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[Continued on next page]

(54) Title: FUSION PEPTIDES COMPRISING MULTI-FUNCTIONAL PEPTIDIC SOLUBILITY TAGS FOR EFFICIENT PRODUCTION, PROCESSING AND SURFACE APPLICATIONS

(57) Abstract: Compositions and methods using fusion peptides comprising multi-functional solubility tags are provided. The multi-functional peptidic solubility tags facilitates more efficient fusion peptide production, easier downstream processing of the fusion peptide, and may be used to provide functional surface properties when coupled to a target material.

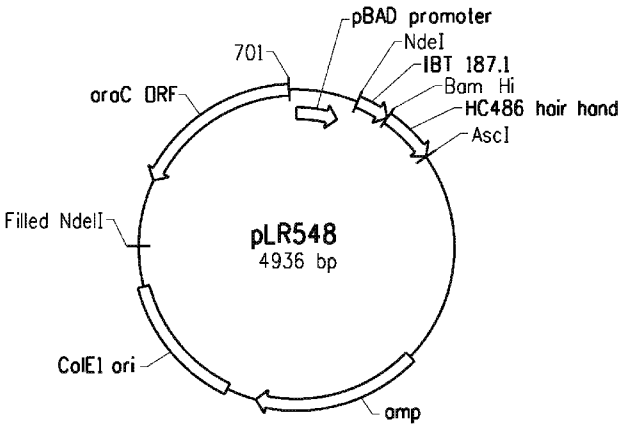

FIG. 1

WO 2012/177868 A1

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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TITLE

FUSION PEPTIDES COMPRISING MULTI-FUNCTIONAL PEPTIDIC SOLUBILITY TAGS FOR EFFICIENT PRODUCTION, PROCESSING AND SURFACE APPLICATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Patent Application No. 61/499,380, filed June 21, 2011, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

This invention relates to the field of recombinant production of fusion peptides comprising at least one multi-functional peptidic solubility tag coupled to a peptide of interest. In addition to inducing inclusion body formation for production in an expression host, the solubility tag facilitates controlled solubilization for more efficient downstream processing and provides surface functionalizing activity when associated with a target material.

BACKGROUND OF THE INVENTION

The efficient production of bioactive proteins and peptides has become a hallmark of the biomedical and industrial biochemical industry. Bioactive peptides and proteins are used as curative agents in a variety of diseases such as diabetes (insulin), viral infections and leukemia (interferon), diseases of the immune system (interleukins), and red blood cell deficiencies (erythropoietin) to name a few. Additionally, large quantities of proteins and peptides are needed for various industrial applications including, for example, the pulp and paper industries, textiles, food industries, personal care and cosmetics industries, sugar refining, wastewater treatment, production of alcoholic beverages and as catalysts for the generation of new pharmaceuticals.

In some cases commercially-useful proteins and peptides may be synthetically produced or isolated from natural sources. However, these

methods are often expensive, time consuming, and characterized by limited production capacity. Recombinant microbial production may be use to commercially produce the desired peptide/protein. Although preferable to synthesis or isolation, recombinant expression of peptides has a number of obstacles to be overcome in order to be a cost-effective means of production. For example, peptides (and in particular short peptides) produced in a cellular environment are susceptible to degradation from the action of native cellular proteases. Additionally, purification can be difficult, resulting in poor yields depending on the nature of the protein or peptide of interest.

One means to mitigate the above difficulties is the use of a genetic chimera for protein and peptide expression. A chimeric protein or "fusion protein" is a polypeptide comprising at least one portion of the desired protein product fused to at least one portion comprising a peptide tag. The peptide tag may be used to assist protein folding, assist post expression purification, protect the protein from the action of degradative enzymes, and/or assist the protein in passing through the cell membrane.

In many cases it is useful to express a peptide in an insoluble form, particularly when the peptide of interest is rather short, soluble, and/or subject to proteolytic degradation within the host cell. Production of the peptide in insoluble form both facilitates simple recovery and protects the peptide from the undesirable proteolytic degradation. One means to produce the peptide in insoluble form is to recombinantly produce the peptide as part of an insoluble fusion peptide by including within the fusion peptide at least one peptidic solubility tag (*i.e.*, an "inclusion body tag" or "IBT") that induces inclusion body formation. Typically, the fusion peptide is designed to include at least one cleavable peptide linker separating the inclusion body tag from the peptide of interest so that the peptide of interest can be subsequently recovered from the fusion peptide. The fusion peptide may be designed to include a plurality of inclusion body tags, cleavable peptide linkers, and regions encoding the peptide of interest.

Fusion proteins comprising a peptide tag that facilitate the expression of insoluble proteins are well known in the art. The solubility tag of the chimeric or fusion protein is often large, increasing the likelihood that the

fusion protein will be insoluble. Example of large peptide solubility tags include, but are not limited to chloramphenicol acetyltransferase (Dykes *et al.*, *Eur. J. Biochem.*, 174:411 (1988), β-galactosidase (Schellenberger *et al.*, *Int. J. Peptide Protein Res.*, 41:326 (1993); Shen *et al.*, *Proc. Nat. Acad. Sci. USA* 281:4627 (1984); and Kempe *et al.*, *Gene*, 39:239 (1985)), glutathione-S-transferase (Ray *et al.*, *Bio/Technology*, 11:64 (1993) and Hancock *et al.* (WO94/04688)), the N-terminus of L-ribulokinase (U.S. Patent 5,206,154 and Lai *et al.*, *Antimicrob. Agents & Chemo.*, 37:1614 (1993), bacteriophage T4 gp55 protein (Gramm *et al.*, *Bio/Technology*, 12:1017 (1994), bacterial ketosteroid isomerase protein (Kuliopulos *et al.*, *J. Am. Chem. Soc.* 116:4599 (1994), ubiquitin (Pilon *et al.*, *Biotechnol. Prog.* 13:374-79 (1997), bovine prochymosin (Haught *et al.*, *Biotechnol. Bioengin.* 57:55-61 (1998), and bactericidal/permeability-increasing protein ("BPI"; U.S. Patent 6,242,219 to Better *et al.*). The art is replete with specific examples of this technology, see for example U.S. Patent 6,613,548, describing fusion protein of proteinaceous tag and a soluble protein and subsequent purification from cell lysate; U.S. Patent 6,037,145, teaching a tag that protects the expressed chimeric protein from a specific protease; U.S. Patent 5,648,244, teaching the synthesis of a fusion protein having a tag and a cleavable linker for facile purification of the desired protein; and U.S. Patents 5,215,896; 5,302,526; 5,330,902; and 7,501,484, describing fusion tags containing amino acid compositions specifically designed to increase insolubility of the chimeric protein or peptide.

Shorter inclusion body tags have been developed from the *Zea mays* zein protein (U.S. Patent 7,732,569), the *Daucus carota* cystatin (U.S. Patent 7,662,913), an amyloid-like hypothetical protein from *Caenorhabditis elegans* (U.S. Patents 7,427,656 and 7,795,382), and a ketosteroid isomerase-derived solubility tag modified to be more acid resistant (U.S. Patent 7,829,311 to DeCarolis *et al.*). The use of short inclusion body tags increases the yield of the target peptide produced within the recombinant host cell.

U.S. Patent Application Publication No. 2009-0043075A1 to Alsop *et al.* discloses fusion proteins comprising cross-linkable inclusion body tags.

After cleaving the recovered fusion protein, oxidative cross-linking is used to separate the inclusion body tag from the polypeptide of interest.

U.S. Patents 7,678,883 and 7,794,979 to Cheng *et al*. disclose inclusion body tags derived from an 11 amino acid synthetic peptide (i.e. peptide "PII-2"; also known as peptide "DN1") capable of self-assembly into β-sheet tapes, ribbons, fibrils, and fibers in water has been described (Aggeli *et al*., *J. Amer. Chem. Soc.,* 125:9619-9628 (2003); Aggeli *et al*., *PNAS*, 98(21):11857-11862 (2001); Aggeli *et al*., *Nature*, 386:259-262 (1997); and Aggeli *et al*., *J. Mater Chem*, 7(7):1135-1145 (1997).

Fusion peptides comprising a solubility tag are typically subjected to a cleavage step to separate the solubility tag from the peptide of interest. Separating the solubility tag from the peptide of interest may be particularly desirable if the presence of the tag adversely impacts the properties of the peptide of interest. However, cleavage of the fusion peptide followed by one or more purification steps adds significant cost to the overall process.

One way to reduce the cost of producing a fusion peptide comprising a solubility tag is to use an inclusion body tag that does not need to be removed during downstream processing. Preferably, the "leave on" solubility tag is capable of providing additional functionality (beyond inclusion body formation) to the peptide of interest. Such multi-functional "leave on" solubility tags would significantly reduce the cost of manufacture and provide a higher value product

Small peptides may be used in material science applications based on their ability to bind to a target material. These "target surface-binding peptides" may be used to prepare peptide-based reagents (*e.g*., peptides of interest) designed to couple or deliver a benefit agent to the target material. Peptide-based reagents have been used to target cosmetic benefit agents to a variety of body surfaces (U.S. Patents 7,220,405; 7,309,482; 7,285,264 and 7,807,141; U.S. Patent Application Publication Nos. 2005-0226839 A1; 2007-0196305 A1; 2006-0199206 A1; 2007-0065387 A1; 2008-0107614 A1; 2007-0110686 A1; 2006-0073111 A1; 2010-0158846; 2010-0158847; and 2010-0247589; and published PCT applications WO2008/054746; WO2004/048399; and WO2008/073368).

The problem to be solved is to provide a solubility tag that is (1) effective in preparing fusion proteins which accumulate in an insoluble form within the host cell (*i.e.*, forming inclusion bodies), and (2) does not need to be cleaved from the fusion proteins prior to the intended use (*i.e.*, the presence of the solubility tag in the fusion peptide/protein does not adversely impact the desired functionality of the polypeptide/protein of interest). In a preferred embodiment of the problem to be solved, the solubility tag is not removed and also provides an additional beneficial property (in addition to driving the formation of inclusion bodies) to the fusion peptide comprising a peptide of interest. Preferably the additional or enhanced property is the ability to control and/or enhance deposition and/or the binding properties of the fusion peptide for the desired target surface.

SUMMARY OF THE INVENTION

Methods and compositions are provided comprising a fusion peptide having a peptide of interest (POI) coupled to at least one multi-functional peptide solubility tag ("multi-functional inclusion body tag") that when present in the fusion protein (1) promotes inclusion body formation within the host cell, and (2) provides an additional functionality to the fusion protein comprising the peptide of interest. As such, the present multi-functional includion body tags are not removed from the fusion peptide. In one aspect, the additional functionality provided by the "leave-on tag" is a controllable and reversible solubility enabling easier downstream processing, a binding functionality for a target material, or a combination of both. In a preferred aspect, the additional functionality of the leave-on tag is a binding affinity for a first target material while the peptide of interest provide a binding functionality for a second target material, wherein the first and second target materials are not the same.

In one embodiment, a method is provided comprising:

a) providing a microbial host cell comprising a heterologous nucleic acid molecule encoding an insoluble fusion peptide comprising at least one first portion and at least one second portion, wherein said first portion comprises a multi-functional solubility tag and said second portion comprises a peptide of interest; wherein the multi-functional solubility tag

has the general formula of:

i) SEQ ID NO: 1 – *Spacer* – [[SEQ ID NO: 1]-[*Spacer*]$_m$]$_n$

or

ii) SEQ ID NO: 2 - *Spacer*-[[SEQ ID NO: 2]-[*Spacer*]$_m$]$_n$

wherein

SEQ ID NO: 1 is

Xaa1-Gln-[Xaa2]$_p$-[Phe-Xaa3-Xaa4-Xaa5]$_s$-Phe-Xaa6-[Xaa7]$_q$-[Gln]$_r$; and

SEQ ID NO: 2 is

Xaa1-Gln-Xaa8-[Xaa4--Xaa8]$_s$-Phe-[Glu-Gln-Gln]$_r$;

wherein

Xaa1 = Gln or His

Xaa2 = Gln, Arg, His, or Lys;

Xaa3 = Gln, His, Lys, Arg, or Glu;

Xaa4 = Trp or Phe

Xaa5 = Gln, His, Lys, Arg or Glu;

Xaa6 = Glu, Gln, or Arg;

Xaa7 = Gln or Lys;

Xaa8 = Asp, Glu, Gln, His, Lys, or Arg;

p, q, and r are independently 0 or 1;

s is an integer ranging from 1 to 5;

n is an integer ranging from 1 to 10;

m = n-1; and

Spacer = a peptide linker ranging from 1 to 100 amino acids in length.

b) growing the microbial host cell under conditions whereby the insoluble fusion peptide is produced within the microbial host cell in the form of at least one inclusion body;

c) recovering the insoluble fusion peptide from the microbial host cell;

d) subjecting the insoluble fusion peptide to an aqueous medium having a first set of conditions whereby the insoluble fusion peptide becomes a soluble fusion peptide; and

e) contacting the soluble fusion peptide with a first target material having a surface whereby the fusion peptide non-covalently associates with the surface of the first target material; wherein the association between the fusion peptide and the first target material is dependent upon, or enhanced by, the presence of the multi-functional solubility tag.

