hanes and Pluckthun, Proc Natl Acad Sci USA, 94, 4937-4942, 1997), mRNA display (Roberts and Szostak, Proc Natl Acad Sci USA, 94, 12297-12302, 1997), or other cell-free systems (U.S. Pat. No. 5,643,768) can be used. The libraries of the

FN3 domain variants may be expressed as fusion proteins displayed on the surface for example of any suitable bacteriophage. Methods for displaying fusion polypeptides on the surface of a bacteriophage are well known (U.S. Pat. Publ. No. 2011/0118144; Int. Pat. Publ. No. WO2009/085462; U.S. Pat. No. 6,969,108; U.S. Pat. No. 6,172,197; U.S. Pat. No. 5,223,409; U.S. Pat. No. 6,582,915; U.S. Pat. No. 6,472,147).

In some embodiments described herein, the FN3 domain specifically binding human serum albumin is based on Tencon sequence of SEQ ID NO: 1 or Tencon27 sequence of SEQ ID NO: 4, the SEQ ID NO: 1 or the SEQ ID NO: 4, optionally having substitutions at residues positions 11, 14, 17, 37, 46, 73, and/or 86.

The FN3 domains specifically binding human serum albumin of the disclosure may be modified to improve their properties such as improve thermal stability and reversibility of thermal folding and unfolding. Several methods have been applied to increase the apparent thermal stability of proteins and enzymes, including rational design based on comparison to highly similar thermostable sequences, design of stabilizing disulfide bridges, mutations to increase alpha-helix propensity, engineering of salt bridges, alteration of the surface charge of the protein, directed evolution, and composition of consensus sequences (Lehmann and Wyss, Curr Opin Biotechnol, 12, 371-375, 2001). High thermal stability may increase the yield of the expressed protein, improve solubility or activity, decrease immunogenicity, and minimize the need of a cold chain in manufacturing. Residues that may be substituted to improve thermal stability of Tencon (SEQ ID NO: 1) are residue positions 11, 14, 17, 37, 46, 73, or 86, and are described in US Pat. Publ. No. 2011/0274623. Substitutions corresponding to these residues may be incorporated to the FN3 domain containing molecules of the invention.

Measurement of protein stability and protein lability can be viewed as the same or different aspects of protein integrity. Proteins are sensitive or "labile" to denaturation caused by heat, by ultraviolet or ionizing radiation, changes in the ambient osmolarity and pH if in liquid solution, mechanical shear force imposed by small pore-size filtration, ultraviolet radiation, ionizing radiation, such as by gamma irradiation, chemical or heat dehydration, or any other action or force that may cause protein structure disruption. The stability of the molecule can be determined using standard methods. For example, the stability of a molecule can be determined by measuring the thermal melting ("T_m") temperature, the temperature in ° Celsius (°C) at which half of the molecules become unfolded, using standard methods. Typically, the higher the T_m, the more stable the

molecule. In addition to heat, the chemical environment also changes the ability of the protein to maintain a particular three dimensional structure.

In one embodiment, the FN3 domain specifically binding human serum albumin of the disclosure may exhibit increased stability by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% or more compared to the same domain prior to engineering measured by the increase in the T_m .

Chemical denaturation can likewise be measured by a variety of methods. Chemical denaturants include guanidinium hydrochloride, guanidinium thiocyanate, urea, acetone, organic solvents (DMF, benzene, acetonitrile), salts (ammonium sulfate, lithium bromide, lithium chloride, sodium bromide, calcium chloride, sodium chloride); reducing agents (e.g. dithiothreitol, beta-mercaptoethanol, dinitrothiobenzene, and hydrides, such as sodium borohydride), non-ionic and ionic detergents, acids (e.g. hydrochloric acid (HCl), acetic acid (CH_3COOH), halogenated acetic acids), hydrophobic molecules (e.g. phospholipids), and targeted denaturants. Quantitation of the extent of denaturation can rely on loss of a functional property, such as ability to bind a target molecule, or by physiochemical properties, such as tendency to aggregation, exposure of formerly solvent inaccessible residues, or disruption or formation of disulfide bonds.

The FN3 domain of the disclosure may be generated as monomers, dimers, or multimers, for example, as a means to increase the valency and thus the avidity of target molecule binding, or to generate bi- or multispecific scaffolds simultaneously binding two or more different target molecules. The dimers and multimers may be generated by linking monospecific, bi- or multispecific protein scaffolds, for example, by the inclusion of an amino acid linker, for example a linker containing poly-glycine, glycine and serine, or alanine and proline. Exemplary linker include $(\text{GS})_2$, (SEQ ID NO: 54), $(\text{GGGS})_2$ (SEQ ID NO: 55), $(\text{GGGGS})_5$ (SEQ ID NO: 56), $(\text{AP})_2$ (SEQ ID NO: 57), $(\text{AP})_5$ (SEQ ID NO: 58), $(\text{AP})_{10}$ (SEQ ID NO: 59), $(\text{AP})_{20}$ (SEQ ID NO: 60) and $\text{A(EAAAK)}_5\text{AAA}$ (SEQ ID NO: 61). The dimers and multimers may be linked to each other in a N-to C-direction. The use of naturally occurring as well as artificial peptide linkers to connect polypeptides into novel linked fusion polypeptides is well known in the literature (Hallewell *et al.*, *J Biol Chem* 264, 5260-5268, 1989; Alfthan *et al.*, *Protein Eng.* 8, 725-

731, 1995; Robinson & Sauer, *Biochemistry* 35, 109-116, 1996; U.S. Pat. No. 5,856,456).

Human Serum Albumin binders

FN3 domains are cleared rapidly from circulation via renal filtration and degradation due to their small size of ~10 kDa. In certain aspects, the disclosure provides FN3 domains that bind specifically to serum albumin, e.g., human serum albumin (HSA) to prolong the half-life of the FN3 domain.

The described albumin-specific FN3 domains bind to domain I or III of human serum albumin and have at least 10-fold higher serum half-life compared to the serum half-life of Tencon sequence of SEQ ID NO: 67, thereby providing an efficient way to extend the *in vivo* serum half-life of drugs or proteins conjugated thereto.

In some embodiments, the isolated FN3 domain specifically binds to domain I of human serum albumin of SEQ ID NO: 62. In some embodiments, the isolated FN3 domain specifically binds to domain III of human serum albumin of SEQ ID NO: 62. In some embodiments the albumin-specific antibodies have at least an 10-fold higher serum half-life compared to the serum half-life of Tencon sequence of SEQ ID NO: 67.

In some embodiments, the described FN3 domains cross-react with *Macaca Fascicularis* (cynomolgus monkey) serum albumin of SEQ ID NO: 63.

In some embodiments, the human serum albumin-binding FN3 domains comprises an initiator methionine (Met) linked to the N-terminus of the molecule.

In some embodiments, the human serum albumin-binding FN3 domains comprise a cysteine (Cys) linked to a C-terminus of the FN3 domain.

The addition of the N-terminal Met and/or the C-terminal Cys may facilitate expression and/or conjugation of half-life extending molecules.

In some embodiments, the isolated FN3 domain comprises the amino acid sequence of SEQ ID NOs: 51, 52, or 53.

In some embodiments, the isolated FN3 domain comprises an amino acid sequence that is 90% identical to the amino acid sequence of SEQ ID NO: 51.

In some embodiments, the isolated FN3 domain comprises an amino acid sequence that has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 substitutions when compared to the amino acid sequence of SEQ ID NO: 51.

In some embodiments, the isolated FN3 domain that specifically binds human serum albumin comprises a cysteine residue in at least one residue position corresponding to residue positions 6, 11, 22, 25, 26, 52, 53, 61 of SEQ ID NO 1, or at a C-terminus.

In certain embodiments, the isolated FN3 domain as described herein may comprise the sequence as set forth in SEQ ID NO: 51-53, wherein the C-strand, the CD loop, the F-strand and the FG-loops are replaced with a respective set of specified C-strand, the CD loop, the F-strand and the FG-loops from any of the described albumin-specific FN3 domain sequences (i.e., SEQ ID NOs: 51-53), or sequences at least 85%, 90%, 95%, 97%, 98%, or 99% identical to the C-strand, the CD loop, the F-strand and the FG-loop sequences of the four core FN3 domain sequences.

In some embodiments, the isolated albumin-specific FN3 domains comprise a sequence as set forth in SEQ ID NO: 51. This albumin-specific FN3 domain may bind to domain I or III of human serum albumin. This albumin-specific FN3 domain may have at least a 10-fold higher serum half-life compared to the serum half-life of Tencon sequence of SEQ ID NO: 67.

In some embodiments, the isolated albumin-specific FN3 domains comprise a sequence as set forth in SEQ ID NO: 52. This albumin-specific FN3 domain may bind to domain I or III of human serum albumin. This albumin-specific FN3 domain may have at least a 10-fold higher serum half-life compared to the serum half-life of Tencon sequence of SEQ ID NO: 67.

In some embodiments, the isolated albumin-specific FN3 domains comprise a sequence as set forth in SEQ ID NO: 53. This albumin-specific FN3 domain may bind to domain I or III of human serum albumin. This albumin-specific FN3 domain may have at least a 10-fold higher serum half-life compared to the serum half-life of Tencon sequence of SEQ ID NO: 67.

Fusions of Human Serum Albumin-specific FN3 domains

One aspect of the present disclosure provides for conjugates comprising a serum albumin binding FN3 domain and at least one additional moiety. The additional moiety may be useful for any diagnostic, imaging, or therapeutic purpose.

In certain embodiments, the serum half-life of the moiety fused to the described FN3 domain is increased relative to the serum half-life of the moiety when not

conjugated to the FN3 domain. In certain embodiments, the serum half-life of the FN3 domain fusion is at least 20, 40, 60, 80, 100, 120, 150, 180, 200, 400, 600, 800, 1000, 1200, 1500, 1800, 1900, 2000, 2500, or 3000% longer relative to the serum half-life of the moiety when not fused to the described FN3 domain. In other embodiments, the serum half-life of the FN3 domain fusion is at least 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5 fold, 4-fold, 4.5- fold, 5-fold, 6-fold, 7-fold, 8-fold, 10-fold, 12-fold, 13-fold, 15-fold, 17-fold, 20-fold, 22-fold, 25 -fold, 27-fold, 30-fold, 35 -fold, 40-fold, or 50-fold greater than the serum half-life of the moiety when not fused to the described FN3 domain. In some embodiments, the serum half-life of the FN3 domain fusion is at least 2 hours, 2.5 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 15 hours, 20 hours, 25 hours, 30 hours, 35 hours or 40 hours in cynomolgus monkey.

Accordingly, the described FN3 domain fusion molecules described herein are useful for increasing the half-life of a therapeutic moiety by creating a fusion between the therapeutic moiety and the described FN3 domain. Such fusion molecules may be used to treat conditions which respond to the biological activity of the therapeutic moiety contained in the fusion. The present disclosure contemplates the use of the described FN3 domain fusion molecules in diseases caused by the dysregulation of any of the following proteins or molecules.