In another embodiment, the present method(s) comprise a multi-functional inclusion body tag selected from the group consisting of SEQ ID NOs: IBT187.1 (SEQ ID NO: 4), IBT139 (SEQ ID NO: 14), IBT201 (SEQ ID NO: 15), IBT202 (SEQ ID NO: 16), IBT203 (SEQ ID NO: 17), IBT204 (SEQ ID NO: 18), IBT205 (SEQ ID NO: 19), IBT206 (SEQ ID NO: 20), IBT207 (SEQ ID NO: 21), IBT208 (SEQ ID NO: 22), IBT209 (SEQ ID NO: 23), IBT210 (SEQ ID NO: 24), IBT212 (SEQ ID NO: 25), IBT214 (SEQ ID NO: 26), IBT216 (SEQ ID NO: 27), IBT218 (SEQ ID NO: 28), IBT220 (SEQ ID NO: 29), IBT222 (SEQ ID NO: 30), IBT223 (SEQ ID NO: 31), IBT224 (SEQ ID NO: 32), IBT225 (SEQ ID NO: 33), IBT229 (SEQ ID NO: 34), IBT230 (SEQ ID NO: 35), IBT232 (SEQ ID NO: 36), IBT233 (SEQ ID NO: 37), IBT239 (SEQ ID NO: 38), IBT241 (SEQ ID NO: 39), IBT242 (SEQ ID NO: 40), IBT247 (SEQ ID NO: 41), IBT248 (SEQ ID NO: 42), IBT249 (SEQ ID NO: 43), IBT254 (SEQ ID NO: 44), IBT255 (SEQ ID NO: 45), IBT258 (SEQ ID NO: 46), IBT259 (SEQ ID NO: 47), IBT260 (SEQ ID NO: 48), IBT261 (SEQ ID NO: 49), IBT262 (SEQ ID NO: 50, IBT263 (SEQ ID NO: 51), IBT282 (SEQ ID NO: 52), IBT283 (SEQ ID NO: 53), IBT284 (SEQ ID NO: 54), IBT287 (SEQ ID NO: 55), IBT289 (SEQ ID NO: 56), IBT290 (SEQ ID NO: 57), IBT294 (SEQ ID NO: 58), IBT295 (SEQ ID NO: 59), IBT297 (SEQ ID NO: 60), IBT298 (SEQ ID NO: 61), IBT299 (SEQ ID NO: 62), IBT310 (SEQ ID NO: 63), IBT311 (SEQ ID NO: 64), IBT312 (SEQ ID NO: 65), IBT313 (SEQ ID NO: 66), IBT314 (SEQ ID NO: 67), IBT315 (SEQ ID NO: 68), IBT316 (SEQ ID NO: 69), IBT317 (SEQ ID NO: 70), IBT320 (SEQ ID NO: 71), IBT321 (SEQ ID NO: 72), IBT326 (SEQ ID

NO: 73), IBT327 (SEQ ID NO: 74), IBT332 (SEQ ID NO: 75), IBT334 (SEQ ID NO: 76), and IBT340 (SEQ ID NO: 77).

In another embodiment, a multi-function solubility tag is provided selected from the group consisting of: IBT205 (SEQ ID NO: 19), IBT206 (SEQ ID NO: 20), IBT207 (SEQ ID NO: 21), IBT208 (SEQ ID NO: 22), IBT209 (SEQ ID NO: 23), IBT210 (SEQ ID NO: 24), IBT212 (SEQ ID NO: 25), IBT214 (SEQ ID NO: 26), IBT216 (SEQ ID NO: 27), IBT218 (SEQ ID NO: 28), IBT220 (SEQ ID NO: 29), IBT222 (SEQ ID NO: 30), IBT233 (SEQ ID NO: 37), IBT242 (SEQ ID NO: 40), IBT248 (SEQ ID NO: 42), IBT249 (SEQ ID NO: 43), IBT254 (SEQ ID NO: 44), IBT255 (SEQ ID NO: 45), IBT258 (SEQ ID NO: 46), IBT259 (SEQ ID NO: 47), IBT261 (SEQ ID NO: 49), IBT262 (SEQ ID NO: 50, IBT263 (SEQ ID NO: 51), IBT299 (SEQ ID NO: 62), IBT310 (SEQ ID NO: 63), IBT311 (SEQ ID NO: 64), IBT312 (SEQ ID NO: 65), IBT313 (SEQ ID NO: 66), IBT314 (SEQ ID NO: 67), IBT315 (SEQ ID NO: 68), IBT316 (SEQ ID NO: 69), IBT317 (SEQ ID NO: 70), IBT320 (SEQ ID NO: 71), IBT321 (SEQ ID NO: 72), IBT326 (SEQ ID NO: 73), IBT327 (SEQ ID NO: 74), IBT332 (SEQ ID NO: 75), IBT334 (SEQ ID NO: 76), and IBT340 (SEQ ID NO: 77).

In another embodiment, a multi-functional inclusion body tag is provided comprising at least one core motif having an amino acid sequence selected form the group consisting of SEQ ID NOs: 80, 85, 86, 87, 88, 91, 92, 93, 94, 95, 96, 97, 98, 100, and 101.

Fusion peptides comprising the present multi-functional solubility tags and/or core motifs may be incorporated into various compositions. As such, a composition is also provided comprising a fusion peptide comprising at least one multi-functional solubilty tag as defined above.

In another embodiment, a composition comprising a particle comprising the at least one of the present fusion peptides is also provided. In a preferred embodiment, the particle comprises an average particle size of 100 nm to 10 μm as measured by a light scattering method. In a further aspect, the particle is partially or completely coated with one or more of the present fusion peptides.

In another embodiment, a personal care product is provided comprising a fusion peptide comprising at least one multi-functional

solubilty tag as defined above.

In another embodiment, the personal care product is selected from the group consisting of a shampoos, hair gels, hair sprays, mousses, hair coloring products, hair bleaching products, hair conditioners, body washes, skin creams, lotions, skin moisturizer, sunscreens, tonics, toothpastes, dental creams, tooth gels, tooth powders, mouth washes, breath fresheners, and dental floss.

In another embodiment, the multi-functional inclusion body tag (IBT) has affinity for a first target material while the peptide of interest (POI) has affinity for a second target material, wherein the first and second target materials are not the same. As such, the fusion peptide acts as an interfacial peptide bridge coupling the first target material to a second target material. The target material(s) may vary and may include in certain embodiments films, particles, and coated particles. In one embodiment, the target material is a pigment (such as lakes, insoluble organic pigment, insoluble inorganic pigment), a body tissue, a cell surface receptor, a body tissue suitable for cosmetic personal care products (*e.g.*, hair, skin, nail, teeth and other oral cavity tissues), a body tissue for medical or veterinary applications, a plant tissue (*e.g.*, seeds, leaves, stems, flowers, fruit, etc.), a polymer (such as polystyrene, polyethylene, polypropylene, polytetrafluoroethylene, polyester, polyvinyl chloride, poly (methyl methacrylate), polyethersulfone, polyimide, polyamide, aramids (*e.g.*, poly *para*-phenylene terephthalamide; "KEVLAR®", poly *meta*-phenylene terephthalamide; "NOMEX®", poly urethanes),and polysaccharides (*e.g.* cellulose, cellulosic materials, starch, chitin)), minerals (*e.g.* silica, silicates, micas, titanium dioxide, alumina, iron oxides, clays), and metals (*e.g.,* gold, silver), carbon surfaces such as carbon black, graphite and carbon nanotubes, to name a few.

In another embodiment, an affinity media is provided comprising a fusion peptide having at least one multi-functional solubilty tag as defined above. In a further aspect, the affinity media comprises a solid support comprising a material selected from the group consisting of a pigment, a plant tissue, a polymer, polystyrene, polyethylene, polypropylene, polytetrafluoroethylene, polyester, polyvinyl chloride, poly (methyl

methacrylate), polyethersulfone, polyimide, polyamide, aramids, poly *para*-phenylene terephthalamide, poly *meta*-phenylene terephthalamide, poly urethane, polysaccharides, cellulose, starch, chitin, minerals, silica, silicates, micas, titanium dioxide, alumina, iron oxides, clays, metals, gold, silver, carbon, carbon black, graphite and carbon nanotubes. In a further aspect, the solid support is in the form of a resin, a membrane, a filter, a bead, a fiber, a foam, a film or any combination thereof.

The affinity media comprising at least one of the present fusion peptides may be used in a method to obtain a material from an aqueous matrix. As such, a method is provided comprising:

a) providing an aqueous matrix comprising a target material to be obtained from the aqueous matrix; and

b) contacting the aqueous matrix with the affinity media described above whereby the target material binds to the fusion protein. In a preferred aspect, the above method further comprises step (c), eluting the bound target material from the affinity media.

In another embodiment, a biomedical or tissue engineering composition comprising one or more of the present fusion peptides or a composition comprising a particle comprising one or the present fusion peptides is also provided. In one embodiment, the portion of the fusion peptide comprising the multi-functional inclusion body tag is capable of forming a hydrogel and the second portion of the fusion peptide has affinity for a body tissue and/or a cell surface receptor. In a further embodiment, the second portion having affinity for the cell surface receptor comprises at least one RGD peptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the map of plasmid pLR548 (Example 1, SEQ ID NO: 78)

Figure 2 is the map of plasmid pLD001-233 (Example 1, SEQ ID NO: 79)

Figure 3 comprises an image of spins tubes illustrating the effects of the various fusion peptides on binding two different surfaces together non-

covalently. Only peptides with specific binding domains for the two respective surfaces can anchor the particles together.

BRIEF DESCRIPTION OF THE BIOLOGICAL SEQUENCES

The following sequences comply with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPC and PCT (Rules 5.2 and 49.5(a bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NO: 1 is the amino acid sequence a structural formula for multi-functional inclusion body tags.

SEQ ID NO: 2 is the amino acid sequence a structural formula for multi-functional inclusion body tags.

SEQ ID NO: 3 is the nucleic acid sequence of a polynucleotide encoding IBT187.1.

SEQ ID NO: 4 is the amino acid sequence of IBT187.1.

SEQ ID NO: 5 is the nucleic acid sequence encoding the peptide of interest HC263.

SEQ ID NO: 6 is the amino acid sequence of the peptide of interest HC263.

SEQ ID NO: 7 is the nucleic acid sequence encoding fusion peptide IBT187.HC263.

SEQ ID NO: 8 is the amino acid sequence of fusion peptide IBT187.HC263.

SEQ ID NO: 9 is the nucleic acid sequence encoding fusion peptide IBT187.H2-TonB.G3.

SEQ ID NO: 10 is the amino acid sequence of fusion peptide IBT187.H2-TonB.G3.

SEQ ID NO: 11 is the nucleic acid sequence of a polynucleotide encoding IBT233.

SEQ ID NO: 12 is the nucleic acid sequence encoding fusion peptide IBT233.HC263.

SEQ ID NO: 13 is the amino acid sequence of fusion peptide IBT233.HC263.

SEQ ID NO: 14 is the amino acid sequence of IBT139.