Heterologous Moiety

In some embodiments, the described FN3 domains are fused to a second moiety that is a small organic molecule, a nucleic acid, or a protein. In some embodiments, the described FN3 domains is fused to a therapeutic moiety that targets receptors, receptor ligands, viral coat proteins, immune system proteins, hormones, enzymes, antigens, or cell signaling proteins. The fusion may be formed by attaching the second moiety to either end of the described FN3 domains, i.e., FN3 domain-therapeutic molecule or therapeutic molecule-FN3 domain arrangements.

In other exemplary embodiments, the described FN3 domain is fused to one or more additional FN3 domains. For example, the described FN3 domain may be fused to one, two, three, four or more additional FN3 domains. The additional FN3 domains may bind to the same or different targets other than serum albumin.

In certain embodiments, the application provides a FN3-Y fusion that may be represented by the formula: FN3-X Y or Y-X FN3, wherein FN3 is an FN3 domain as

described herein (including any N-terminal and/or C-terminal extensions), Xi is a polypeptide linker (suitable linkers include, for example, any one of SEQ ID NOs: 54-61), and Y is a therapeutic moiety as described herein.

In certain embodiments, the application provides a FN3-Y fusion that may be represented by the formula: FN3-Xi-Cys-X₂-Y or Y-Xi-Cys-X₂-FN3, wherein FN3 is an isolated FN3 domain as described herein (including any N-terminal and/or C-terminal extensions), Xi is an optional polypeptide linker (suitable linkers include, for example, any one of SEQ ID NOs: 54-61), Cys is a cysteine residue, X₂ is a chemically derived spacer, and Y is a therapeutic moiety as described herein. In exemplary embodiments, the chemically derived spacer contains a maleimide moiety which may be used to conjugate the therapeutic moiety to the C-terminal Cys of the described FN3 domains, or to conjugate the described FN3 domains to the C-terminal Cys of the therapeutic moiety, by Michael addition as described further herein. In other aspects, a described FN3 domain may be bound to two or more therapeutic moieties. For example, two moieties can be bound to a described FN3 domain in various arrangements, such as for example, from N-terminus to C-terminus of a fusion sequence, as follows: X-Y-FN3, X-FN3-Y, or FN3-X-Y, wherein X and Y represent two different therapeutic moieties. The two different therapeutic moieties may be selected from any of the moieties disclosed herein.

Deimmunization of binding polypeptides

The amino acid sequences of serum albumin binders and their fusions may be altered to eliminate one or more B- or T-cell epitopes. A protein, including the described FN3 domain fusions described herein, may be deimmunized to render it non-immunogenic, or less immunogenic, to a given species. Deimmunization can be achieved through structural alterations to the protein. Any deimmunization technique known to those skilled in the art can be employed, see e.g., WO 00/34317, the disclosure of which is incorporated herein in its entirety.

In one embodiment, the sequences of the serum albumin binders and their fusions can be analyzed for the presence of MHC class II binding motifs. For example, a comparison may be made with databases of MHC-binding motifs such as, for example, by searching the "motifs" database on the worldwide web at sitewehil.weihi.edu.au. Alternatively, MHC class II binding peptides may be identified using computational threading methods such as those devised by Altuvia et al. (J. Mol. Biol. 249 244-250

(1995)) whereby consecutive overlapping peptides from the polypeptide are testing for their binding energies to MHC class II proteins. Computational binding prediction algorithms include iTope™, Tepitope, SYFPEITHI, EpiMatrix (EpiVax), and MHCpred. In order to assist the identification of MHC class II-binding peptides, associated sequence features which relate to successfully presented peptides such as amphipathicity and Rothbard motifs, and cleavage sites for cathepsin B and other processing enzymes can be searched for.

Having identified potential (e.g. human) T-cell epitopes, these epitopes are then eliminated by alteration of one or more amino acids, as required to eliminate the T-cell epitope. Usually, this will involve alteration of one or more amino acids within the T-cell epitope itself. This could involve altering an amino acid adjacent the epitope in terms of the primary structure of the protein or one which is not adjacent in the primary structure but is adjacent in the secondary structure of the molecule. The usual alteration contemplated will be amino acid substitution, but it is possible that in certain circumstances amino acid addition or deletion will be appropriate. All alterations can be accomplished by recombinant DNA technology, so that the final molecule may be prepared by expression from a recombinant host, for example by well established methods, but the use of protein chemistry or any other means of molecular alteration may also be used.

Once identified T-cell epitopes are removed, the deimmunized sequence may be analyzed again to ensure that new T-cell epitopes have not been created and, if they have, the epitope(s) can be deleted.

Not all T-cell epitopes identified computationally need to be removed. A person skilled in the art will appreciate the significance of the "strength" or rather potential immunogenicity of particular epitopes. The various computational methods generate scores for potential epitopes. A person skilled in the art will recognize that only the high scoring epitopes may need to be removed. A skilled person will also recognize that there is a balance between removing potential epitopes and maintaining binding affinity or other biological activity of the protein. Therefore, one strategy is to sequentially introduce substitutions into the described FN3 domains or FN3 domain fusion protein and then test for target binding or other biological activity and immunogenicity.

Additional Modifications

In certain embodiments, the serum albumin binders and their fusions may further comprise post-translational modifications. Exemplary post-translational protein modification include phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination, glycosylation, carbonylation, sumoylation, biotinylation or addition of a polypeptide side chain or of a hydrophobic group. As a result, the modified serum albumin binders and their fusions may contain non-amino acid elements, such as lipids, poly- or mono-saccharide, and phosphates. A preferred form of glycosylation is sialylation, which conjugates one or more sialic acid moieties to the polypeptide. Sialic acid moieties improve solubility and serum half-life while also reducing the possible immunogenicity of the protein. See, e.g., Raju et al. *Biochemistry*. 2001 Jul 31;40(30):8868-76. Effects of such non-amino acid elements on the functionality of the serum albumin binders or their fusions may be tested for their ability to bind a particular serum albumin (e.g., HSA or RhSA) and/or the functional role conferred by a specific non-FN3 moiety in the context of a fusion (e.g., the effect of FGF21 on glucose uptake).

Vectors & Polynucleotides Embodiments

Also included in the present disclosure are nucleic acid sequences encoding any of the proteins described herein. As appreciated by those skilled in the art, because of third base degeneracy, almost every amino acid can be represented by more than one triplet codon in a coding nucleotide sequence. In addition, minor base pair changes may result in a conservative substitution in the amino acid sequence encoded but are not expected to substantially alter the biological activity of the gene product. Therefore, a nucleic acid sequence encoding a protein described herein may be modified slightly in sequence and yet still encode its respective gene product. Certain exemplary nucleic acids encoding the serum albumin binders and their fusions described herein include nucleic acids having the sequences set forth in SEQ ID Nos. 69-72.

Nucleic acids encoding any of the various proteins or polypeptides disclosed herein may be synthesized chemically. Codon usage may be selected so as to improve expression in a cell. Such codon usage will depend on the cell type selected. Specialized codon usage patterns have been developed for *E. coli* and other bacteria, as well as mammalian cells, plant cells, yeast cells and insect cells. See for example: Mayfield et al, *Proc Natl Acad Sci U S A*. 2003 100(2):438- 42; Sinclair et al. *Protein Expr Purif*. 2002

(1):96-105; Connell ND. *Curr Opin Biotechnol.* 2001 (5):446-9; Makrides et al. *Microbiol Rev.* 1996 60(3):512-38; and Sharp et al. *Yeast.* 1991 7(7):657-78.

General techniques for nucleic acid manipulation are within the purview of one skilled in the art and are also described for example in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Vols. 1-3, Cold Spring Harbor Laboratory Press, 2 ed., 1989, or F. Ausubel et al, *Current Protocols in Molecular Biology* (Green Publishing and Wiley-Interscience: New York, 1987) and periodic updates, herein incorporated by reference. The DNA encoding a protein is operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, viral, or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants are additionally incorporated. Suitable regulatory elements are well-known in the art.

The proteins and fusion proteins described herein may be produced as a fusion protein with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process a native signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion, the native signal sequence may be substituted by, e.g., the yeast invertase leader, a factor leader (including *Saccharomyces* and *Kluyveromyces* alpha-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in PCT Publication No. WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor regions may be ligated in reading frame to DNA encoding the protein.

Expression vectors used in eukaryotic host cells (e.g., yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA.

Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the multivalent antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See PCT Publication No. WO 94/11026 and the expression vector disclosed therein.

The recombinant DNA can also include any type of protein tag sequence that may be useful for purifying the protein. Examples of protein tags include but are not limited to a histidine tag, a FLAG tag, a myc tag, an HA tag, or a GST tag. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts can be found in *Cloning Vectors: A Laboratory Manual*, (Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference. The expression construct is introduced into the host cell using a method appropriate to the host cell, as will be apparent to one of skill in the art. A variety of methods for introducing nucleic acids into host cells are known in the art, including, but not limited to, electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is an infectious agent).

Suitable host cells include prokaryotes, yeast, mammalian cells, or bacterial cells. Suitable bacteria include gram negative or gram positive organisms, for example, *E. coli* or *Bacillus* spp. Yeast, preferably from the *Saccharomyces* species, such as *S. cerevisiae*, may also be used for production of polypeptides. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, (*Bio/Technology*, 6:47, 1988). In some instance it will be desired to produce proteins in vertebrate cells, such as for glycosylation, and the propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of suitable mammalian host cell lines include endothelial cells, COS-7 monkey kidney cells, CV-1, L cells, C127, 3T3, Chinese hamster ovary (CHO), human embryonic kidney cells, HeLa, 293, 293T, and BHK cell lines. For many applications, the small size of the protein multimers described herein would make *E. coli* the preferred method for expression.

Protein Production

Host cells are transformed with the herein-described expression or cloning vectors for protein production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the proteins of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al, Meth. Enz. 58:44 (1979), Barnes et al, Anal. Biochem. 102:255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Proteins disclosed herein can also be produced using cell-translation systems. For such purposes, the nucleic acids encoding the proteins must be modified to allow in vitro transcription to produce mRNA and to allow cell-free translation of the mRNA in the particular cell-free system being utilized. Exemplary eukaryotic cell-free translation systems include, for example, mammalian or yeast cell-free translation systems, and exemplary prokaryotic cell-free translation systems include, for example, bacterial cell-free translation systems.

Proteins disclosed herein can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). Modifications to the protein can also be produced by chemical synthesis.

The proteins disclosed herein can be purified by isolation/purification methods for proteins generally known in the field of protein chemistry. Non-limiting examples include extraction, recrystallization, salting out (e.g., with ammonium sulfate or sodium sulfate), centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion exchange chromatography, hydrophobic chromatography, normal phase chromatography, reversed-phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, countercurrent distribution or any combinations of these. After purification, proteins may be exchanged into different buffers and/or concentrated by any of a variety of methods known to the art, including, but not limited to, filtration and dialysis.

The purified proteins are preferably at least 85% pure, more preferably at least 95% pure, and most preferably at least 98% pure. Regardless of the exact numerical value of the purity, the proteins are sufficiently pure for use as a pharmaceutical product.

Imaging, Diagnostic and Other Applications

The FN3 domain fusions provided herein may be used to treat a variety of diseases and disorders, based on the identity of the heterogenous molecule fused to the described FN3 domains. The applications for the FN3 domain fusions may be determined by the skilled artisan based on the knowledge in the art and the information provided herein. Uses for various FN3 domain fusion proteins are described in detail herein. FN3 domain fusions may be administered to any mammalian subject or patient, including both human and non-human organisms.

The serum albumin binders and fusion molecules described herein can be detectably labeled and used to contact cells expressing, e.g., a protein bound by the fusion molecule for imaging or diagnostic applications. Any method known in the art for conjugating a protein to the detectable moiety may be employed, including those methods described by Hunter, et al, *Nature* 144:945 (1962); David, et al, *Biochemistry* 13: 1014 (1974); Pain, et al, *J. Immunol. Meth.* 40:219 (1981); and Nygren, J. *Histochem. and Cytochem.* 30:407 (1982).

In certain embodiments, the serum albumin binders and fusion molecules described herein are further attached to a label that is able to be detected (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor). The label may be a radioactive agent, such as: radioactive heavy metals such as iron chelates, radioactive chelates of gadolinium or manganese, positron emitters of oxygen, nitrogen,

iron, carbon, or gallium, ^{43}K , ^{52}Fe , ^{57}Co , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{123}I , ^{125}I , ^{13}T , ^{132}I , or ^{99}Tc . A serum albumin binder or fusion molecule affixed to such a moiety may be used as an imaging agent and is administered in an amount effective for diagnostic use in a mammal such as a human and the localization and accumulation of the imaging agent is then detected. The localization and accumulation of the imaging agent may be detected by radioscintigraphy, nuclear magnetic resonance imaging, computed tomography or positron emission tomography. As will be evident to the skilled artisan, the amount of radioisotope to be administered is dependent upon the radioisotope. Those having ordinary skill in the art can readily formulate the amount of the imaging agent to be administered based upon the specific activity and energy of a given radionuclide used as the active moiety.

Serum albumin binders and fusion molecules also are useful as affinity purification agents. In this process, the proteins are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The proteins can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays (Zola, Monoclonal Antibodies: A Manual of Techniques, pp. 147-158 (CRC Press, Inc., 1987)). Exemplary

Therapeutic Formulations and Modes of Administration

The present invention provides methods for administering a therapeutic moiety fused to a described FN3 domain, wherein the half-life of the therapeutic moiety is extended when fused to the described FN3 domains. Techniques and dosages for administration of the fusion constructs will vary depending on the type of therapeutic moiety fused to the described FN3 domains and the specific condition being treated but can be readily determined by the skilled artisan. In general, regulatory agencies require that a protein reagent to be used as a therapeutic is formulated so as to have acceptably low levels of pyrogens. Accordingly, therapeutic formulations will generally be distinguished from other formulations in that they are substantially pyrogen free, or at least contain no more than acceptable levels of pyrogen as determined by the appropriate regulatory agency (e.g., FDA). In certain embodiments, pharmaceutical formulations of described FN3 domains and their fusion molecules comprise, e.g., 1-20 mM succinic acid, 2-10% sorbitol, and 1-10% glycine at pH 4.0-7.0. In an exemplary embodiment,

pharmaceutical formulations of the described FN3 domain and their fusion molecules comprise, e.g., 10 mM succinic acid, 8% sorbitol, and 5% glycine at pH 6.0.

In some embodiments, the described FN3 domains and fusions thereof are pharmaceutically acceptable to a mammal, in particular a human. A "pharmaceutically acceptable" polypeptide refers to a polypeptide that is administered to an animal without significant adverse medical consequences. Examples of pharmaceutically acceptable FN3 domains disclosed herein and fusions thereof include FN3 domains that lack the integrin-binding domain (RGD) and compositions that are essentially endotoxin free or have very low endotoxin levels.

Therapeutic compositions may be administered with a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form. Administration may be parenteral (e.g., intravenous, subcutaneous), oral, or topical, as non-limiting examples. The composition can be in the form of a pill, tablet, capsule, liquid, or sustained release tablet for oral administration; a liquid for intravenous, subcutaneous or parenteral administration; or a gel, lotion, ointment, cream, or a polymer or other sustained release vehicle for local administration.

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" (20th ed., ed. A.R. Gennaro AR., 2000, Lippincott Williams & Wilkins, Philadelphia, PA). Formulations for parenteral administration may, for example, contain excipients, sterile water, saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene- polyoxypropylene copolymers may be used to control the release of the compounds. Nanoparticulate formulations (e.g., biodegradable nanoparticles, solid lipid nanoparticles, liposomes) may be used to control the biodistribution of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. The concentration of the compound in the formulation varies depending upon a number of factors, including the dosage of the drug to be administered, and the route of administration.

The polypeptide may be optionally administered as a pharmaceutically acceptable salt, such as non-toxic acid addition salts or metal complexes that are commonly used in the pharmaceutical industry. Examples of acid addition salts include organic acids such

as acetic, lactic, pamoic, maleic, citric, malic, ascorbic, succinic, benzoic, palmitic, suberic, salicylic, tartaric, methanesulfonic, toluenesulfonic, or trifluoroacetic acids or the like; polymeric acids such as tannic acid, carboxymethyl cellulose, or the like; and inorganic acid such as hydrochloric acid, hydrobromic acid, sulfuric acid phosphoric acid, or the like. Metal complexes include zinc, iron, and the like. In one example, the polypeptide is formulated in the presence of sodium acetate to increase thermal stability. Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients. These excipients may be, for example, inert diluents or fillers (e.g., sucrose and sorbitol), lubricating agents, glidants, and anti-adhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc).

Formulations for oral use may also be provided as chewable tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium.

A therapeutically effective dose refers to a dose that produces the therapeutic effects for which it is administered. The exact dose will depend on the disorder to be treated, and may be ascertained by one skilled in the art using known techniques. In general, the FN3domain fusion is administered at about 0.01 $\mu\text{g/kg}$ to about 50 mg/kg per day, preferably 0.01 mg/kg to about 30 mg/kg per day, most preferably 0.1 mg/kg to about 20 mg/kg per day. The polypeptide may be given daily (e.g., once, twice, three times, or four times daily) or less frequently (e.g., once every other day, once or twice weekly, or monthly). In addition, as is known in the art, adjustments for age as well as the body weight, general health, sex, diet, time of administration, drug interaction, and the severity of the disease may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

Kits for Detecting Human Serum Albumin

Provided herein are kits for detecting human serum albumin in a biological sample. These kits include one or more of the serum albumin-specific FN3 domains described herein and instructions for use of the kit.

The provided serum albumin-specific FN3 domain may be in solution; lyophilized; affixed to a substrate, carrier, or plate; or detectably labeled.

The described kits may also include additional components useful for performing the methods described herein. By way of example, the kits may comprise means for obtaining a sample from a subject, a control or reference sample, *e.g.*, a sample from a subject having slowly progressing cancer and/or a subject not having cancer, one or more sample compartments, and/or instructional material which describes performance of a method of the invention and tissue specific controls or standards.

The means for determining the level of serum albumin can further include, for example, buffers or other reagents for use in an assay for determining the level of serum albumin. The instructions can be, for example, printed instructions for performing the assay and/or instructions for evaluating the level of serum albumin.

The described kits may also include means for isolating a sample from a subject. These means can comprise one or more items of equipment or reagents that can be used to obtain a fluid or tissue from a subject. The means for obtaining a sample from a subject may also comprise means for isolating blood components, such as serum, from a blood sample. Preferably, the kit is designed for use with a human subject.

EXAMPLES

The following examples are provided to supplement the prior disclosure and to provide a better understanding of the subject matter described herein. These examples should not be considered to limit the described subject matter. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be apparent to persons skilled in the art and are to be included within, and can be made without departing from, the true scope of the invention.

EXAMPLE 1. CONSTRUCTION OF TENCON LIBRARIES WITH RANDOMIZED LOOPS

Tencon (SEQ ID NO: 1) is an immunoglobulin-like scaffold, fibronectin type III (FN3) domain, designed from a consensus sequence of fifteen FN3 domains from human tenascin-C (Jacobs *et al.*, Protein Engineering, Design, and Selection, 25:107-117, 2012; U.S. Pat. No. 8,278,419). The crystal structure of Tencon shows six surface-exposed loops that connect seven beta-strands. These loops, or selected residues within each

loop, can be randomized in order to construct libraries of fibronectin type III (FN3) domains that can be used to select novel molecules that bind to specific targets.

Tencon:

LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSFLIQYQESEKVGAINLTVPGSERS
YDLTGLKPGTEYTVSIYGVKGGHRSNPLSAEFTT (SEQ ID NO 1):

Various libraries were generated using the tencon scaffold and various design strategies. In general, libraries TCL1 and TCL2 produced good binders. Generation of TCL1 and TCL2 libraries are described in detail in Int. Pat. Publ. No. WO2014081944A2.

Construction of TCL1 library

A library designed to randomize only the FG loop of Tencon (SEQ ID NO: 1), TCL1, was constructed for use with the *cis*-display system (Jacobs *et al.*, Protein Engineering, Design, and Selection, 25:107-117, 2012). In this system, a double-stranded DNA incorporating sequences for a Tac promoter, Tencon library coding sequence, RepA coding sequence, cis-element, and ori element is produced. Upon expression in an *in vitro* transcription/translation system, a complex is produced of the Tencon-RepA fusion protein bound in *cis* to the DNA from which it is encoded. Complexes that bind to a target molecule are then isolated and amplified by polymerase chain reaction (PCR), as described below.

Construction of the TCL1 library for use with *cis*-display was achieved by successive rounds of PCR to produce the final linear, double-stranded DNA molecules in two halves; the 5' fragment contains the promoter and Tencon sequences, while the 3' fragment contains the *repA* gene and the cis- and ori elements. These two halves are combined by restriction digest in order to produce the entire construct. The TCL1 library was designed to incorporate random amino acids only in the FG loop of Tencon, KGGHRSN (SEQ ID NO: 32). NNS codons were used in the construction of this library, resulting in the possible incorporation of all 20 amino acids and one stop codon into the FG loop. The TCL1 library contains six separate sub-libraries, each having a different randomized FG loop length, from 7 to 12 residues, in order to further increase diversity.

TCL1 library (SEQ ID NO: 2)

LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSFLIQYQESEKVGGEAINLTVPGSERS
YDLTGLKPGTEYTVSIYGVX₇₋₁₂PLSAEFTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₇ is any amino acid; and

X₈, X₉, X₁₀, X₁₁ and X₁₂ are any amino acid or deleted

Construction of TCL2 Library

TCL2 library was constructed in which both the BC and the FG loops of Tencon were randomized and the distribution of amino acids at each position was strictly controlled. Table 2 shows the amino acid distribution at desired loop positions in the TCL2 library. The designed amino acid distribution had two aims. First, the library was biased toward residues that were predicted to be structurally important for Tencon folding and stability based on analysis of the Tencon crystal structure and/or from homology modeling. For example, position 29 was fixed to be only a subset of hydrophobic amino acids, as this residue was buried in the hydrophobic core of the Tencon fold. A second layer of design included biasing the amino acid distribution toward that of residues preferentially found in the heavy chain HCDR3 of antibodies, to efficiently produce high-affinity binders (Birtalan *et al.*, J Mol Biol 377:1518-28, 2008; Olson *et al.*, Protein Sci 16:476-84, 2007). Towards this goal, the “designed distribution” in Table 1 refers to the distribution as follows: 6% alanine, 6% arginine, 3.9% asparagine, 7.5% aspartic acid, 2.5% glutamic acid, 1.5% glutamine, 15% glycine, 2.3% histidine, 2.5% isoleucine, 5% leucine, 1.5% lysine, 2.5% phenylalanine, 4% proline, 10% serine, 4.5% threonine, 4% tryptophan, 17.3% tyrosine, and 4% valine. This distribution is devoid of methionine, cysteine, and STOP codons.