SEQ ID NO: 15 is the amino acid sequence of IBT201.

SEQ ID NO: 16 is the amino acid sequence of IBT202.

SEQ ID NO: 17 is the amino acid sequence of IBT203.

SEQ ID NO: 18 is the amino acid sequence of IBT204.

SEQ ID NO: 19 is the amino acid sequence of IBT205.

SEQ ID NO: 20 is the amino acid sequence of IBT206.

SEQ ID NO: 21 is the amino acid sequence of IBT207.

SEQ ID NO: 22 is the amino acid sequence of IBT208.

SEQ ID NO: 23 is the amino acid sequence of IBT209.

SEQ ID NO: 24 is the amino acid sequence of IBT210.

SEQ ID NO: 25 is the amino acid sequence of IBT212.

SEQ ID NO: 26 is the amino acid sequence of IBT214.

SEQ ID NO: 27 is the amino acid sequence of IBT216.

SEQ ID NO: 28 is the amino acid sequence of IBT218.

SEQ ID NO: 29 is the amino acid sequence of IBT220.

SEQ ID NO: 30 is the amino acid sequence of IBT222.

SEQ ID NO: 31 is the amino acid sequence of IBT223.

SEQ ID NO: 32 is the amino acid sequence of IBT224.

SEQ ID NO: 33 is the amino acid sequence of IBT225.

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SEQ ID NO: 35 is the amino acid sequence of IBT230.

SEQ ID NO: 36 is the amino acid sequence of IBT232.

SEQ ID NO: 37 is the amino acid sequence of IBT233.

SEQ ID NO: 38 is the amino acid sequence of IBT239.

SEQ ID NO: 39 is the amino acid sequence of IBT241.

SEQ ID NO: 40 is the amino acid sequence of IBT242.

SEQ ID NO: 41 is the amino acid sequence of IBT247.

SEQ ID NO: 42 is the amino acid sequence of IBT248.

SEQ ID NO: 43 is the amino acid sequence of IBT249.

SEQ ID NO: 44 is the amino acid sequence of IBT254.
SEQ ID NO: 45 is the amino acid sequence of IBT255.
SEQ ID NO: 46 is the amino acid sequence of IBT258.
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SEQ ID NO: 49 is the amino acid sequence of IBT261.
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SEQ ID NO: 52 is the amino acid sequence of IBT282.
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SEQ ID NO: 63 is the amino acid sequence of IBT310.
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SEQ ID NO: 68 is the amino acid sequence of IBT315.
SEQ ID NO: 69 is the amino acid sequence of IBT316.
SEQ ID NO: 70 is the amino acid sequence of IBT317.
SEQ ID NO: 71 is the amino acid sequence of IBT320.
SEQ ID NO: 72 is the amino acid sequence of IBT321.
SEQ ID NO: 73 is the amino acid sequence of IBT326.
SEQ ID NO: 74 is the amino acid sequence of IBT327.
SEQ ID NO: 75 is the amino acid sequence of IBT332.
SEQ ID NO: 76 is the amino acid sequence of IBT334.
SEQ ID NO: 77 is the amino acid sequence of IBT340.

SEQ ID NO: 78 is the nucleic acid sequence of plasmid pLR548.

SEQ ID NO: 79 is the nucleic acid sequence of plasmid pLD001.233.

SEQ ID NOs: 80-104 are the amino acid sequences of core motifs found in at least one of the multi-functional inclusion body tags.

SEQ ID NOs: 105-392 are the amino acid sequences of exemplary peptides that to bind at least one body surface including hair (SEQ ID NOs: 105-231), skin (SEQ ID NOs: 227-279), nail (SEQ ID NOs: 280-281), and tooth (SEQ ID NOs: 282-392).

SEQ ID NOs: 393 – 421 are the amino acid sequences of exemplary antimicrobial peptides.

SEQ ID NOs: 422-446 are the amino acid sequences of exemplary pigment binding peptides.

SEQ ID NOs: 447-452 are exemplary cellulose-binding peptides.

SEQ ID NOs: 453 – 479 are the amino acid sequences of exemplary polymer binding peptides. Specifically, SEQ ID NO: 453 binds to poly(ethylene terephthalate), SEQ ID NOs: 454-464 bind to poly(methyl methacrylate), SEQ ID NOs: 465-470 bind to Nylon, and SEQ ID NOs: 471-479 bind to poly(tetrafluoroethylene).

SEQ ID NOs: 480 – 495 are the amino acid sequences of exemplary clay binding peptides.

SEQ ID NO: 496 is the amino acid sequence of “AuBD”, a peptide having affinity for gold.

SEQ ID NO: 497 is the amino acid sequence of fusion peptide IBT255.AuBD.

SEQ ID NO: 498 is the amino acid sequence of synthetic peptide HC353 (U.S. Patent Application Publication No. 2010-0158837; hereby incorporated by reference).

SEQ ID NO: 499 is the amino acid sequence of fusion peptide IBT255.HC263.

DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods directed to the use of a multi-functional solubility tag as a component of fusion peptide/protein are provided herein,

wherein the presence of the multi-functional solubility tag in the fusion peptide facilitates:

(i) producing the fusion peptide in an insoluble form (*i.e.*, induces inclusion body formation) within a recombinant host cell;

(ii) controlling fusion peptide solubility (once recovered from the recombinant host cell) upon modulation of physio-chemical conditions;

(iii) providing new or enhanced surface active properties; and

(iv) increasing the overall efficiency of production.

The presence of the solubility tag in a fusion peptide provides additional beneficial functionality beyond inclusion body formation. As such, the present multi-functional solubility tags are not removed ("leave-on" peptide tags; "LOTs") from the fusion peptide and remain part of the desired peptidic fusion product.

In one embodiment, the methods and present compositions are useful for the recombinant expression and recovery of any bioactive peptides and proteins. Such peptides/proteins typically have high value in any number of applications including, but not limited to, medical, biomedical, diagnostic, personal care, and affinity applications where the peptides of interest are used as linkers to various surfaces.

In this disclosure, a number of terms and abbreviations are used. The following definitions apply unless specifically stated otherwise. Unless otherwise noted, all U.S. Patents and U.S. Patent Applications referenced herein are incorporated by reference in their entirety.

As used herein, the articles "a", "an", and "the" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances (*i.e.*, occurrences) of the element or component. Therefore "a", "an" and "the" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

The term "comprising" means the presence of the stated features, integers, steps, or components as referred to in the claims, but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof. The term "comprising" is intended to include embodiments encompassed by the terms "consisting essentially of"

and “consisting of”. Similarly, the term “consisting essentially of” is intended to include embodiments encompassed by the term “consisting of”.

As used herein, the term "about" modifying the quantity of an ingredient or reactant employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or use solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or carry out the methods; and the like. The term “about” also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities.

Where present, all ranges are inclusive and combinable. For example, when a range of “1 to 5” is recited, the recited range should be construed as including ranges “1 to 4”, “1 to 3”, “1-2”, “1-2 & 4-5”, “1-3 & 5”, and the like.

As used herein, an "inclusion body" is an intracellular deposit comprising aggregated protein found in the cytoplasm of a cell. A peptide of interest that is typically soluble under the normal physiological conditions within the host cell and/or cell lysates can be fused to one or more of the multi-functional inclusion body tags to facilitate formation of an insoluble fusion protein. In another embodiment, the peptide of interest may be partially insoluble in the host cell, but produced at relatively lows levels where significant inclusion body formation does not occur. As such, the formation of inclusion bodies will increase peptide production. In a further embodiment, fusion of the peptide of interest to one or more multi-functional inclusion body tags increases the amount of protein produced in the host cell. Formation of the inclusion body facilitates simple and efficient purification of the fusion peptide from the cell lysate using techniques well known in the art such as centrifugation and filtration.

As used herein, the terms “inclusion body tag” and “solubility tag” will be abbreviated “IBT” and will refer a peptide/polypeptide that facilitates formation of inclusion bodies when fused to a peptide of interest. The

peptide of interest is typically soluble within the host cell and/or host cell lysate when not fused to an inclusion body tag. Fusion of the peptide of interest to the inclusion body tag produces a fusion protein that agglomerates into intracellular bodies (inclusion bodies) within the host cell.

As used herein, the terms "multi-functional tag", "muti-functional solubility tag", "multi-functional inclusion body tag", "leave-on tag" , and "LOT" will refer to an inclusion body tag having at least one beneficial functionality beyond inclusion body formation when present in a fusion peptide. As such, the "leave-on tag" is not removed during downstream processing and becomes part of the desired peptidic fusion product. The functionality beyond inclusion body formation may include, but is not limited to, medical, biomedical, diagnostic, personal care, and affinity applications (where the peptides of interest are used as linkers to various surfaces). In one embodiment, the presence of the multi-functional solubility tag in the fusion protein provides the ability to control solubility of the fusion peptide under different physio-chemical conditions. In another embodiment, the presence of the multi-functional solubility tag in the fusion protein provides new or enhanced surface active properties. For example, the "leave on tag" may provide a binding affinity for a first target material or may enhance the binding properties of the peptide of interest (POI) for a first target material. In another embodiment, the multi-functional IBT has affinity for a first target material while the peptide of interest (POI) portion of the fusion peptide has affinity for a second target material. As such, the fusion peptide may be used as a peptidic bridge to couple a first target material to a second target material, wherein the first and second target materials are compositionally different.

As used herein, the term "spacer" will refer to a peptide within the present inclusion body tags used to separate the core motif sequences (SEQ ID NOs: 1, 2, and 80-101). In one embodiment, the spacer is 1-100 amino acids in length, preferably 3 to 60 amino acids in length, and most preferably 3 to 30 amino acids in length and is comprised of any naturally occurring L-amino acids. In one embodiment, the "spacer" is comprised of one or more L-amino acids selected from the group consisting of proline, glycine, arginine, asparagine, glutamic acid, glutamine, serine, tyrosine,

tryptophan, alanine, leucine, isoleucine, threonine, histidine, lysine, aspartic acid, and combinations thereof.

As used herein, the term "peptide of interest" or "POI" is any peptide that provides a defined desired functionality (that by itself is distinct from the functionality of the multi-functional inclusion body tag), such as a binding affinity for a target material, surface or a specific molecule; a biological activity such as a growth factor or an antimicrobial; a physical modification of a surface to provide charge, hydrophobicity, chelation, dispersion, optical properties such as refractive index, color, UV protection or fluorescence or conditioning agent, and the ability to self assemble in macromolecular structures. The peptide of interest may comprise one or more distinct surface binding domains. The peptide of interest is soluble and/or difficult to accumulate within the microbial host cell when not produced in the form of a fusion peptide comprising at least one of the present multi-functional inclusion body tags.

As used herein, the terms "fusion protein", "fusion peptide", "chimeric protein", and "chimeric peptide" will be used interchangeably and will refer to a polymer of amino acids (peptide, oligopeptide, polypeptide, or protein) comprising at least two portions, each portion comprising distinct function(s). The present fusion peptides comprise at least one multi-functional solubility tag and at least one peptide of interest, wherein the presence of the mulit-functional inclusion body tag provides additional functionality (beyond inclusion body formation) to the fusion peptide. Means to prepare peptides synthetically at small scale are well known in the art (see, for example, Stewart *et al.*, Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL, 1984; Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, New York, 1984; and Pennington *et al.*, Peptide Synthesis Protocols, Humana Press, Totowa, NJ, 1994). The various components of the fusion peptides (inclusion body tag, peptide of interest, and the optional peptide "spacers") described herein can be combined using carbodiimide coupling agents (see for example, Hermanson, Greg T., Bioconjugate Techniques, Academic Press, New York (1996)), diacid chlorides, diisocyanates and other difunctional coupling reagents that are reactive to terminal amine and/or carboxylic acid groups on the peptides.

However, chemical synthesis is often limited to peptides of less than about 50 amino acids length due to cost and/or impurities.In a preferred embodiment, the biological molecules described herein are prepared using standard recombinant DNA and molecular cloning techniques.