TCL2 library (SEQ ID NO: 3)

LPAPKNLVVSEVTEDSLRLSWX₁X₂X₃X₄X₅X₆X₇X₈SFLIQYQESEKVGGEAINLTVPGSERSYDLTGLKPGTEYTVSIYGVX₉X₁₀X₁₁X₁₂X₁₃SX₁₄X₁₅LSAEFTT; wherein

X₁ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₃ Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₅ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₆ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₇ is Phe, Ile, Leu, Val or Tyr;

X₈ is Asp, Glu or Thr;

X₉ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₀ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₁ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₃ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; and

X₁₅ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val.

Table 2.

Residue Position*	WT residues	Distribution in the TCL2 library
22	T	designed distribution
23	A	designed distribution
24	P	50% P + designed distribution
25	D	designed distribution
26	A	20% A + 20% G + designed distribution
27	A	designed distribution
28	F	20% F, 20% I, 20% L, 20% V, 20% Y
29	D	33% D, 33% E, 33% T
75	K	designed distribution
76	G	designed distribution
77	G	designed distribution
78	H	designed distribution
79	R	designed distribution
80	S	100% S
81	N	designed distribution
82	P	50% P + designed distribution

*residue numbering is based on Tencon sequence of SEQ ID NO: 1

Subsequently, these libraries were improved by various ways, including building of the libraries on a stabilized Tencon framework (U.S. Pat. No. 8,569,227) that incorporates substitutions E11R/L17A/N46V/E86I (Tencon27; SEQ ID NO: 4) when compared to the wild type tencon as well as altering of the positions randomized in the BC and FG loops. Tencon27 is described in Int. Pat. Appl. No. WO2013049275. From this, new libraries designed to randomize only the FG loop of Tencon (library TCL9), or a combination of the BC and FG loops (library TCL7) were generated. These libraries were constructed for use with the cis-display system (Odegrip et al., Proc Natl Acad Sci U S A 101: 2806-2810, 2004). The details of this design are shown below:

Stabilized Tencon (Tencon27) (SEQ ID NO: 4)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFLIQYQESEKVGGEAIVLTVPGSERS
YDLTGLKPGTEYTVSIYGVKGGHRSNPLSAIFTT

TCL7 (randomized FG and BC loops) (SEQ ID NO: 5)

LPAPKNLVVSRVTEDSARLSWX₁X₂X₃X₄X₅X₆X₇X₈X₉FDSFLIQYQESEKVGGEAIV
LTVPGSERSYDLTGLKPGTEYTVSIYGVX₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈X₁₉SNPLSA
IFTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂, X₁₃, X₁₄, X₁₅ and X₁₆ is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; and

X₇, X₈, X₉, X₁₇, X₁₈ and X₁₉, is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted.

TCL9 (randomized FG loop) (SEQ ID NO: 6)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFLIQYQESEKVGGEAIVLTVPGSERS
YDLTGLKPGTEYTVSIYGV X₁X₂X₃X₄X₅X₆X₇X₈X₉ X₁₀X₁₁X₁₂SNPLSAIFTT;

X₁, X₂, X₃, X₄, X₅, X₆ and X₇, is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; and

X₈, X₉, X₁₀, X₁₁ and X₁₂ is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted.

For library construction, DNA fragments encoding randomized BC loops (lengths 6-9 positions) or FG loops (lengths 7-12 positions) were synthesized using Slonomics technology (Sloning Biotechnology GmbH) so as to control the amino acid distribution of the library and to eliminate stop codons. Two different sets of DNA molecules randomizing either the BC loop or the FG loops were synthesized independently and later combined using PCR to produce the full library product.

Construction of FG loop libraries (TCL9)

A set of synthetic DNA molecules consisting of a 5' Tac promoter followed by the complete gene sequence of Tencon with the exception of randomized codons in the FG loop was produced (SEQ ID NOs: 26-31). For FG loop randomization, all amino

acids except cysteine and methionine were encoded at equal percentages. The lengths of the diversified portion are such that they encode for 7, 8, 9, 10, 11, or 12 amino acids in the FG loop. Sub-libraries of each length variation were synthesized individually at a scale of 2 μ g and then amplified by PCR using oligos Sloning-FOR (SEQ ID NO: 9) and Sloning-Rev (SEQ ID NO: 10).

The 3' fragment of the library is a constant DNA sequence containing elements for display, including a PspOMI restriction site, the coding region of the repA gene, and the cis- and ori elements. PCR reactions were performed to amplify this fragment using a plasmid (pCR4Blunt) (Invitrogen) as a template with M13 Forward and M13 Reverse primers. The resulting PCR products were digested by PspOMI overnight and gel-purified. To ligate the 5' portion of library DNA to the 3' DNA containing repA gene, 2 pmol (~540ng to 560ng) of 5' DNA was ligated to an equal molar (~1.25 μ g) of 3' repA DNA in the presence of NotI and PspOMI enzyme and T4 ligase at 37°C overnight. The ligated library product was amplified by using 12 cycles of PCR with oligos POP2250 (SEQ ID NO: 11) and DigLigRev (SEQ ID NO: 12). For each sub-library, the resulting DNA from 12 PCR reactions were combined and purified by Qiagen spin column. The yield for each sub-library of TCL9 ranged from 32-34 μ g.

Construction of FG/BC Loop libraries (TCL7)

The TCL7 library provides for a library with randomized Tencon BC and FG loops. In this library, BC loops of lengths 6-9 amino acids were mixed combinatorially with randomized FG loops of 7-12 amino acids in length. Synthetic Tencon fragments BC6, BC7, BC8, and BC9 (SEQ ID No. 13-16) were produced to include the Tencon gene encoding for the N-terminal portion of the protein up to and including residue VX such that the BC loop is replaced with either 6, 7, 8, or 9 randomized amino acids. These fragments were synthesized prior to the discovery of L17A, N46V and E83I mutations (CEN5243) but these mutations were introduced in the molecular biology steps described below. In order to combine this fragment with fragments encoding for randomized FG loops, the following steps were taken.

First, a DNA fragment encoding the Tac promoter and the 5' sequence of Tencon up to the nucleotide encoding for amino acid A17 (130mer-L17A, SEQ ID No. 17) was produced by PCR using oligos POP2222ext (SEQ ID No. 18) and LS1114 (SEQ ID No. 19). This was done to include the L17A mutation in the library (CEN5243). Next,

DNA fragments encoding for Tencon residues R18-V75 including randomized BC loops were amplified by PCR using BC6, BC7, BC8, or BC9 as a templates and oligos LS1115 (SEQ ID No. 20) and LS1117 (SEQ ID No. 21). This PCR step introduced a BsaI site at the 3' end. These DNA fragments were subsequently joined by overlapping PCR using oligos POP2222ext and LS1117 as primers. The resulting PCR product of 240bp was pooled and purified by Qiagen PCR purification kit. The purified DNA was digested with BsaI-HF and gel purified.

Fragments encoding the FG loop were amplified by PCR using FG7 (SEQ ID No. 31), FG8 (SEQ ID No. 30), FG9 (SEQ ID No. 29), FG10 (SEQ ID No. 28), FG11 (SEQ ID No. 27), and FG12 (SEQ ID No. 26) as templates with oligonucleotides SDG10 (SEQ ID No. 22) and SDG24 (SEQ ID No. 23) to incorporate a BsaI restriction site and N46V and E86I variations (CEN5243).

The digested BC fragments and FG fragments were ligated together in a single step using a 3-way ligation. Four ligation reactions in the 16 possible combinations were set up, with each ligation reaction combining two BC loop lengths with 2 FG loop lengths. Each ligation contained ~300 ng of total BC fragment and 300 ng of the FG fragment. These 4 ligation pools were then amplified by PCR using oligos POP2222 (SEQ ID No. 24) and SDG28 (SEQ ID No. 25). 7.5 µg of each reaction product were then digested with NotI and cleaned up with a Qiagen PCR purification column. 5.2 µg of this DNA, was ligated to an equal molar amount of RepA DNA fragment (~14 µg) digested with PspOMI and the product amplified by PCR using oligos POP2222.

EXAMPLE 2: GENERATION OF TENCON LIBRARIES HAVING ALTERNATIVE BINDING SURFACES

The choice of residues to be randomized in a particular library design governs the overall shape of the interaction surface created. X-ray crystallographic analysis of an FN3 domain containing scaffold protein selected to bind maltose binding protein (MBP) from a library in which the BC, DE, and FG loops were randomized was shown to have a largely curved interface that fits into the active site of MBP (Koide et al., Proc Natl Acad Sci U S A 104: 6632-6637, 2007). In contrast, an ankyrin repeat scaffold protein that was selected to bind to MBP was found to have a much more planar interaction surface and to bind to the outer surface of MBP distant from the active (Binz et al., Nat Biotechnol 22: 575-582, 2004). These results suggest that the shape of the binding

surface of a scaffold molecule (curved vs. flat) may dictate what target proteins or specific epitopes on those target proteins are able to be bound effectively by the scaffold. Published efforts around engineering protein scaffolds containing FN3 domains for protein binding has relied on engineering adjacent loops for target binding, thus producing curved binding surfaces. This approach may limit the number of targets and epitopes accessible by such scaffolds.

Tencon and other FN3 domains contain two sets of CDR-like loops lying on the opposite faces of the molecule, the first set formed by the BC, DE, and FG loops, and the second set formed by the AB, CD, and EF loops. The two sets of loops are separated by the beta-strands that form the center of the FN3 structure. If the image of the Tencon is rotated by 90 degrees, an alternative surface can be visualized. This slightly concave surface is formed by the CD and FG loops and two antiparallel beta- strands, the C and the F beta-strands, and is herein called the C-CD-F-FG surface. The C-CD-F-FG surface can be used as a template to design libraries of protein scaffold interaction surfaces by randomizing a subset of residues that form the surface. Beta-strands have a repeating structure with the side chain of every other residue exposed to the surface of the protein. Thus, a library can be made by randomizing some or all surface exposed residues in the beta strands. By choosing the appropriate residues in the beta-strands, the inherent stability of the Tencon scaffold should be minimally compromised while providing a unique scaffold surface for interaction with other proteins.

Library TCL14 (SEQ ID NO: 7), was designed into Tencon27 scaffold (SEQ ID NO: 4).

A full description of the methods used to construct this library is described in US. Pat. Publ. No. US2013/0226834.

TCL14 library (SEQ ID NO: 7):

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFX₁IX₂YX₃EX₄X₅X₆X₇GEAIVLTVPG
SERSYDLTGLKPGTEYX₈VX₉IX₁₀GVKGGX₁₁X₁₂SX₁₃PLSAIFTT;

Wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂ and X₁₃ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y, or M.