As used herein, the term "altering the physio-chemical conditions" will refer to making alterations to the pH, temperature, ionic composition, ionic strength of the aqueous matrix, and any combination thereof that facilitates controlled solubility of the fusion peptide comprising the multi-functional inclusion body tag. Under normal physiological conditions within the recombinant microbial host cell (typically an intracellular pH of about 6.5-about 8.0, preferably about 7.5 to about 8.0 for most neutrophiles) the multi-functional inclusion body tag induces inclusion body formation. The fusion peptide may be subjected to altered physio-chemical conditions (typically after the fusion peptide has been recovered from the host cell) to control the solubiility, wherein the presence of the leave-on tag alters the solubility profile of the fusion peptide. The physio-chemcial conditions may be altered one or more times to dissolve or precipitate the fusion peptide. In one embodiment, solubilized fusion peptide (in the presence of a target material) may be exposed to physio-chemical conditions that induces or enchances deposition/binding of the fusion peptide to the surface of the target material. Physico-chemical conditions that can be modified to promote the solubility or the insolubility of a fusion peptide (typically once recoved from the cell) may include pH, medium conductivity, salts, metal ions, polyvalent ions, chaotropes, surfactants, solvents, fusion peptide concentration, rheology modifiers, thickeners and temperature.

As used herein, the term "medium" may mean fermetation medium or a suspension medium. The fermentation medium is the medium which supports the growth of fusion peptide-producing cells. The medium is typically aqueous but may also include a water–oil emulsion. As used herein, the term "aqueous medium" refers to a medium comprising water. The solubility state of the fusion peptides recovered from the host cell is controlled by altering the physio-chemcial conditions within an aqueous medium comprising the fusion peptide. Different sets of physio-chemical conditions may used and may be generally separated into three general

categories: (1) a set of conditions that promotes solubility, (2) a set of conditions that promotes insolubility, and (3) a set of conditions thay may promote or enhance deposition of the fusion peptide to a target material surface (*i.e.*, the target material is contacted with a medium comprising the fusion peptide).

As used herein, the term "benefit agent" refers to a material that promotes or enhances a useful advantage, a favorable/desirable effect or benefit. The benefit agent may be a fusion peptide or a molecule or a material associated with (or coupled to) a portion of the fusion peptide. Examples of benefits agents may include, but are not limited to conditioners, pigments, dyes, fragrances, whitening agents, bleaching agents, enzymes, pharmaceutical agents (*e.g.*, targeted delivery of cancer treatment agents), diagnostic/labeling agents, ultraviolet light blocking agents (*i.e.*, active agents in sunscreen protectants), affinity media, particles, and antimicrobial agents (*e.g.,* antimicrobial peptides), to name a few.

As used herein, the term "target material" will refer to a material that receives or delivers a beneficial or desired property upon the presence of the fusion peptide. In one embodiment, the fusion peptide has affinity for a target material wherein the binding functionality is dependent upon the presence of the multi-functional inclusion boy tag (IBT), the peptide of interest (POI), or a combination of the IBT and POI. In one embodiment, the presence of the multi-functional IBT enhances the binding affinity of a POI having affinity for a target material. In another embodiment, the multi-functional IBT has affinity for a target surface and the portion of the fusion peptide comprising the peptide of interest is a benefit agent, such as a conditioning agent or antimicrobial agent (*e.g.*, antimicrobial peptide).

The target material(s) may vary and may include in certain embodiments films, particles, and coated particles. In one embodiment, the target material may include a pigment (such as lakes, insoluble organic pigment, insoluble inorganic pigment), a body tissue suitable for cosmetic personal care products (*e.g.*, hair, skin, nail, teeth and other oral cavity tissues), a body tissue for medical or veterinary applications, a plant tissue (*e.g.*, seeds, leaves, stems, flowers, fruit, etc.), a polymer (such as polystyrene, polyethylene, polypropylene, polytetrafluoroethylene,

polyester, polyvinyl chloride, poly (methyl methacrylate), polyethersulfone, polyimide, polyamide, aramids (*e.g.*, poly *para*-phenylene terephthalamide; "KEVLAR®", poly *meta*-phenylene terephthalamide; "NOMEX®", poly urethanes), and polysaccharides (*e.g.* cellulose, cellulosic materials, starch, chitin)), minerals (*e.g.* silica, silicates, micas, titanium dioxide, alumina, iron oxides, clays), and metals (*e.g.,* gold, silver), carbon surfaces such as carbon black, graphite and carbon nanotubes, to name a few.

As used herein, the term "pigment" refers to an insoluble, organic or inorganic colorant.

As used herein, the term "hair" as used herein refers to mammalian hair. In one embodiment, the "hair" is preferably human hair, eyebrows, and eyelashes.

As used herein, the term "skin" as used herein refers to mammalian skin. In one embodiment, the "skin" is preferably human skin, or substitutes for human skin, such as pig skin, VITRO-SKIN® and EPIDERM™.

As used herein, the term "nails" as used herein refers to mammalian nails, such as fingernails, toenails and other keratinaceious nail materials. In one embodiment, the "nails" are preferably human fingernails and/ortoenails.

As used herein, the term "strong affinity" or "high affinity" will refer to a binding affinity having a K_D or MB_{50} value of less than or equal to about 10^{-5} M, preferably less than or equal to about 10^{-6} M, more preferably less than or equal to about 10^{-7} M, more preferably less than or equal to about 10^{-8} M, even more preferably less than or equal to about 10^{-9} M, or most preferably less than or equal to about 10^{-10} M.

As used herein, "K_D" corresponds to the concentration of peptide at which the binding site on the target is half occupied, *i.e.*, when the concentration of target with peptide bound (bound target material) equals the concentration of target with no peptide bound. The smaller the dissociation constant, the more tightly the peptide is bound. For example, a peptide with a nanomolar (nM) dissociation constant binds more tightly than a peptide with a micromolar (μM) dissociation constant. Certain embodiments of the invention will have a K_D value of 10^{-5} or less.

As used herein, "MB_{50}" refers to the concentration of the binding peptide that gives a signal that is 50% of the maximum signal obtained in an ELISA-based binding assay. See, *e.g.*, Example 3 of U.S. Patent Application Publication 2005/022683; hereby incorporated by reference. The MB_{50} provides an indication of the strength of the binding interaction or affinity of the components of the complex. The lower the value of MB_{50}, the stronger, *i.e.*, "better," the interaction of the peptide with its corresponding substrate. For example, a peptide with a nanomolar (nM) MB_{50} binds more tightly than a peptide with a micromolar (μM) MB_{50}. Certain embodiments of the invention will have a MB_{50} value of 10^{-5} M or less.

As used herein, the terms "bind", "binding", and "coupling" are used interchangeably and is meant to convey an association between a fusion peptide and a surface on a target material though a variety of non-covalent interactions, such as ionic bond-based (electrostatic interaction), hydrogen bond-based, hydrophobic bond-based, chelation based, biological specific affinity, a general aggregation or the formation of tertiary or quaternary structures or a combination thereof, but does not include, by proviso, covalent binding.

As used hereink, the term "affinity peptide" or "target surface-binding peptide" refers to a peptide having strong affinity for a particular target material or compound. Examples may include, but are not limited to, body surface-binding peptides, pigment-binding peptides, polymer-binding peptides, various mineral-binding peptides, clay-binding peptides, cotton-binding peptides, cellulose-binding peptides, and metal-binding peptides.

As used herein, a "body surface-binding peptide" is an affinity peptide that binds with high affinity to a specified body surface. Examples may include, but are not limited to, hair-binding peptides, skin-binding peptides, nail-binding peptides, and tooth-binding peptides.

As used herein, a "hair-binding peptide" is an affinity peptide that binds with high affinity to hair.

As used herein, a "skin-binding peptide" is an affinity peptide that binds with high affinity to skin.

As used herein, a "nail-binding peptide" is an affinity peptide that

binds with high affinity to fingernail or toenail.

As used herein, a "tooth-binding peptide" is an affinity peptide that binds with high affinity to tooth enamel and/or tooth pellicle. In a preferred embodiment, the tooth-binding peptide binds with high affinity to tooth enamel.

As used herein, an "antimicrobial peptide" is a peptide having the ability to kill microbial cell populations (U.S. Patent 7,427,656; hereby incorported by reference). The amino acid sequences of exemplary antimicrobial peptides are provided as SEQ ID NOs: 393-421.

As used herein, "cellulose-binding peptide" refers to an affinity peptide that binds with high affinity to cellulose. Exemplary cellulose-bindign peptides are provided as SEQ ID NOs: 447-452.

As used herein, "clay-binding peptide" refers to a peptide that binds with high affinity to clay. Exemplary clay-binding peptides are provided as SEQ ID NOs: 480-495.

As used herein, "pigment-binding peptide" refers to an affinity peptide that binds with high affinity to a pigment, Examples of pigment-binding peptides are provided as SEQ ID NOs: 422-446.

As used herein, "polymer-binding peptide" refers to an affinity peptide that binds with high affinity to at least one specificed polymer. Examples of polymer-binding peptides are provided herein as SEQ ID NOs: 453-479.

As used herein, "metal-binding petpide" refers to an affinity peptide that binds with high affinity to at least one metal. Examples of metal-binding peptides may include, but are not limited to, gold-binding peptides and silver-binding peptides.

As used herein, the term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, that the coding sequence is under the transcriptional control of the promoter). In a further embodiment, the definition of "operably linked" may also be extended to describe the products of chimeric genes, such as fusion peptides. As such, "operably linked" will also refer to the linking of a "leave on" multi-functional solubility

tag to a peptide of interest (POI) to be produced, recovered and used as such or for an aqueous composition or formulation.

As used herein, the term “bioactive” refers to peptides that cause a biological response and may be used in a variety of applications including, but not limited to curative agents for diseases (*e.g.*, insulin, interferon, interleukins, anti-angiogenic peptides) (U.S. Patent 6,815,426), antimicrobial or toxin.

As used herein, the term "solubility" refers to the amount of a substance that can be dissolved in a unit volume of a liquid under specified conditions. In the present application, the term “solubility” is used to describe the ability of a peptide/fusion peptide to be resuspended in a volume of solubilization medium, *e.g.* (an aqueous medium such as a buffer medium) used in biochemical applications and may include Tris, saline, MES, and the like. In one embodiment, a peptide may be defined as soluble in a defined medium when it remains in solution after centrifuging the peptide-containing solution for 5 minutes at 15000 rpm (rcf = 21130 *g*) in a standard tabletop microcentrifuge. The POI, when not coupled to the multi-functional inclusion body tag, is soluble within the host cell and/or cell lysate under normal physiological conditions. As used herein, the term “normal physiological conditions within the host cell” may be defined as an aqueous matrix having a pH of about 6.5 to about 8.0 (typical for neutrophiles) at a temperature suitable for growth of the recombinant host cell. One of ordinary skill in the art can easily determine the physiological conditions within the particule host cell used for recombinant production of the present peptides. Fusion of multi-functional inclusion body tag (IBT) to the POI results in the formation of a fusion peptide that is insoluble under normal physiological conditions within the recombinant host cell, resulting in the formation of at least one inclusion body.

The term “amino acid” refers to the basic chemical structural unit of a protein or polypeptide. The following abbreviations are used herein to identify specific amino acids:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any naturally-occurring amino acid (or as defined by the formulas described herein)	Xaa	X

As used herein, the term “genetic construct” refers to a series of contiguous nucleic acids useful for modulating the genotype or phenotype of an organism. Examples of genetic constructs include but are not limited to a nucleic acid molecule, and open reading frame, a gene, a plasmid and the like.

As used herein, the terms “host cell” and “recombinant host cell” refer to a microbial cell comprising an expressible heterologous nucleic acid molecule encoding a fusion peptide having at least one of the present multi-

functional inclusion body tags.

Expressible Peptides of Interest

The peptide of interest ("POI") targeted for production using the present method is one that is appreciably soluble in the recombinant host cell and/or host cell liquid lysate under normal physiological conditions. In one aspect, the peptides of interest are generally short (< 300 amino acids in length) and difficult to recombinantly produce in sufficient amounts due to their solubility and /or exposure to endogenous proteolytic degradation. Fusion of the POI to at least one multi-functional inclusion body tag creates a fusion peptide that is insoluble in the host cell and/or host cell lysate under normal physiological conditions (*i.e.*, inclusion body formation comprising the fusion peptide is observed within the grown host cell). Production of the fusion peptide is typically increased when expressed and accumulated in the form of an insoluble inclusion body as the peptide is generally more protected from endogenous proteolytic degradation. Furthermore, the insoluble fusion peptide can be easily separated from the host cell lysate using a simple separation technique such as centrifugation or filtration. The fusion peptide is not cleaved to remove multi-functional solubility tag that not only facilitates inclusion body formation but also provides surface interactions in downstream applications for forming adducts between two or more surfaces.