The two beta strands forming the C-CD-F-FG surface in Tencon27 have a total of 9 surface exposed residues that could be randomized; C-strand: S30, L32, Q34, Q36; F-strand: E66, T68, S70, Y72, and V74, while the CD loop has 6 potential residues: S38, E39, K40, V41, G42, and E43 and the FG loop has 7 potential residues: K75, G76, G77, H78, R79, S80, and N81. Select residues were chosen for inclusion in the TCL14 design due to the larger theoretical size of the library if all 22 residues were randomized.

Thirteen positions in Tencon were chosen for randomizing: L32, Q34 and Q36 in C-strand, S38, E39, K40 and V41 in CD-loop, T68, S70 and Y72 in F-strand, H78, R79, and N81 in FG-loop. In the C and F strands S30 and E66 were not randomized as they lie just beyond the CD and FG loops and do not appear to be as apparently a part of the C-CD-F-FG surface. For the CD loop, G42 and E43 were not randomized as glycine, providing flexibility, can be valuable in loop regions, and E43 lies at the junction of the surface. The FG loop had K75, G76, G77, and S80 excluded. The glycines were excluded for the reasons above while careful inspection of the crystal structures revealed S80 making key contacts with the core to help form the stable FG loop. K75 faces away from the surface of the C-CD-F-FG surface and was a less appealing candidate for randomization. Although the above mentioned residues were not randomized in the original TCL14 design, they could be included in subsequent library designs to provide additional diversity for de novo selection or for example for an affinity maturation library on a select TCL14 target specific hit.

Subsequent to the production of TCL14, 3 additional Tencon libraries of similar design were produced. These two libraries, TCL19, TCL21 and TCL23, are randomized at the same positions as TCL14 (see above) however the distribution of amino acids occurring at these positions is altered (Table 3). TCL19 and TCL21 were designed to include an equal distribution of 18 natural amino acids at every position (5.55% of each), excluding only cysteine and methionine. TCL23 was designed such that each randomized position approximates the amino acid distribution found in the HCDR3 loops of functional antibodies (Birtalan et al., J Mol Biol 377: 1518-1528, 2008) as described in Table 2. As with the TCL21 library, cysteine and methionine were excluded.

A third additional library was built to expand potential target binding surface of the other libraries library. In this library, TCL24, 4 additional Tencon positions were

randomized as compared to libraries TCL14, TCL19, TCL21, and TCL23. These positions include N46 and T48 from the D strand and S84 and I86 from the G strand. Positions 46, 48, 84, and 86 were chosen in particular as the side chains of these residues are surface exposed from beta-strands D and G and lie structurally adjacent to the randomized portions of the C and F strand, thus increasing the surface area accessible for binding to target proteins. The amino acid distribution used at each position for TCL24 is identical to that described for TCL19 and TCL21 in Table 2.

TCL24 Library (SEQ ID NO: 8)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFX₁IX₂YX₃EX₄X₅X₆X₇GEAIX₈LX₉VP
GSERSYDLTGLKPGTEYX₁₀VX₁₁IX₁₂GVKGGX₁₃X₁₄SX₁₅PLX₁₆AX₁₇FTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂ and X₁₃ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V or W.

Table 3. Amino acid frequency (%) at each randomized position for TCL21, TCL23, and TCL24.

<u>Amino Acid</u>	<u>TCL19</u>	<u>TCL21</u>	<u>TCL23</u>	<u>TCL24</u>
Ala	5.6	5.6	6.0	5.6
Arg	5.6	5.6	6.0	5.6
Asn	5.6	5.6	3.9	5.6
Asp	5.6	5.6	7.5	5.6
Cys	0.0	0.0	0.0	0.0
Gln	5.6	5.6	1.5	5.6
Glu	5.6	5.6	2.5	5.6
Gly	5.6	5.6	15.0	5.6
His	5.6	5.6	2.3	5.6
Ile	5.6	5.6	2.5	5.6

Leu	5.6	5.6	5.0	5.6
Lys	5.6	5.6	1.5	5.6
Met	0.0	0.0	0.0	0.0
Phe	5.6	5.6	2.5	5.6
Pro	5.6	5.6	4.0	5.6
Ser	5.6	5.6	10.0	5.6
Thr	5.6	5.6	4.5	5.6
Trp	5.6	5.6	4.0	5.6
Tyr	5.6	5.6	17.3	5.6
Val	5.6	5.6	4.0	5.6

Generation of TCL21, TCL23, and TCL24 libraries

The TCL21 library was generated using Colibra library technology (Isogenica) in order to control amino acid distributions. TCL19, TCL23, and TCL24 gene fragments were generated using Slonomics technology (Morphosys) to control amino acid distributions. PCR was used to amplify each library following initial synthesis followed by ligation to the gene for RepA in order to be used in selections using the CIS-display system (Odegrip et al., Proc Natl Acad Sci U S A 101: 2806-2810, 2004) as described above for the loop libraries.

EXAMPLE 3: SELECTION OF FIBRONECTIN TYPE III (FN3) DOMAINS THAT BIND HUMAN SERUM ALBUMIN

Library screening

Cis-display was used to select human serum albumin binding domains from the TCL14, TCL19, TCL21, TCL23, and TCL24 libraries. Human and rabbit serum albumin purified from serum (Sigma-Aldrich, St. Louis, MO) was biotinylated using standard methods and used for panning. For *in vitro* transcription and translation (ITT), 3 µg of library DNA were incubated with 0.1 mM complete amino acids, 1X S30 premix components, and 15 µL of S30 extract (Promega) in a total volume of 50 µL and incubated at 30°C. After 1 hour, 375 µL of blocking solution ((0.1% Casein (Thermo

Fisher, Rockford, IL), 100 mg/ml Herring Sperm DNA (Promega, Madison, WI), 1 mg/mL heparin (Sigma-Aldrich, St. Louis, MO)) was added and the reaction was incubated on ice for 15 minutes. For selection, biotinylated antigen was added at concentrations of 400 nM (Round 1), 200 nM (Rounds 2 and 3) and 100 nM (Rounds 4 and 5). Bound library members were recovered using neutravidin magnetic beads (Thermo Fisher, Rockford, IL) (Rounds 1, 3, and 5) or streptavidin magnetic beads (Promega, Madison, WI) (Rounds 2 and 4) and unbound library members were removed by washing the beads 5-14 times with 500 μ L PBST followed by 2 washes with 500 μ L PBS. Additional selection rounds were performed in order to identify scaffold molecules with improved affinities. Briefly, outputs from round 5 were prepared as described above and subjected to additional iterative rounds of selection with the following changes: incubation with biotinylated antigen was decreased from 1 hour to 15 minutes and bead capture was decreased from 20 minutes to 15 minutes, bt-HSA decreased to 25 nM (Rounds 6 and 7) or 2.5 nM (Rounds 8 and 9), and an additional 1 hour wash was performed in the presence of an excess of non-biotinylated target protein. The goal of these changes was to simultaneously select for binders with a potentially faster on-rate and a slower off-rate yielding a substantially lower K_D .

Following panning, selected FN3 domains were amplified by PCR using oligos Tcon6 (SEQID NO: 33) and Tcon5shortE86I (SEQID NO: 34), subcloned by annealing into a pET15-LIC and transformed into BL21-GOLD (DE3) cells (Agilent, Santa Clara, CA) for soluble expression in *E. coli* using standard molecular biology techniques. Single clones were picked and grown to saturation in 1 mL LB with ampicillin in 96 deepwell plates at 37°C. The following day, 25 μ L was transferred to fresh 1 mL LB-Amp media in 96 deepwell plates and grown at 37 °C for 2 hours. IPTG was added at 1 mM final concentration and protein expression was induced at 30°C for 16 hours. The cells were harvested by centrifugation and subsequently lysed with Bugbuster HT (EMD Chemicals, Gibbstown, NJ) supplemented with 0.2 mg/mL final Chicken Egg White Lysozyme (Sigma- Aldrich, St. Louis, MO). Bacterial lysates were clarified by centrifugation and supernatants were transferred to new 96 deepwell plates and tested for binding to the target protein by ELISA.

Selection of FN3 domains that Bind Human Serum Albumin

An enzyme linked immunosorbant assay (ELISA) was performed on individual clones from selected panning outputs to identify Human Serum Albumin Binders. Maxisorp plates (Nunc, Rochester, NY) were coated with either 5 ug HSA, rabSA or 5 ug/ml ovalbumin overnight (Sigma-Aldrich, St. Louis, MO), washed with Tris-Buffered Saline, pH 7.4 with 0.05% Tween-20 (TBST) and blocked using Starting Block T20 (Thermo Fisher, Rockford, IL). Clarified bacterial lysates (described above) were applied onto the wells of the coated HSA, rabSA and ovalbumin plates. Plates were incubated for 1 hour, washed with TBST and bound Centyrin was detected with pAb25-HRP (Janssen R&D, Springhouse PA) and POD chemiluminescent substrate (Roche, Indianapolis, IN) using a Molecular Devices M5 plate reader. Hits were defined as Binding Signal for Human Serum Albumin over Binding Signal for Ovalbumin > 10.

Clones were further characterized in the ELISA assay described above for binding to distinct human serum albumin domain constructs and for cross-reactivity to albumins from various species: Albumin Domain I (Albumin Biosciences, Huntsville, AL), Domain II (Albumin Biosciences, Huntsville, AL), Domain III (Albumin Biosciences, Huntsville, AL), Domain I-II (Albumin Biosciences, Huntsville, AL), rhesus serum albumin (Sigma-Aldrich, St. Louis, MO) and mouse serum albumin (Sigma-Aldrich, St. Louis, MO). Domains I, II, and III were coated at a concentration of 1 ug/ml, Domain I-II at a concentration of 2 ug/ml and the full length rhesus and mouse albumins at 3 ug/ml with human serum albumin serving as the positive binding control at 3 ug/ml. Albumin binding domain specificities of selected FN3 domains are shown in Table 4. Table 5 shows the full amino sequences of the selected FN3 domains.

Table 4. Albumin-binding domain specificities of the Selected FN3 domains

Clone ID	Albumin binding Domain Specificity
ALB-E05	III
ALB-E07	I
ALB-H9	III

Table 5. Amino Acid Sequences of the Selected FN3 domains

Clone ID	SEQ ID NO:	Sequence
ALB-E05	51	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFQ IEYWEDDVGGGEAIVLTVPGSERSYDLTGLKPG TEYDVYILGVKGGWESGPLSAIFTT
ALB-E07	52	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFK ILYEEYLVFGGEAIVLTVPGSERSYDLTGLKPGT EYWVAIWGVKGGQVSGTLSAIFTT
ALB-H9	53	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF HIEYWEQSIVGEAIVLTVPGSERSYDLTGLKP GTEYRVWIYGVKGGNDSWPLSAIFTT

Small Scale Expression and Purification identified FN3 domains binding Human Serum Albumin

Select FN3 domain clones were picked and grown to saturation in 1 mL Luria Broth (LB) supplemented with 100 ug/mL ampicillin (LB-Amp media) in 96 deep well plates at 37°C. The following day, 25 uL was transferred to fresh 5 mL LB-Amp media in 24 deep well plates and grown at 37 °C for 2 hours. IPTG was added at 1 mM final concentration and protein expression was induced at 30°C for 16 hours. Cells were harvested by centrifugation and lysed with Bugbuster HT (EMD Chemicals, Gibbstown, NJ) supplemented with 0.2 mg/mL final chicken egg white lysozyme (Sigma-Aldrich, St. Louis, MO). Bacterial lysates were clarified by centrifugation and supernatants were transferred to new 96 deepwell plates. The His-tagged FN3 domains were purified using a 96 well Ni-NTA Multitrap Plate following the manufacturers recommendation (GE Lifesciences, Piscataway, NJ).