In general, the present multi-functional inclusion body tags can be used in a process to produce any peptide of interest that is (1) typically soluble in the cell and/or cell lysate under typical physiological conditions and/or (2) those that can be produced at significantly higher levels when expressed in the form of LOT. In one embodiment, the POI is appreciably soluble in the host cell and/or corresponding cell lysate under normal physiological and/or process conditions.

The length of the POI may vary as long as (1) the POI is appreciably soluble in the host cell and/or cell lysate, and/or (2) the amount of the POI produced is increased when expressed in the form of an insoluble fusion peptide/inclusion body.

The function of the POI is not limited by the present methods and

may include, but is not limited to bioactive molecules such as curative agents for diseases (*e.g.*, insulin, interferon, interleukins, peptide hormones, anti-angiogenic peptides, peptide toxins, and peptides (with the proviso that the peptide is not an antibody or an F_{ab} portion of an antibody) that bind to and affect defined cellular targets such as receptors, channels, lipids, cytosolic proteins, and membrane proteins; see U.S. Patent 6,696,089), peptides having an affinity for a particular material such as hair, skin, nail or tooth (see WIPO International Patent Application Publication Nos. WO01/079479, WO04/048399, and WO04/000257; U.S. Patent Application Publication Nos. 2005-0226839, 2010-0247457, and 2010-0247589; U.S. Patents 7,736,633; 7,427,656; 7,309,482; 7,749,957; and 7,807,141), cellulose, iron oxides (U.S. Patent Applicatoin Publication No. 2010-0158837), silica (U.S. Patent Application Publication No. 2010-0158822), various polymers (U.S. Patents 7,709,601; 7,700,716; 7,906,617; and 7,632,919; and U. S. Patent Application Publication No. 2007-0264720), calcium carbonate (U.S. Patent 7,754,680), and clay binding peptides (U.S. Patent 7,749,957), for targeted delivery of at least one benefit agent.

Examples of various affinity peptides and/or anti-microbial peptides are provided herein as amino acid sequence: SEQ ID NOs: 105-392 are exemplary body surface-binding peptides, SEQ ID NOs: 393-421 are exemplary antimicrobial peptides, SEQ ID NOs: 422-446 are exemplary pigment-binding peptides, SEQ ID NOs: 447-452 are exemplary cellulose-binding peptides, SEQ ID NOs: 453 – 479 are exemplary polymer-binding peptides, and SEQ ID NOs: 480 – 495 are exemplary clay binding peptides.

Manipulations of Physio-chemical Conditions to Control Solubility of Fusion Peptides

The solubility of the fusion peptides may vary from peptide to peptide. Anyone skilled in the practice of protein chemistry can determine conditions necessary to control solubility by carrying out systematic titrations of independent parameters (pH, salt, ionic strength, fusion peptide concentration, etc.) or by a sampling of conditions involving multiple parameters using a statistical design of experiments (Atkinson, A. C. and Donev, A. N. and Tobias, R. D. (2007) Optimum Experimental

Designs, With SAS. Oxford University Press, New York, NY). Assays to characterize the physical state of the peptide can include observation for turbidity by eye or with a spectrophotometer at a chosen UV or visible wavelength, sedimentation behavior following centrifugation at a chosen centrifugal force followed by the detection of the peptide in the supernatant or the pellet with a dye assay or polyacrylamide gel electrophoresis, or by dynamic light scattering measurement with an instrument like the Malvern Zetasizer (Malvern Instruments Ltd, England). In all experiments, additional parameters should be taken into consideration such as the speed of the change of state and the effect of the initial peptide concentration.

Fusion Peptides

The multi-functional inclusion body tags are used to create chimeric polypeptides ("fusion peptides") that are insoluble when expressed under the physiological conditions within the host cell, forming inclusion bodies. One of skill in the art will recognize that the elements of the fusion protein can be structured in a variety of ways. The fusion protein will include at least one multi-functional inclusion body tag and at least one peptide of interest (POI). The fusion peptide may include one or more peptide spacers separating the core motif sequences within the multi-functional IBT or may include peptdie spacers separating the IBT from the POI. The multi-functional inclusion body tag may be positioned as a leader sequence or a terminator sequence relative to the position of the peptide of interest within the fusion peptide. In another embodiment, a plurality of IBTs and POIs are used when engineering the fusion peptide.

The fusion peptide will typically be insoluble in an aqueous environment at a temperature of 0 °C to 50 °C using a pH range from pH 5 to pH 10, preferably 6 to 10, and most preferably 6 to 8. The temperature, pH, and/or ionic strength of the aqueous environment may be adjusted to obtain the desired solubility characteristics of the fusion peptide/inclusion body.

Method to Make a Peptide of Interest Using Insoluble Fusion Peptides

A genetic construct is prepared encoding a fusion peptide comprising at least one "leave-on" multi-functional inclusoin body tag and at least one peptide of interest, wherein the tag(s) and the POI(s) may be separated by optional peptide spacers. Expression of the genetic construct encoding the fusion protein produces an insoluble form of the peptide of interest that accumulates in the form of inclusion bodies within the host cell. The host cell is grown for a period of time sufficient for the insoluble fusion peptide to accumulate within the cell.

The host cell is subsequently lysed using any number of techniques well known in the art. The insoluble fusion peptide/inclusion bodies are then separated from the soluble components of the cell lysate using a simple and economical technique, such as centrifugation and/or filtration.

Transformation and Expression

Construction of genetic cassettes and vectors that may be transformed into an appropriate expression host is common and well known in the art. Typically, the vector or cassette contains sequences directing transcription and translation of the relevant chimeric construct, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are derived from genes homologous to the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

Transcription initiation control regions or promoters, which are useful to drive expression of the genetic constructs encoding the fusion peptides in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these constructs is suitable for the present invention including, but not limited to *CYC1*, *HIS3*, *GAL1*, *GAL10*, *ADH1*, *PGK*, *PHO5*, *GAPDH*, *ADC1*, *TRP1*, *URA3*, *LEU2*, *ENO*, *TPI* (useful for expression in *Saccharomyces*); *AOX1* (useful for expression in *Pichia*); and *lac*, *ara* (*pBAD*), *tet*, *trp*, *IPL*, *IPR*, *T7*, *tac*, and *trc* (useful for expression

in *Escherichia coli*) as well as the *amy*, *apr*, *npr* promoters and various phage promoters useful for expression in *Bacillus*.

Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

Preferred host cells for expression of the fusion peptides are microbial hosts that can be found broadly within the fungal or bacterial families and which grow over a wide range of temperature, pH values, and solvent tolerances. Because the transcription, translation, and the protein biosynthetic apparatus is the same irrespective of the cellular feedstock, genes are expressed irrespective of the carbon feedstock used to generate the cellular biomass. Large-scale microbial growth and functional gene expression may utilize a wide range of simple or complex carbohydrates, organic acids and alcohols, saturated hydrocarbons such as methane or carbon dioxide in the case of photosynthetic or chemoautotrophic hosts. The functional genes may be regulated, repressed or depressed by specific growth conditions, which may include the form and amount of nitrogen, phosphorous, sulfur, oxygen, carbon or any trace micronutrient including small inorganic ions. In addition, the regulation of functional genes may be achieved by the presence or absence of specific regulatory molecules that are added to the culture and are not typically considered nutrient or energy sources. Growth rate may also be an important regulatory factor in gene expression. Examples of host strains include, but are not limited to fungal or yeast species such as *Aspergillus, Trichoderma, Saccharomyces, Pichia, Yarrowia, Candida, Hansenula, or bacterial species such as Salmonella, Bacillus, Acinetobacter, Zymomonas, Agrobacterium, Erythrobacter, Chlorobium, Chromatium, Flavobacterium, Cytophaga, Rhodobacter, Rhodococcus, Streptomyces, Brevibacterium, Corynebacteria, Mycobacterium, Deinococcus, Escherichia, Erwinia, Pantoea, Pseudomonas, Sphingomonas, Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylomicrobium, Methylocystis, Alcaligenes, Synechocystis, Synechococcus, Anabaena, Thiobacillus, Methanobacterium, Klebsiella, and Myxococcus.*

Preferred bacterial host strains include *Escherichia, Pseudomonas*,

and *Bacillus.* In a highly preferred aspect, the bacterial host strain is *Escherichia coli*.

Fermentation Media

Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include, but are not limited to, monosaccharides such as glucose and fructose, disaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. It is contemplated that the source of carbon utilized may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism. Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are glucose, fructose, and sucrose.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the expression of the fusion peptides.

Culture Conditions

Suitable culture conditions can be selected dependent upon the chosen production host. Typically, cells are grown at a temperature in the range of about 25 °C to about 40 °C in an appropriate medium. Suitable growth media may include common, commercially-prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, *e.g.,* cyclic adenosine 2':3'-monophosphate, may also be incorporated into the fermentation medium. Suitable pH ranges for the fermentation are typically between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred. Fermentations may be performed under aerobic or anaerobic conditions

where aerobic conditions are generally preferred.

Industrial Batch and Continuous Fermentations

A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism or organisms, and fermentation is permitted to occur without adding anything to the system. Typically, a "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA. (hereinafter "Brock"), or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227 (1992).

Although biomass production may be performed in batch mode, it is

contemplated that the production method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to the medium being drawn off must be balanced against the cell growth rate in the fermentation.

It is contemplated that the present methods may be practiced using either batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

The meaning of abbreviations used is as follows: “min” means minute(s), “h” means hour(s), “µL” means microliter(s), “mL” means milliliter(s), “L” means liter(s), “nm” means nanometer(s), “mm” means millimeter(s), “cm” means centimeter(s), “µm” means micrometer(s), “mM” means millimolar, “M” means molar, “mmol” means millimole(s), “µmol” means micromole(s), “pmol” means picomole(s), “g” means gram(s), “µg”

means microgram(s), "mg" means milligram(s), "*g*" means the gravitation constant, "rpm" means revolutions per minute, "DTT" means dithiothreitol, and "cat#" means catalog number.

GENERAL METHODS:

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described by Sambrook, J. and Russell, D., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory Cold Press Spring Harbor, NY (1984); and by Ausubel, F. M. et. al., Short Protocols in Molecular Biology, 5th Ed. Current Protocols and John Wiley and Sons, Inc., N.Y., 2002.

Materials and methods suitable for the maintenance and growth of bacterial cultures are also well known in the art. Techniques suitable for use in the following Examples may be found in Manual of Methods for General Bacteriology, Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, DC., 1994, or in Brock (*supra*). All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from BD Diagnostic Systems (Sparks, MD), Invitrogen (Carlsbad, CA), Life Technologies (Rockville, MD), QIAGEN (Valencia, CA) or Sigma-Aldrich Chemical Company (St. Louis, MO), unless otherwise specified.

EXAMPLE 1

Expression Plasmid Constructs

The purpose of this example is to describe a generic construction of a plasmid encoding for the expression of a LOT peptide.

The region downstream of the promoter in each of the vectors was designed to facilitate and simplify swapping of the DNA constructs encoding multi-functional solubility tags (alone or in combination with the peptide of interest (POI). In general, the nucleic acid molecules were designed to include the appropriate *Nde*I/*Bam*HI (for region encoding the multi-functional

solubility tags) and *Bam*HI/*Asc*I restriction sites (for region encoding POI) to facilitate insertion in the expression vector. More specific description of the construction of the various constructs is given below.

Construction of pLR557, the expression plasmid encoding a fusion peptide comprising the multi-functional solubility tag IBT187 coupled to the peptide of interest HC263 (IBT187.HC263).

Plasmid pLR557 was derived from the commercially available plasmid pBAD-HisA (Invitrogen). pLR557 harbors the gene for the *ara*C regulator. *ara*C is critical for arabinose promoter function. Additionally the plasmid pLR557 comprises a ColE1 type origin of replication, a *bla* gene to confer ampicillin resistance and *aad*A-1 gene to confer spectinomycin (Spec) resistance.

To construct pLR557, a modified multiple cloning site (MCS) was cloned in pBAD-HisA. An *Nde*I restriction site at position 2844 was eliminated to create a single *Nde*I site downstream of the *pBAD* promoter and the resulting plasmid was named pBAD-HisA_MCSmod. The *Nde*I/*Eco*RI fragment of plasmid pKSIC4-HC77623 (U.S. Patent 7,285,264) was inserted into the *Nde*I/*Eco*RI site of pBAD-HisA_MCSmod, generating plasmid pSF004_pBAD-KSIC4-HC77623. The *Hin*dIII fragment of plasmid pCL1920 (Lerner and Inouye, *Nucleic Acids Research*, 18:4631 (1990); GENBANK® Accession No. AB236930) comprising the spectinomycin resistance gene (*aad*A-1) was inserted into pSF004_pBAD-KSI4-HC77623, creating plasmid pLR042. Plasmid pLR557 was created from plasmid pLR042 by removing the coding region for the KSIC4-HC77623 fusion peptide and inserting the coding region (SEQ ID NO: 7) for fusion peptide IBT187-HC263 (SEQ ID NO: 8) comprising the multi-functional solubility tag IBT187 linked in tandem to the peptide of interest (POI) HC263.

Construction of pLR642, the expression plasmid encoding a fusion peptide comprising solubility tag IBT233 coupled to the peptide of interest HC263 (IBT233.HC263)

pLR642 was derived from plasmid pLR042 by removing the coding region for the KSIC4-HC77623 fusion peptide and inserting the coding

region (SEQ ID NO: 12) for fusion peptide IBT233.HC263 (SEQ ID NO: 13). Many additional plasmids encoding variant IBTs were constructed using similar strategy.

Construction of pLR562, the expression plasmid for IBT187.H2-TonB-G3

pLR562 was derived from plasmid pLR042 by removing the coding region for the KSIC4-HC77623 fusion peptide and inserting the coding region (SEQ ID NO: 9) for a multi-functional solubility tag 187 linked to the polypeptide H2-TonB-G3 (*i.e.*, IBT187.H2-TonB-G3; SEQ ID NO: 10).

Construction of pLR565, the expression plasmid for LOT comprising IBT (IBT187.1)

The plasmid pLR565 was constructed to drive the expression of the gene IBT187.1 (SEQ ID NO: 3) in *E. coli* KK2000 for the production of multi-functional peptide tag IBT187.1 (SEQ ID NO: 4). pLR565 was derived from pLR548 by introducing a stop codon after the IBT 187.1 coding sequence. pLR548 is a derivative of the pBAD expression vector (See Figure 1 for a map of pLR548, SEQID NO: 78).

Construction of pLD001-233, the expression plasmid for IBT233

The plasmid pLD001-233 was used to drive the expression of a polynucleotide encoding IBT233 (SEQ ID: 11) in *E. coli* BL21-AI (Invitrogen) for the production of the multi-functional inclusion body tag IBT233 (SEQ ID NO: 37). pLD001-233 is a derivative of the T7 expression vector. (See Figure 2 for a map of pLD001-233, SEQ ID NO: 79).

EXAMPLE 2

Accumulation of the Multi-functional Solubility Tags as Inclusion Bodies

The purpose of this example is to describe the production of a peptide (a multi-functional solubility tag) in a bacterial host by its accumulation as insoluble inclusion bodies.

Strain KK2000 is a derivative of *E. coli* MG1655 (ATCC 46076™) strain wherein the endogenous chromosomal copy of the *ara*BAD operon has been deleted. The expression plasmids pLR565, pLR557, pLR642 and

pLR562 described in Example 1 were individually transformed into the *E. coli* strain KK2000. Plasmid pLD001-233, containing a T7 promoter that drives expression of the gene of interest, was transformed into *E. coli* BL21-AI (Invitrogen). Approximately 30 µL of overnight cultures of individual *E. coli* transformants (both KK2000 and BL21-AI) were inoculated in 3 mL of LB medium (plus 100 µg/mL of ampicillin). The culture was grown to an OD_{600} of about 0.4 and the recombinant proteins were produced by the addition of 0.2% arabinose and grown for 3 hours. To determine soluble versus insoluble cell content, the cells were lysed with CelLytic Express (a mixture of non-denaturing detergents and enzymes available from Sigma, St. Louis, USA) and soluble and insoluble fractions were analyzed on an SDS-PAGE gel. The multi-functional solubility tags IBT187.1 and IBT233 were each produced in the form of insoluble inclusion bodies.

In a similar fashion, plasmids pLR557, pLR642, and pLR562 were used to recombinantly produce insoluble fusion peptides IBT187.HC263 (SEQ ID NO: 8), IBT233.HC263 (SEQ ID NO: 13) and IBT187.H2-TonB-G3 (SEQ ID NO: 10), respectively.

EXAMPLE 3

Production and Purification of Inclusion Bodies Produced from IBT187.1, IBT233, IBT187.HC263, IBT233.HC263, and IBT187.H2-TonB-G3

The purpose of this example is to describe a general process for the production and recovery of the insoluble peptides from the fermentation of the microbial host to the recovery and purification of the inclusion bodies.

The cells containing the two different inclusion body tags and three different fusion peptide constructs were each grown at 37 ºC for 2 hr in 1 liter autoinduction media (Na_2HPO_4, 7.1 g; KH_2PO_4, 6.8 g; $(NH_4)_2SO_4$, 3.3 g; $MgSO_4$, 0.36 g; Tryptone, 9.2 g; Yeast Extract, 4.6 g; NaCl, 4.6 g; glycerol, 7.5 g; D-glucose, 0.75 g; and L-arabinose, 0.5 g).

The cells were harvested by centrifugation and lysed in 200 mL lysis buffer (50 mM Tris pH 7.5,10 mM EDTA, 50 mg lysozyme), followed by sonication on ice for 40 seconds. The suspensions were incubated at 37 °C with shaking for 30 min, followed by being frozen overnight at -20 ºC. Upon

thawing, the inclusion bodies were harvested by centrifugation. The inclusion body pellets were resuspended and washed with 200 mL of wash solution (50 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA), and harvested by centrifugation. The inclusion body pellets were washed with 200 mL of water, harvested by centrifugation, and lyophilized.

All the above constructs formed inclusion bodies and remained as pellets under the conditions of harvest and washes.

EXAMPLE 4

Solubilization of Solubility Tags IBT187.1, IBT233, and Fusion Peptides IBT187.HC263, IBT233.HC263, and IBT187.H2-TonB-G3 at pH 10

The purpose of this example is to describe the solubilization of representative peptides by resuspension of inclusion bodies at alkaline pH.

Constructs comprising multi-functional solubility tags IBT187.1 and IBT233 and fusion peptides IBT187.HC263, IBT233.HC263, and IBT187.H2-TonB-G3 were individually expressed as inclusion bodies in *E. coli*. This indicates that under normal physiological conditions (around neutral pH and in the presence of salt as found in the cells) these peptides would be in insoluble form as observed in Example 3. The recovered inclusion bodies were tested for solubility in de-ionized water adjusted to pH 10 with sodium hydroxide.

In each case the inclusion bodies were solubilized at 20 μM concentration of the inclusion bodies.

EXAMPLE 5

Additional Multifuntional Inclusion Body Tags Designed to Have Controllable Solubility

The purpose of this example is to demonstrate that additional peptide solubility tags and/or fusion peptides comprising such multi-functional solubility tags can be prepared from structurally similar peptide tags. The present solubility tags and/or fusion peptides comprising at

least one of the solubility tags are processable by controlling their solubility via changing of the physio-chemical conditions of the medium.

Variants of inclusion body tag IBT139 (SEQ ID NO: 14; see U.S. Patents 7,678,883 and 7,794,979) were prepared. IBT139 and the variant peptide tags derived from IBT139 were prepared and tested for controllable solubility. Table 1 provides data on the various multi-functional solubility tags.

Table 1.

Peptide Tag Name	Amino Acid Sequence[1]	SEQ ID NO:	Core Motif(s)	SEQ ID NO(s): of Core Motif(s)
IBT139	MQQRFQWQFEQQPRGQQRFQWQFEQQPRGQQRFQWQFEQQPEG QQRFQWQFEQQGSDP	14	QQRFQWQFEQQ	89
IBT187.1	MQQKFKWKFQQQPRGQQKFKWKFQQQPRGQQKFKWKFQQQPEGQ QKFKWKFQKQ	4	QQKFKWKFQQQ QQKFKWKFQKQ	99 102
IBT201	MASQQRFQWQFQQQPRGQQRFQWQFQQQPEGQQRFQWQFQQQGPGSDP	15	QQRFQWQFQQQ	90
IBT202	MASQQRFQWQFQQQPRGQQRFQWQFQQQPRGQQRFQWQFQQQPEGQQRFQWQ FQQQGPGSDP	16	QQRFQWQFQQQ	90
IBT203	MASQQRFQWQFQQQPRGQQRFQWQFQQQPEGQQRFQWQFQQQGPGSGGAGSPG SAGGPGSDP	17	QQRFQWQFQQQ	90
IBT204	MASQQRFQWQFQQQPRGQQRFQWQFQQQPRGQQRFQWQFQQQPEGQQRFQWQ FQQQGPGSGGAGSPGSAGGPGSDP	18	QQRFQWQFQQQ	90
IBT205	MASQQRFQWQFRQQPRGQQQFRWQFQQQPEGQQRFQWQFRQQGPGSGGAGSPG SAGGPGSDP	19	QQRFQWQFRQQ QQQFRWQFQQQ	92 86
IBT206	MASQQQFRWQFQQQPRGQQRFQWQFRQQPEGQQQFQWRFQQQGPGSGGAGSPG SAGGPGSDP	20	QQQFRWQFQQQ QQRFQWQFRQQ	86 92

IBT207	MASQQRFRWRFQQQPRGQQRFEWEFQQQPRGQQRFRWRFQQQPEGQQRFEWEF QQQGPGSDP	21	QQRFRWRFQQQ QQRFEWEFQQQ	98 100
IBT208	MASQQRFRWRFQQQPRGQQRFEWEFQQQPRGQQRFRWRFQQQP EGQQRFEWEFQQQGPGSGGAGSPGSAGGPGSDP	22	QQRFRWRFQQQ QQRFEWEFQQQ	98 100
IBT209	MASQQRFQWQFQWQFEQQPRGQQRFQWQFQWQFEQQPEGQQRFQWQFQWQFE QQGPGSDP	23	QQRFQWQFQWQFEQQ	91
IBT210	MASQQRFQWQFQWQFEQQPRGQQRFQWQFQWQFEQQPEGQQRFQWQFQWQFE QQGPGSGGAGSPGSAGGPGSDP	24	QQRFQWQFQWQFEQQ	91
IBT212	MASQQRFRWQFQWQFEQQPRGQQRFRWQFQWQFEQQPEGQQRFRWQFQWQFE QQGPGSGGAGSPGSAGGPGSDP	25	QQRFRWQFQWQFEQQ	96
IBT214	MASQQRFRWQFRWQFEQQPRGQQRFQWQFRWQFEQQPEGQQRFRWQFRWQFE QQGPGSGGAGSPGSAGGPGSDP	26	QQRFRWQFRWQFEQQ QQRFQWQFRWQFEQQ	97 93
IBT216	MASQQRFQWQFRWQFEQQPRGQQRFRWQFRWQFEQQPEGQQRFQWQFRWQFE QQGPGSGGAGSPGSAGGPGSDP	27	QQRFQWQFRWQFEQQ QQRFRWQFRWQFEQQ	93 97
IBT218	MASQQRFQFQFEQQPRGQQRFQFQFEQQPEGQQRFQFQFEQQGPGSGG AGSPGSAGGPGSDP	28	QQRFQFQFQFEQQ	88
IBT220	MASQQRFRFRFQFEQQPRGQQRFRFQFEQQPEGQQRFRFRFQFEQQGPGSGGA GSPGSAGGPGSDP	29	QQRFRFRFQFEQQ QQRFRFQFQFEQQ	95 94
IBT222	MASQQRFRFQFQFEQQPRGQQRFRFRFQFQFEQQPEGQQRFRFQFQFEQQGPGSGGA GSPGSAGGPGSDP	30	QQRFRFQFQFEQQ QQRFRFRFQFEQQ	94 95
IBT223	MASQQRFQWQFEQQPRGQQRFQWQFEQQPRGQQRFQWQFEQQPEGQQRFQWQF EQQGSDP	31	QQRFQWQFEQQ	89