Size Exclusion Chromatography Analysis

Size exclusion chromatography was used to determine the aggregation state of the FN3 domains binding Human Serum Albumin. Aliquots (10 µL) of each purified FN3 domain were injected onto a Superdex 75 5/150 column (GE Healthcare) at a flow rate of 0.3 mL/min in a mobile phase of PBS pH 7.4. Elution from the column was

monitored by absorbance at 280 nm. FN3 domains that exhibited high levels of aggregation by SEC were excluded from further analysis.

EXAMPLE 4: CHARACTERIZATION OF HUMAN SERUM ALBUMIN-BINDING FN3 DOMAINS

Immunoprecipitation of Human Serum Albumin-FN3 domain complexes

To evaluate the functionality of binders, selected FN3 domains were tested for their ability to complex with endogenous albumin in normal serum. To ensure that albumin binding was preserved when selected FN3 domains were attached at either the amino or carboxy terminus of another FN3 domain, bivalent FN3 genes were prepared to generate constructs ALB18, ALB 30, ALB 21, ALB33, ALB34 and ALB35 (Table 6).

Table 6: Bivalent constructs examined for albumin binding preservation

Clone ID	FN3 Domain 1	Linker	FN3 Domain 2
ALB18	Tc25	(G4S)4 (SEQ ID NO: 71)	ALB-E05
ALB30	ALB-E05	(G4S)4 (SEQ ID NO: 71)	Tc25
ALB21	ALB-E07	(G4S)4 (SEQ ID NO: 71)	Tc25
ALB33	Tc25	(G4S)4 (SEQ ID NO: 71)	ALB-E07
ALB34	Tc25	(G4S)4 (SEQ ID NO: 71)	ALB-H9
ALB35	ALB-H9	(G4S)4 (SEQ ID NO: 71)	Tc25

C-terminally His₆-tagged (SEQ ID NO: 72) bivalent FN3 domains (0.25–2.0 mg/mL in 1X DPBS without calcium or magnesium) were mixed in a 1:1 volumetric ratio with normal serum and incubated for 1 hour at room temperature to allow binding to endogenous albumin in the serum. His₆-tagged (SEQ ID NO: 72) FN3 domain-albumin complexes were recovered from serum *via* immobilized metal affinity chromatography (IMAC) using 200+ PhyTip columns containing a 5 µL resin bed (PureSpeed™ Protein Tip IMAC from Rainin Instrument LLC; Oakland, CA) pre-equilibrated with 50 mM Tris, pH 7.0 containing 500 mM NaCl and 10 mM imidazole. Unbound serum proteins were removed by exhaustively washing the resin with equilibration buffer. Bound

protein was eluted with 25–100 μ L of elution buffer (50 mM Tris, pH 7.0 containing 500 mM NaCl and 250 mM imidazole, pH 7.5). Eluates were analyzed by SDS-PAGE for the co-precipitation of albumin. SDS-PAGE gels were stained with colloidal Coomassie. Lanes containing albumin binding FN3 domains have intense bands consistent with the molecular weight of serum albumin plus FN3 domains. In contrast, albumin was not complexed when Tencon25 (SEQ ID No. 73), an FN3 domain with no affinity for serum albumin, was extracted from serum *via* IMAC purification (see Figure 2).

Pharmacokinetics of Albumin Binding FN3 Domains in Cynomolgus Monkeys:

In-vivo studies were performed with ALB18 and ALB33. Test articles were supplied to the testing facility at 2.0 mg/mL and stored at $-70 \pm 10^{\circ}\text{C}$. FN3 domains were thawed at room temperature 24 hours prior to dosing and stored at $2-8^{\circ}\text{C}$. On the day of dosing test articles were warmed to room temperature. Nine naïve female cynomolgus monkeys ranging from 2.0 - 4.0 kg at time of dosing were divided into 3 groups. FN3 domains were administered by intravenous (IV) bolus injection (target time 1-3 minutes based on overall dose volume) on Day 1 at 2 mg/kg. The volume of each dose delivered (mL) was based on individual animal's body weight as recorded 1 to 2 days prior to dosing. Approximately 1 mL of whole blood was collected from a peripheral vessel on day 1 pre-dose, 10 minutes, and 6 and 24 hours post dose. Additional samples were collected on Days 3, 4, 5, 6, 7, 9, 11, 14, and 21 at approximately the same time of day. All blood samples were collected in serum separator tubes with no anti-coagulant. Blood samples were clotted for 30 minutes and then centrifuged in a refrigerated centrifuge for 15 minutes. Approximately 1mL serum was transferred to two Eppendorf tubes (0.5 mL each) and stored at -65°C until shipment to sponsor.

H9 was evaluated as follows. Three naïve male cynomolgus monkeys, ranging from 2.2-4.4kg, were dosed with 1mg/kg H9 (0.5mg/mL prepared in PBS) at 2 mL/kg IV bolus injection. Blood samples (approximately 1 mL) were collected from a cephalic or femoral vein into BD Vacutainer® SST™ 3.5-mL tubes (Cat. No. 367957) without anticoagulant. Samples were taken at the following time points: predose ($t=0$), and at 10 min, 1h, 6h, 24h, 48h, 72h (Day 4), 168h (Day 8), 240h (Day 11), 336h (Day 15) and 504h (Day 22) hours post dose. Blood samples at each time point were clotted for 30 to

60 minutes at room temperature followed by centrifugation for 15 minutes at room temperature. Approximately 0.5 mL serum was transferred into a pre-labeled polypropylene micro-tube and immediately placed on dry ice prior to storage at -70°C or below. Serum samples were shipped on dry ice.

To determine the concentration of FN3 domains present in serum samples, standard curves for albumin binding FN3 domains H9, ALB18, and ALB33 were prepared at 300 ng/ml and serially diluted 3-fold in assay buffer (10% Control naïve cyno serum (Bioreclamation) in Super Block T20 (TBS) Blocking Buffer (Thermo Scientific)) for an 11pt curve. All study serum samples were thawed at room temperature and prepared in assay buffer at a 1:10 dilution and further serially diluted using 5-fold dilutions in assay buffer. A homogenous master mix for capture and detection was generated using biotinylated capture and ruthenium labeled detection antibodies (polyclonal anti-FN3 domain antibody pab139, Janssen Pharmaceuticals) prepared in Super Block to 0.625µg/ml. 40µL of master mix and 10µL diluted samples, standards or controls were added to 96-well Streptavidin Gold plates (MesoScale Discovery). Plates were incubated for 2 hours at room temperature, protected from light. Plates were washed using 300 µL TBS-T, 0.05% Tween-20 (Sigma) and blotted dry. A 1X solution of Read Buffer T with surfactant (MesoScale Discovery) was prepared in distilled water. Read buffer was added to assay plates at 150µL/well and electrochemiluminescence was measured using the QuickPlex SQ 120 reader (MesoScale Discovery).

Standard curves were generated in GraphPad Prism by transforming the X and Y axes to $X=\log(X)$ and $Y=\log(Y)$. A nonlinear regression (curve fit) was performed using a sigmoidal dose-response (variable slope); unknowns were interpolated off of the standard curve. To correct for the initial transformation, the mean interpolated results were transformed using $X=10^X$ and $Y=10^Y$. The linear portion of the standard curve was determined by eye. All interpolated concentrations were corrected for the dilution factor used in the assay. The final concentration was determined by averaging valid results for all dilutions and converting from ng/ml to nM.

The PKSolver add-in for excel (Zhang, Y. et al. (2010) Comput. Methods Programs Biomed., 99 306-314) was used for PK analysis. A non-compartmental analysis was performed for each animal. The half-life and clearance for the three animals for each construct were averaged to determine the final results. Pharmacokinetic data for

albumin binding FN3 domains are shown in Fig. 2 and Table 7. Albumin domain 3 binders, H9 and ALB18 showed similar exposure profiles and terminal half-life values (33-47 hours). The albumin domain 1 binder ALB33 demonstrates intermediate exposure and a terminal half-life of approximately 26 hours.

Table 7. Pharmacokinetic properties for albumin binding FN3 domains in cynomolgous monkeys presented as mean +/- standard deviation.

Clone ID	Albumi-Binding Domain Specificity	Half-life (hr)	Clearance (mg/kg)/(nmol/L)/h
ALB18	III	46.7 ± 3.6	1.08E-05 ± 1.07E-06
ALB33	I	25.8 ± 3.4	7.59E-05 ± 1.20E-05
ALB-H9	III	32.9 ± 2.8	9.74E-02 ± 5.30E-03
Tencon25	na	2.5 ± 0.7	1.26E-03 ± 1.0E-03

Sequence information

SEQ ID No. 1= Original Tencon Sequence

LPAPKNLVVSEVTEDSLRLSWTAPDAAFD SFLIQYQESEKVGEAINLTVPGSERS
YDLTGLKPGTEYTVSIYGVKGGHRSNPLSAEFTT

SEQ ID No. 2= TCL1 library

LPAPKNLVVSEVTEDSLRLSWTAPDAAFD SFLIQYQESEKVGEAINLTVPGSERS
YDLTGLKPGTEYTVSIYGV(X)₇₋₁₂PLSAEFTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₇ is any amino acid; and

X₈, X₉, X₁₀, X₁₁ and X₁₂ are any amino acid or deleted

SEQ ID No. 3=TCL2 library

LPAPKNLVVSEVTEDSLRLSWX₁X₂X₃X₄X₅X₆X₇X₈SFLIQYQESEKVGEAINLTVP
GSERSYDLTGLKPGTEYTVSIYGVX₉X₁₀X₁₁X₁₂X₁₃SX₁₄X₁₅LSAEFTT;

wherein

X₁ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₃ Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₅ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₆ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₇ is Phe, Ile, Leu, Val or Tyr;

X₈ is Asp, Glu or Thr;

X₉ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₀ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₁ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₃ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X₁₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; and

X₁₅ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val.

SEQ ID No. 4= Stabilized Tencon (Tencon 27)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFLIQYQESEKVGAEIVLTVPGSERS
YDLTGLKPGTEYTVSIYGVKGGHRSNPLSAIFTT

SEQ ID No. 5= TCL7 (FG and BC loops)

LPAPKNLVVSRVTEDSARLSWX₁X₂X₃X₄X₅X₆X₇X₈X₉FDSFLIQYQESEKVGAEIV
LTVPGSERSYDLTGLKPGTEYTVSIYGVX₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈X₁₉SNPLSA
IFTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂, X₁₃, X₁₄, X₁₅ and X₁₆ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; and

X₇, X₈, X₉, X₁₇, X₁₈ and X₁₉, are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted

SEQ ID No. 6= TCL9 (FG loop)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFLIQYQESEKVGAEIVLTVPGSERS
YDLTGLKPGTEYTVSIYGV X₁X₂X₃X₄X₅X₆X₇X₈X₉ X₁₀X₁₁X₁₂SNPLSAIFTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆ and X₇, is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y;

and

X₈, X₉, X₁₀, X₁₁ and X₁₂ is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted.