IBT224	MASQQRFQWQFQQQPRGQQRFQWQFQQQPRGQQRFQWQFQQQPEGQQRFQWQ FQQQGSGSDP	32	QQRFQWQFQQQ	90
IBT225	MASQQRFQWQFQQQPRGQQRFQWQFQQQPRGQQRFQWQFQQQPEGQQRFQWQ FQQQGPGSGGAGSPGSAGGPGSGSDP	33	QQRFQWQFQQQ	90
IBT229	MASQQHFQWQFEQQPRGQQHFQWQFEQQPRGQQHFQWQFEQQPEGQQHFQWQ FEQQGSDP	34	QQHFQWQFEQQ	83
IBT230	MASQQHFQWQFEQQPRGQQHFQWQFEQQPRGQQHFQWQFEQQPEGQQHFQWQ FEQQGPGSGGAGSPGSAGGPGSDP	35	QQHFQWQFEQQ	83
IBT232	MASQQHFHWQFEQQPRGQQHFHWQFEQQPRGQQHFHWQFEQQPEGQQHFHWQF EQQGSDP	36	QQHFHWQFEQQ	82
IBT233	MASHQHFHWQFEQQPRGHQHFHWQFEQQPRGHQHFHWQFEQQPEGHQHFHWQF EQQGS	37	HQHFHWQFEQQ	80
IBT239	MASQQRFQWQFEQQPRGQQRFQWQFEQQPRGQQRFQWQFEQQPEGQQRFQWQ FEQQGPGSGGAGSPGSAGGPGSDP	38	QQRFQWQFEQQ	89
IBT241	MASQQHFHWQFEQQPRGQQHFHWQFEQQPRGQQHFHWQFEQQPEGQQHFHWQF EQQGPGSGGAGSPGSAGGPGSDP	39	QQHFHWQFEQQ	82
IBT242	MASHQHFHWQFEQQPRGHQHFHWQFEQQPRGHQHFHWQFEQQPEGHQHFHWQF EQQGPGSGGAGSPGSAGGPGSDP	40	HQHFHWQFEQQ	80
IBT247	MASQQHFHWQFEQQPRGQQHFHWQFEQQPRGQQHFHWQFEQQPRGQQHFHWQF EQQPRRGPGSGGAGSPGSAGGPGS	41	QQHFHWQFEQQ	82
IBT248	MASHQHFHWQFEQQPRGHQHFHWQFEQQPRGHQHFHWQFEQQPRGHQHFHWQF EQQPRRGPGSGGAGSPGSAGGPGS	42	HQHFHWQFEQQ	80

IBT249	MASQQRFQWQFEQQGGAGQQGGLGSQGAGQQGAGQQRFQWQFEQQGGAGQQGGYG GLGSQGAGRGGQGAGQQRFQWQFEQQGGAGQQGGYGGLGSQGAGRGGLGGQGAG QQRFQWQFEQQGPGSGGAGSPGSAGGPGSDP	43	QQRFQWQFEQQ	89
IBT254	MASQQHFHWQFEQQGGAGQQGGLGSQGAGQQGAGQQHFHWQFEQQGGAGQQGGYGG LGSQGAGRGGQGAGQQHFHWQFEQQGGAGQQGGYGGLGSQGAGRGGLGGQGAGQ QHFHWQFEQQGPGSGGAGSPGSAGGPGSDP	44	QQHFHWQFEQQ	82
IBT255	MASHQHFHWQFEQQGGAGQQGGLGSQGAGQQGAGHQHFHWQFEQQGGAGQQGGYGG LGSQGAGRGGQGAGHQHFHWQFEQQGGAGQQGGYGGLGSQGAGRGGLGGQGAGH QHFHWQFEQQGPGSGGAGSPGSAGGPGSDP	45	HQHFHWQFEQQ	80
IBT258	MASQQQFQWQFEQQPEGQQQFQWQFEQQPEGQQQFQWQFEQQGPGSGGAGSPG SAGGPGS	46	QQQFQWQFEQQ	85
IBT259	MASQQQFRWRFEQQPEGQQQFEWEFEQQPRGQQQFRWRFEQQPEGQQQFEWEF EQQGPGSGGAGSPGSAGGPGS	47	QQQFRWRFEQQ QQQFEWEFEQQ	87 101
IBT260	MASQQHFHWQFEQQPEGQQHFHWQFEQQPRGQQHFHWQFEQQPEGQQHFHWQF EQQGPGSGGAGSPGSAGGPGS	48	QQHFHWQFEQQ	82
IBT261	MASHQHFHWQFEQQPEGHQHFHWQFEQQPRGHQHFHWQFEQQPEGHQHFHWQF EQQGPGSGGAGSPGSAGGPGS	49	HQHFHWQFEQQ	80
IBT262	MASQQRFRWRFQQQGGAGQGGLGSQGAGQQGAGQQRFEWEFQQQGGAGQGGYGG LGSQGAGRGGQGAGQQRFRWRFQQQGGAGQGGYGGLGSQGAGRGGLGGQGAGQ QRFEWEFQQQGPGSGGAGSPGSAGGPG SDP	50	QQRFRWRFQQQ QQRFEWEFQQQ	98 100
IBT263	MASQQRFRWRFQQQGGAGQGGLGSQGAGQQGAGQQRFEWEFQQQGGAGQGGYGG		QQRFRWRFQQQ	

	LGSQGAGRGGQGAGQQRFRWRFQQQGGAGQGGYGGLGSQGAGRGGLGGQGAGQ QRFEWEFQQQGPGSGGAGSPGSAGGPGSGPGSGGAGSPGSAGGPGSDP	51	QQRFEWEFQQQ	98 100
IBT282	MASQQHFHWHFQQQPRGQQHFHWHFQQQPEGQQHFHWHFQQQGPGSGGAGSPG SAGGPGS	52	QQHFHWHFQQQ	82
IBT283	MASQQHFHWHFQQQPRGQQKFKWKFQQQPEGQQHFHWHFQQQGPGSGGAGSPG SAGGPGS	53	QQHFHWHFQQQ QQKFKWKFQQQ	82 99
IBT284	MASQQKFHWHFQQQPRGQQKFHWHFQQQPEGQQKFHWHFQQQGPGSGGAGSPG SAGGPGS	54	QQKFHWHFQQQ	84
IBT287	MASQQKFKWKFQQQPRGQQKFKWKFQQQPEGQQKFKWKFQQQGPGSGGAGSPGS AGGPGS	55	QQKFKWKFQQQ	99
IBT289	MASQQKFKWKFQQQPRGQQHFHWHFQQQPEGQQKFKWKFQQQGPGSGGAGSPGS AGGPGS	56	QQKFKWKFQQQ QQHFHWHFQQQ	99 81
IBT290	MASQQKFHWKFQQQPRGQQKFHWKFQQQPEGQQKFHWKFQQQGPGSGGAGSPGS AGGPGS	57	QQKFHWKFQQQ	103
IBT294	MASGPCGQQHFHWHFQQQPRGQQKFKWKFQQQPEGQQHFHWHFQQQGPGSGGA GSPGSAGGPGS	58	QQHFHWHFQQQ QQKFKWKFQQQ	82 99
IBT295	MASGPCGQQKFHWHFQQQPRGQQKFHWHFQQQPEGQQKFHWHFQQQGPGSGGA GSPGSAGGPGS	59	QQKFHWHFQQQ	84
IBT297	MASGPCGQQKFKWKFQQQPRGQQKFKWKFQQQPRGQQKFKWKFQQQPEGQQKFK WKFQKQGPGSGGAGSPGSAGGPGS	60	QQKFKWKFQQQ QQKFKWKFQKQ	99 102
IBT298	MASGPCGQQRFQWQFEQQPRGQQRFQWQFEQQPRGQQRFQWQFEQQPEGQQRF	61	QQRFQWQFEQQ	

	QWQFEQQGPGSGGAGSPGSAGGPGS			89
IBT299	MASGPCGQQRFQWQFEQQGGAGQGGLGSQGAGQGAGQQRFQWQFEQQGGAGQG GYGGLGSQGAGRGGQGAGQQRFQWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQ GAGQQRFQWQFEQQGPGSGGAGSPGSAGGPGS	62	QQRFQWQFEQQ	89
IBT310	MASHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGSQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGH QHFHWQFEQQGPRSGGAGSPGSAGGPGSGPGSGGAGSPGSAGGPGSDP	63	HQHFHWQFEQQ	80
IBT311	MASHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGSQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGH QHFHWQFEQQGPRSGAHSPGHSAGHPGSDP	64	HQHFHWQFEQQ	80
IBT312	MASHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGSQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGH QHFHWQFEQQGPRSGGAHSPGSAHGPGSHPGSGHAGSPHSAGHPGSDP	65	80 HQHFHWQFEQQ	
IBT313	MASHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGSQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGH QHFHWQFEQQGPGSGASPGSDP	66	HQHFHWQFEQQ	80
IBT314	MASHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGSQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGH QHFHWQFEQQGPHSGHSHGSDP	67	HQHFHWQFEQQ	80
IBT315	MASHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGQGAGHQHFHWQFEQQGPGS GGAGSPGSAGGPGS	68	HQHFHWQFEQQ	80

IBT316	MASHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGQGAGHQHFHWQFEQQGGAG QGGYGGLGSQGAGRGGLGGQGAGHQHFHWQFEQQGPGSGGAGSPGSAGGPGS	69	HQHFHWQFEQQ	80
IBT317	MASHQKFHWQFEQQGGAGQGGLGSQGAGQGAGHQKFHWQFEQQGGAGQGGYGG LGSQGAGRGGQGAGHQKFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGH QKFHWQFEQQGPGSGGAGSPGSAGGPGS	70	HQKFHWQFEQQ	104
IBT320	MASHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGGAGQGGLGS QGAGQGAGHQHFHWQFEQQGGAGQGGLGSQGAGQGAGHQHFHWQFEQQGPGSG GAGSPGSAGGPGS	71	HQHFHWQFEQQ	80
IBT321	MASHQHFHWQFEQQGGAGQGGYGGLGSQGAGRGGLGGQGAGHQHFHWQFEQQG GAGQGGYGGLGSQGAGRGGLGGQGAGHQHFHWQFEQQGGAGQGGYGGLGSQGA GRGGLGGQGAGHQHFHWQFEQQGPGSGGAGSPGSAGGPGS	72	HQHFHWQFEQQ	80
IBT326	MQQRFQWQFEQQNGKTQQRFQWQFEQQGS	73	QQRFQWQFEQQ	89
IBT327	MQQRFQWQFEQQYNGKQQRFQWQFEQQGS	74	QQRFQWQFEQQ	89
IBT332	MASHQHFHWQFEQQGGAGQGGLGCQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGCQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGCQGAGRGGLGGQGAGH QHFHWQFEQQGPRSGAHSPGHSAGHPGS	75	HQHFHWQFEQQ	80
IBT334	MASHQHFHWQFEQQGPGSGGAGSPGSAGGPGSHDHKNQKETHQRHAAGPGSGGA GSPGSAGGPGSHQHFHWQFEQQGPGSGGAGSPGSAGGPGSTAEIQSSKNPNPHPQ RSWTNGPGSGGAGSPGSAGGPGSHQHFHWQFEQQGPGSGGAGSPGSAGGPGSTP PELAHTPHHLAQTRLTDRGPGSGGAGSPGSAGGPGSHQHFHWQFEQQGPGSGGAG	76	HQHFHWQFEQQ	

	SPGSAGGPGS			80
IBT340	MASHQHFHWQFEQQGGAGQGGLGCQGAGQGAGHQHFHWQFEQQGGAGQGGYGG LGCQGAGRGGQGAGHQHFHWQFEQQGGAGQGGYGGLGCQGAGRGGLGGQGAGH QHFHWQFEQQGPGSGGAGSPGSAGGPGS	77	HQHFHWQFEQQ	80

[1]= core motif(s) underlined.