SEQ ID No. 7= TCL14 library

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFX₁IX₂YX₃EX₄X₅X₆X₇GAEIVLTVPG
SERSYDLTGLKPGTEYX₈VX₉IX₁₀GVKGGX₁₁X₁₂SX₁₃PLSAIFTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂ and X₁₃ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y, or M.

SEQ ID No. 8= TCL24 Library

TCL24 Library (SEQ ID NO: 8)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFX₁IX₂YX₃EX₄X₅X₆X₇GAEIX₈LX₉VP
GSERSYDLTGLKPGTEYX₁₀VX₁₁IX₁₂GVKGGX₁₃X₁₄SX₁₅PLX₁₆AX₁₇FTT;

wherein

X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂ and X₁₃ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V or W.

SEQ ID No. 9 = Sloning-FOR

GTGACACGGCGGTAGAAC

SEQ ID No. 10 = Sloning-REV

GCCTTTGGGAAGCTTCTAAG

SEQ ID No. 11 = POP2250

CGGCGGTAGAACGCGGCTACAATTAATAC

SEQ ID No. 12 = DigLigRev

CATGATTACGCCAAGCTCAGAA

SEQ ID No. 13 = BC9

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNN
NNNNNNNNNNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTG
AAAAAGTTGGTGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTA
CGACCTGACCGGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGT
GTTCTTAGAAGCTTCCCAAAGGC

SEQ ID No. 14 = BC8

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNN
NNNNNNNNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAA
AAGTTGGTGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGA
CCTGACCGGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTT
CTTAGAAGCTTCCCAAAGGC

SEQ ID No. 15 = BC7

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNN
NNNNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGT
TGGTGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTG
ACCGGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTCTTA
GAAGCTTCCCAAAGGC

SEQ ID No. 16 = BC6

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNN
NNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGG
TGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACC
GGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTCTTAGAA
GCTTCCCAAAGGC

SEQ ID No. 17 = 130mer-L17A

CGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCTGTTGAC
AATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTC
ACACAGGAAACAGGATCTACCATGCTG

SEQ ID No. 18 = POP222ext

CGG CGG TTA GAA CGC GGC TAC AAT TAA TAC

SEQ ID No. 19 = LS1114

CCA AGA CAG ACG GGC AGA GTC TTC GGT AAC GCG AGA AAC AAC CAG
GTT TTT CGG CGC CGG CAG CAT GGT AGA TCC TGT TTC

SEQ ID No. 20 = LS1115
CCG AAG ACT CTG CCC GTC TGT CTT GG

SEQ ID No. 21 = LS1117
CAG TGG TCT CAC GGA TTC CTG GTA CTG GAT CAG GAA AGA GTC GAA

SEQ ID No. 22 = SDG10
CATGCGGTCTCTTCCGAAAAAGTTGGTGAAGCGATCGTCCTGACCGTTCCGG
GT

SEQ ID No. 23 = SDG24
GGTGGTGAAGATCGCAGACAGCGGGTTAG

SEQ ID No. 24 = POP2222
CGGCGGTTAGAACGCGGCTAC

SEQ ID No. 25 = SDG28
AAGATCAGTTGCGGCCGCTAGACTAGAACCGCTGCCACCGCCGGTGGTGAA
GATCGCAGAC

SEQ ID No. 26 = FG12
GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGACCGCGCCGGA
CGCGGCGTTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGT
GAAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCG
GTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTT
CACCACCGGCGGTCACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCG
GCCGCAACTGATCTTGGC

SEQ ID No. 27 = FG11
GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGACCGCGCCGGA
CGCGGCGTTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGT
GAAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCG
GTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTT
CACCACCGGCGGTCACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCC
GCAACTGATCTTGGC

SEQ ID No. 28 = FG10

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGACCGCGCCGGA
CGCGGCGTTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGT
GAAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCG
GTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCAC
CGGCGGTACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCA
ACTGATCTTGGC

SEQ ID No. 29 = FG9

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGACCGCGCCGGA
CGCGGCGTTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGT
GAAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCG
GTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGG
CGGTACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACT
GATCTTGGC

SEQ ID No. 30 = FG8

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGACCGCGCCGGA
CGCGGCGTTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGT
GAAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCG
GTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNN
NNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGGCGGT
CACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACTGATC
TTGGC

SEQ ID No. 31 = FG7

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTG
GTTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGACCGCGCCGGA
CGCGGCGTTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGT
GAAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCG
GTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNN
NNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGGCGGT
CATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACTGATCTT
GC

SEQ ID NO: 32 FG loop of Tencon
KGGHRSN

SEQ ID No. 33 = Tcon 6
AAGAAGGAGAACCGGTATGCTGCCGGCGCCGAAAAAC

SEQ ID No. 34 = Tcon5E86Ishort
GAG CCG CCG CCA CCG GTT TAA TGG TGA TGG TGA
TGG TGA CCA CCG GTG GTG AAG ATC GCA GAC AG

SEQ ID No. 35 Original tencon C-strand
sfliqyqe

SEQ ID No. 36 ALB-E05 C-strand
sfQiEyWe

SEQ ID No. 37 ALB-E07 C-strand
sfKiLyEe

SEQ ID No. 38 ALB-H9 C-strand
sfHiEyWe

SEQ ID No. 39 Original tencon CD-loop
sekvge

SEQ ID No. 40 ALB-E05 CD-loop
DDVGge

SEQ ID No. 41 ALB-E07 CD-loop
YLVFge

SEQ ID No. 42 ALB-H9 CD-loop
QSIVge

SEQ ID No. 43 Original tencon F-strand
eytvsygvk

SEQ ID No. 44 ALB-E05 F-strand
eyDvYiLgvk

SEQ ID No. 45 ALB-E07 F-strand
eyWvAiWgvk

SEQ ID No. 46 ALB-H9 F-strand
eyRvWiYgvk

SEQ ID No. 47 Original tencon FG-loop
gghrsnp

SEQ ID No. 48 ALB-E05 FG-loop
ggWEsGP

SEQ ID No. 49 ALB-E07 FG-loop
ggQVsGT

SEQ ID No. 50 ALB-H9 FG-loop
ggNDsWP

SEQ ID No. 51 ALB-E05
LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFQIEYWEDDVGGEAIVLTVPGSERS
YDLTGLKPGTEYDVYILGVKGGWESGPLSAIFTT

SEQ ID No. 52 ALB-E07
LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFKILYEEYLVFGEAIVLTVPGSERSY
DLTGLKPGTEYWVAIWGVKGGQVSGTLSAIFTT

SEQ ID No. 53 ALB-H9
LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFHIEYWEQSIVGEAIVLTVPGSERS
YDLTGLKPGTEYRVWIYGVKGGNDSWPLSAIFTT

SEQ ID No. 54 (GS)₂
GSGS

SEQ ID No. 55 (GGGS)₂
GGSGGGGS

SEQ ID No. 56 (GGGS)₂
GGGGSGGGSGGGSGGGSGGGGS

SEQ ID No. 57 (AP)₂
APAP

SEQ ID No. 58 (AP)₅
APAPAPAPAP

SEQ ID No. 59 (AP)₁₀
APAPAPAPAPAPAPAPAPAP

SEQ ID No. 60 (AP)₂₀
AP

SEQ ID No. 61 A(EAAAK)₅AAA
AEAAAKEAAAKEAAAKEAAAKEAAAKAAA

SEQ ID No. 62 Human Serum Albumin

KWVTFISLLFLFSSAYS SRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQ
 QCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGE
 MADCCAKQEPERNECF LQHKDDNP NLPRLVRPEVDVMCTAFHDNEETFLKKYL
 YEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAAACLLPKLDELDEGKASS
 AKQRLKCASLQKFGERAFAKAWAVARLSQRFPKAEFAEVSKLVTDLT KVHTECC
 HGDLLCADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA
 DLPSLAADFVESKDVCKNYAEAKDVF LGMFLYEYARRHPDY SVVLLRLAKTY
 ETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCLEFEQLGEYKFQNAL
 LVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDYLSVVLNQL
 CVLHEKTPVSDRVTKCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICT
 LSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKCKADDKETCF
 AEEGKKLV AASQAALGL

SEQ ID No. 63 Cynomolgus Serum Albumin

KWVTFISLLFLFSSAYS SRGVFRRDTHKSEVAHRFKDLGE EHFKGLVLVA
 FSQYLQCPFEEHV KLVNEVTEFAKTCVADESAENCDKSL HTLFGDKLCT
 VATLRETYGEMADCCAKQEP ERNECF LQHK DDNP NLPPLVRPEVDVMCTA
 FHDNEATFLKKYLYEVARRHPYFYAPELLFFAARYKAAFAECCQAADKAA
 CLLPKLDELDEGKASSAKQ RLKCASLQKFGDRAFAKAWAVARLSQKFPKA
 EFAEVSKLVTDLT KVHTECC HGDLLCADDRADLAKYMCE NQDSISSKLK
 ECCDKPLLEKSHCLAEVENDEMPADLPSLAADYVESKDVCKNYAEAKDVF
 LGMFLYEYARRHPDY SVMLLLRLAKAYEATLEKCCAAADPHECYAKVFDE
 FQPLVEEPQN LVKQNCLEFEQLGEYKFQNAL LVRYTKKVPQVSTPTLVEV
 SRNLGKVGAKCKLPEAKRMPCAEDYLSVVLNRLCVLHEKTPVSEKVTKC
 CTESLVNRRPCFSALELDEAYVPKAFNAETFTFHADMCTLSEKEKQVKKQ
 TALVELVKHKPKATKEQLKGVMDNFAAFVEKCKCKADDKEACFAEEGPKFV
 AASQAALA

SEQ ID No. 64 ALB-E05 nucleic acid sequence

TTGCCGGCCCCGAAGAACCTGGTCGTGAGCCGTGTTACCGAGGACAGCGCGC
 GTCTGAGCTGGACCGCACCGGACGCGGCGTTTGATTCTGTTTACGATTGAGTA
 TTGGGAAGATGACGTGGGTGGTGAGGCTATCGTGCTGACCGTCCCGGGTAGC
 GAGCGCAGCTACGATCTGACGGGTCTGAAACCGGGTACCGAGTACGACGTG
 TACATTTTGGGTGTTAAAGGTGGCTGGGAGAGCGGTCCGCTGTCAGCAATCT
 TTACGACG

SEQ ID No. 65 ALB-E07 nucleic acid sequence

CTGCCGGCCCCGAAGAACCTGGTCGTGAGCCGTGTTACCGAGGACAGCGCGC
 GTCTGAGCTGGACCGCACCGGACGCGGCGTTTGATTCTGTTTAAAGATTCTGTA
 TGAAGAATACCTGGTGTTTCGGTGAGGCTATCGTGCTGACCGTCCCGGGTAGC
 GAGCGCAGCTACGATCTGACGGGTCTGAAACCGGGTACCGAGTACTGGGTG
 GCGATTTGGGGTGTTAAAGGTGGCCAGGTGAGCGGCACCCTGAGCGCAATCT
 TTACGACG

SEQ ID. No. 66 ALB-H9 nucleic acid sequence

ATGTTGCCGGCCCCGAAGAACCTGGTCGTGAGCCGTGTTACCGAGGACAGCG
 CGCGTCTGAGCTGGACCGCACCGGACGCGGCGTTTGATTCTGTTTACATTGA
 GTATTGGGAACAGAGCATCGTGGGTGAGGCTATCGTGCTGACCGTCCCGGGT

AGCGAGCGCAGCTACGATCTGACGGGTCTGAAACCGGGTACCGAGTACCGC
GTGTGGATTACGGTGTTAAAGGTGGCAATGACAGCTGGCCGCTGTCAGCAA
TCTTTACGACG

SEQ ID. No. 67 Tencon25

LPAPKNLVVSEVTEDSARLSWTAPDAAFDSFLIQYQESEKVGEAIVLTVPGSERS
YDLTGLKPGTEYTVSIYGVKGGHRSNPLSAIFTT

SEQ ID. No. 68 3rd FN3 Domain human Tenascin C

DAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSI
GNLKPDEYEVSLISRRGDMSSNPAKETFTT

SEQ ID. No. 69 Fibcon

LDAPTDLQVTNVTDTTSITVSWTPPSATITGYRITYTPSNGPGEPKELTVPPSSTSVT
ITGLTPGVEYVVS LYALKDNQESPLVGTQTT

SEQ ID. No. 70 10th FN3 Domain human Fibronectin

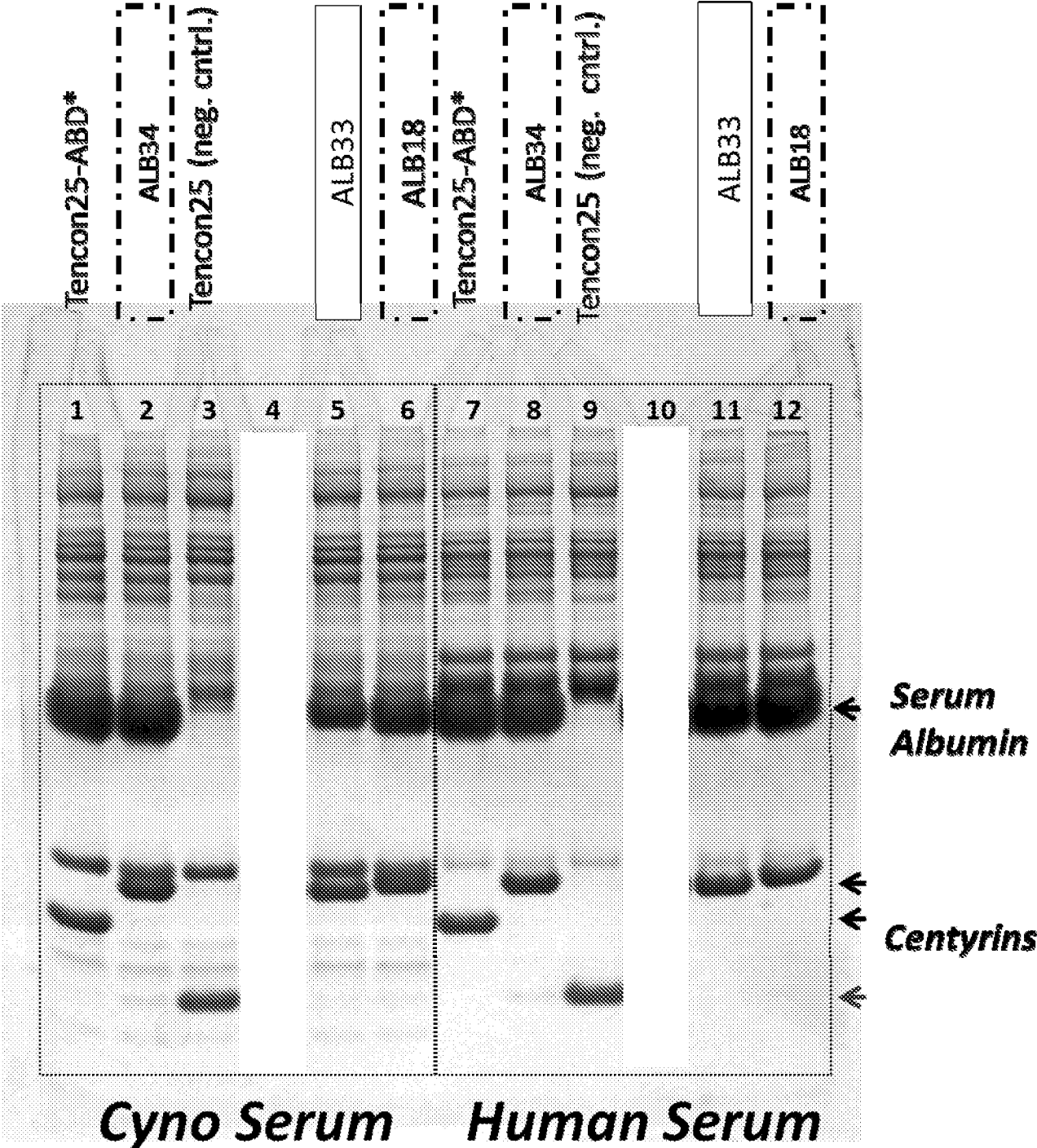
VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSKS
TATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRT

We claim:

1. An isolated fibronectin type III (FN3) domain, wherein the FN3 domain specifically binds to domain I or III of human serum albumin, and wherein the serum half-life of the FN3 domain is at least 10-fold higher than the serum half-life of the Tencon25 protein having the amino acid sequence of SEQ ID NO: 67.
2. The isolated FN3 domain of claim 1, wherein the FN3 domain cross-reacts with *Cynomolgus monkey* serum albumin.
3. The isolated FN3 domain of claim 1, wherein
 - a. the FN3 domain is based on the Tencon amino acid sequence of SEQ ID NO: 1 or the Tencon27 amino acid sequence of SEQ ID NO: 4, the amino acid sequence of SEQ ID NO: 1 or the amino acid sequence of SEQ ID NO: 4 optionally having substitutions at residues positions 11, 14, 17, 37, 46, 73, and/or 86; or
 - b. the FN3 domain is isolated from a library comprising the amino acid sequence of SEQ ID NOs: 2, 3, 5, 6, 7 or 8.
4. The isolated FN3 domain of claim 1, wherein the FN3 domain comprises a modified amino acid sequence in the C-strand, CD-loop, F-strand, and/or FG-loop relative to the Tencon amino acid sequence of SEQ ID NO: 1.
5. The isolated FN3 domain of any of the claims 1-4, wherein the FN3 domain comprises an amino acid sequence that is 90% identical to the amino acid sequence of SEQ ID NO: 51, or that has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 substitutions when compared to the amino acid sequence of SEQ ID NO: 55.
6. The isolated FN3 domain of claim 4, wherein the FN3 domain comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 51-53.
7. The isolated FN3 domain of any one of claims 4-6, wherein the serum half-life of the FN3 domain is at least 10-fold higher than the serum half-life of a protein comprising the Tencon25 amino acid sequence of SEQ ID NO: 67
8. The isolated FN3 domain of any one of claims 4-7, wherein the serum half-life of the FN3 domain is at least about 25 hours in cynomolgus monkey.
9. An isolated polynucleotide encoding the FN3 domain of any one of claims 1-8.
10. A vector comprising the polynucleotide of claim 9.

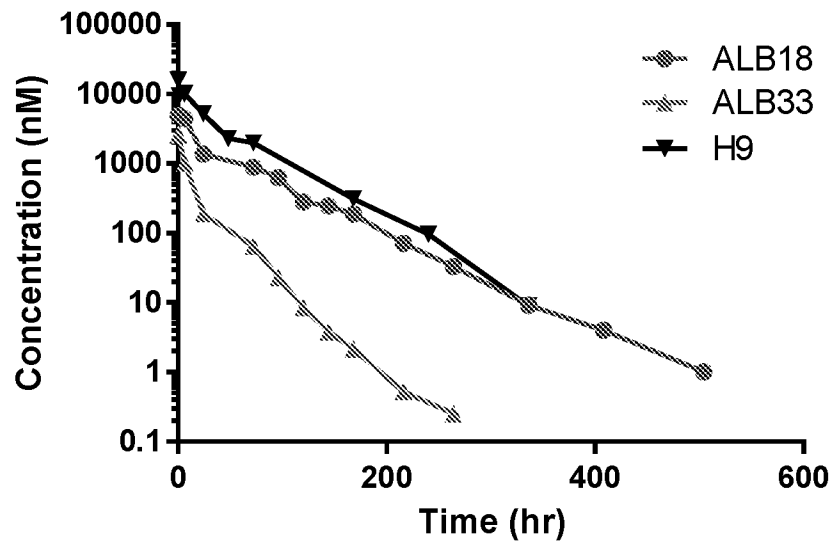
11. An isolated host cell comprising the vector of claim 10.
12. A method of producing an FN3 domain, comprising culturing the isolated host cell of claim 11 under conditions such that the FN3 domain is expressed, and purifying the FN3 domain.
13. A pharmaceutical composition comprising the FN3 domain of any one of claims 1-8 and a pharmaceutically acceptable carrier.
14. A method of detecting the presence of human serum albumin in a biological sample comprising contacting the biological sample with the FN3 domain of any one of claims 1-8 and evaluating the binding of the biological sample to the FN3 domain.
15. A kit comprising the FN3 domain of any of the claims 1-8.
16. An isolated bispecific FN3 molecule comprising a first FN3 domain and a second FN3 domain, wherein the first FN3 domain binds comprises the FN3 domain of any one of claims 1-8 and the second FN3 domain binds to a target protein other than human serum albumin.

Figure 1



2/2

Figure 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/035442

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

- a. ☒ forming part of the international application as filed:
 ☒ in the form of an Annex C/ST.25 text file.
 ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-8, 51-53, 55, and 67 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/035442

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/035442

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/38; A61K 38/39; C07K 14/78 (2017.01)

CPC - A61K 38/385; A61K 38/39; C07K 14/78; C07K 2318/20; G01N 2333/78 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 436/88; 514/9.3; 514/15.2; 530/353 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0041182 A1 (JANSSEN BIOTECH INC) 11 February 2016 (11.02.2016) entire document	1-5
Y	WO 2015/143199 A1 (BRISTOL-MYERS SQUIBB COMPANY) 24 September 2015 (24.09.2015) entire document	1-5
Y	WO 2010/051274 A2 (CENTOCOR ORTHO BIOTECH INC. et al) 06 May 2010 (06.05.2010) entire document	4
Y	US 2015/0210756 A1 (NEW YORK UNIVERSITY et al) 30 July 2015 (30.07.2015) entire document	5
A	US 2015/0152147 A1 (BRISTOL-MYERS SQUIBB COMPANY) 04 June 2015 (04.06.2015) entire document	1-6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 July 2017

Date of mailing of the international search report

11 AUG 2017

Name and mailing address of the ISA/US

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