The multi-functional solubility tags in Table 1 can be defined by the following general structures:

SEQ ID NO: 1 – *Spacer* – [[SEQ ID NO: 1]-[*Spacer*]$_m$]$_n$

or

SEQ ID NO: 2 - *Spacer*-[[SEQ ID NO: 2]-[*Spacer*]$_m$]$_n$

wherein SEQ ID NO:1 and SEQ ID NO: 2 represent core motifs within each peptide tag;

SEQ ID NO: 1 is
Xaa1-Gln-[Xaa2]$_p$-[Phe-Xaa3-Xaa4-Xaa5]$_s$-Phe-Xaa6-[Xaa7]$_q$-[Gln]$_r$; and

SEQ ID NO: 2 is
Xaa1-Gln-Xaa8-[Xaa4--Xaa8]$_s$-Phe-[Glu-Gln-Gln]$_r$;

wherein

Xaa1 = Gln or His;
Xaa2 = Gln, Arg, His, or Lys;
Xaa3 = Gln, His, Lys, Arg, or Glu;
Xaa4 = Trp or Phe;
Xaa5 = Gln, His, Lys, Arg or Glu;
Xaa6 = Glu, Gln, or Arg;
Xaa7 = Gln or Lys;
Xaa8 = Asp, Glu, Gln, His, Lys, or Arg;
p, q, and r are independently 0 or 1;
s is an integer ranging from 1 to 5;
n is an integer ranging from 1 to 10;
m = n-1; and
Spacer = a peptide linker ranging from 1 to 100 amino acids in length.

The IBT139-derived variant inclusion body tags in Table 1 were prepared using standard molecular biology techniques. These multi-functional solubility tags were prepared using several approaches including, but not limited to, varying the amino acid composition of the core motifs, changing the number of core motif sequences, selecting different combinations of core motifs, altering the length and composition of the peptide spacers separating the core motifs.

The solubility and insolubility characteristics of the IBTs fused to the soluble peptide of interest (POI) HC263 were evaluated. All the peptides were expressed in *E. coli* as inclusion bodies indicating their insolubility at neutral pH. Their controllable solubility by using pH (in this case pH 12) was assessed by making a 1-2 mL suspension of fusion peptide in water (~ 1-2 mg / mL), adjusting the pH to 12 with NaOH, separating the insoluble fraction (pellet) from the soluble fraction (supernatant) by centrifugation (10 min at 15,000 x *g*) and assaying the supernatant by polyacrylamide gel electrophoresis to confirm the solubilization of the peptide. The soluble fraction supernatants were transferred to a new tube and the control of re-insolubilization was assessed by shifting the pH to pH 7.5 by 20 mM Tris 50 mM NaCl, separating the insoluble fraction (pellet) from the soluble fraction (supernatant) by centrifugation (10 min at 15,000 x *g*) and assaying the insoluble fraction (pellet) by polyacrylamide gel electrophoresis to confirm that the fusion peptide was once again insoluble.

All of the multi-functional IBTs tested as fusions to peptide HC263 formed inclusion bodies inside *E. coli*, could be solubilized at pH 12 and rendered insoluble at pH 7.5 in presence of 50 mM NaCl, thus satisfying the functional feature of controllable solubility of the multi-functional solublity tags.

EXAMPLE 6

Solubility Tags Providing Additional Functionality

The purpose of this example is to demonstrate that the solubility tags (defined by SEQ ID NO: 1 and SEQ ID NO: 2; Example 5) that are not removed from the peptide of interest ("leave on tags"; LOTs) provide

additional functionality to the fusion protein beyond inclusion body formation and controllable solubility during downstream processing. More specifically, the presence of the multi-functional solubility tag can be used to functionalize a target surface by controlling or enhancing deposition of the fusion peptide on a surface via the presence of the multi-functional solubility tag.

The peptide of interest (POI) may be selected to have a binding affinity for a first target material. When coupled to a multi-functional solubility tag providing a binding affinity for a second target material (wherein the first and second materials are different) the resulting fusion peptide enables one to couple the first and second target materials. In one embodiment, the present example demonstrates the ability of fusion proteins comprising the present multi-functional solubility tags to act as bridging agents between the two target materials.

Seven peptides were subjected to a magnetic bead assay described in this example to illustrate the concept that a “leave-on” solubility tag (multi-functional solubility tags and/or fusion peptides comprising such peptide tags) has targeted surface activity. The peptides and their associated functionalities are listed in Table 2.

Table 2. Peptides and their associated functionalities

Peptide Name (SEQ ID NO:)	Classification	Target material affinity (predicted)	Function(s)
IBT187.1 (SEQ ID NO: 4)	Multi-functional Inclusion Body Tag (IBT)	Silica (*e.g.* silica particles)	Presence in fusion peptides responsible for inclusion body formation when coupled to a peptide of interest.
HC263 (SEQ ID NO: 6)	Peptide of Interest (POI)	Cobalt-nitrilotriacetic acid resin (*e.g.* Co-NTA coated magnetic beads)	HC263 contains a hex-his tag (His6) that is predicted to bind to Co-NTA resins.
IBT187.HC263 (SEQ ID NO: 8)	Fusion peptide (IBT-POI)	Silica particles and Co-NTA coated magnetic beads	IBT187 predicted to bind to silica while HC263 predicted to bind to Co-NTA resin coated magnetic beads
IBT187.H2-TonB-G3 (SEQ ID NO: 10)	Fusion peptide (IBT-POI)	Silica (*e.g.*, silica particles only)	Same sequence as fusion peptide IBT187.HC263 except lacking the His6 tag. Predicted to bind to silica only.
IBT233 (SEQ ID NO: 37)	Multi-functional Inclusion Body Tag (IBT)	Silica (*e.g.*, silica particles only)	Presence in fusion peptides responsible for inclusion body formation when coupled to a peptide of interest.
IBT233.HC263 (SEQ ID NO: 13)	Fusion peptide (IBT-POI)	Silica particles and Co-NTA coated magnetic beads	IBT233 predicted to bind to silica while the His6 portion of HC263 predicted to bind to Co-NTA resin coated magnetic beads.
HC353 (SEQ ID NO: 298)	Peptide of Interest (POI)	Hair and iron oxide particles (with silica coating)	HC353 contains a hair-binding domain (HP2-TonB-Grey3) linked to an iron oxide pigment particle binding domain

The seven peptides were individually dissolved in water adjusted to pH 10 with sodium hydroxide at peptide concentrations of 20 μM each as described in Example 4. One mL of the peptide solution was transferred to 1.8-mL microfuge tubes. Silica particles (*i.e.*, Schott glass, approximately 600 nm average diameter particles) were added to a final concentration of 0.5% (w/v). The mixtures were incubated for 60 minutes with agitation. KCl was added to 50 mM concentration to promote insolubility of the "leave on" multi-functional solubility tag and the mixtures were incubated with agitation for an additional 60 minutes. The particles were spun down for 1 minute at 13000 rpm in a centrifuge. The supernatant was discarded and the resulting particle pellet resuspended in 1 mL of pH 10 water. The particles were spun down for 1 min at 13000 rpm in a centrifuge to remove any remaining unbound peptide. The supernatant was discarded and the resulting particle pellet was resuspended in 1 mL of 10 mM MES, pH 5 buffer. Five microliters of magnetic DYNABEADS® TALON™ (magnetic beads coated with Co-NTA resin; Invitrogen, Carlsbad, CA, Cat # 101.01D) were washed according to manufacturer's instructions. The TALON™ technology is comprised of a tetradentate metal chelator which binds to the imidazole rings of a poly histidine peptide chain resin. Approximately 100 μL of silica particle suspension, 5 μL of TALON™ beads and 500 μL of TALON™ binding buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 0.01% TWEEN®-20) were mixed in a 1.8-mL microfuge tube. After 10 minutes of gentle mixing, the tubes were placed vertically on a magnet (Dynal magnetic particle concentrator, Invitrogen, Cat# 120.20D). The magnetic TALON™ beads travel to the center of the magnet as a globule and unbound silica drops to the bottom of the tube. The tubes were rotated around their axis (while still on magnet) to apply shear force on the particles. In the case of peptide-mediated binding of the two particle types to each other, all the particles would travel to the center of the tubes, and could not be broken up via rotating the tubes. There is also a noticeable color change, silica alone is white, TALON™ beads are brown, and the mixed particles are more orange

in color. Table 3 below shows which peptides in the magnetic bead assay described in Example 6 were able to facilitate binding between the two types of target materials (*i.e.*, silica particles and Co-NTA coated magnetic particles).

Table 3. Description and functions of multi-functional inclusion body tags, fusion peptides, and peptides of interest

Peptide ID (SEQ ID NO:)	Composition	Expected functions	Observed Surface-binding Activity[1]
No peptide	---	control	---
IBT187.1 (SEQ ID NO: 4)	Multi-functional inclusion body tag (IBT)	Silica only	---
HC263 (SEQ ID NO: 6)	Peptide of Interest (POI)	Co-NTA coated magnetic beads	---
IBT187.HC263 (SEQ ID NO: 8)	Fusion peptide (IBT-POI)	Silica (particles) and Co-NTA coated magnetic beads	++++
IBT187.H2-TonB-G3 (SEQ ID NO: 10)	Fusion peptide (IBT-POI)	Silica only	+
IBT233 (SEQ ID NO: 37)	Multi-functional inclusion body tag (IBT)	Silica only	---
IBT233.HC263 (SEQ ID NO: 13)	Fusion peptide (IBT-POI)	Silica (particles) and Co-NTA coated magnetic beads	++++
HC353 (SEQ ID NO: 298)	Peptide of Interest (POI)	Hair and Iron Oxide particles	---

[1] = Note: ++++ refers to strong binding between particle types that cannot be disrupted by rotating tubes. + refers to very weak binding that can largely

be disrupted by rotating tubes. - refers to no observable binding even before rotating tubes.

The results of magnetic bead assay described in Example 6 and Table 3 are also shown in Figure 3 and indicate that both binding domains (IBT & POI) must be present for the peptide to link the two particle types together.

IBT187.1 and IBT233 are excellent examples of multi-functional "leave on" peptidic solubility tags that (when coupled to a peptide of interest having surface binding activity) facilitate insoluble inclusion body formation, exhibit reversible solubility, and have demonstrated surface binding activity.

EXAMPLE 7

Fusion Peptide IBT233.HC263 Can Mediate Binding Between Two Target Materials

The purpose of this example is to demonstrate an example of bridging the surfaces of two target materials using a fusion peptide comprising a multi-functional inclusion body tag having affinity for a first target material (a pigment) and a peptide of interest having affinity for a second target material (a body surface, such as hair).

Fusion peptide IBT233.HC263 (SEQ ID NO: 13) contains two engineered structural domains: an inclusion body tag (IBT233; SEQ ID NO: 37)) and a POI (HC263; SEQ ID NO: 6).

The constructs comprising IBT233 and the physio-chemical environment are described in Examples 1-5 for its use as a multi-functional (tri-functional in this case) inclusion body tag. The three functions of IBT233 (when incorporated in a fusion peptide) are:

1. It promotes fusion peptide insolubility in the *E. coli* cells to facilitate inclusion body formation (Example 2);

2. It provides reversible and controllable solubility for the fusion peptide (Examples 2, 3, 4 and 5); and

3. It provides surface binding activity for silica (Example 6).

In addition to the multi-functional inclusion body tag domain, IBT233.HC263 comprises peptide of interest HC263, which contains two

sub-domains; one of which binds to hair and the other hex-His (His6) tag that binds to Co-NTA coated magnetic beads.

This example shows that fusion peptide IBT233.HC263 (SEQ ID NO: 13) can be used to bind pigments to hair. IBT233.HC263 was dissolved in pH 10 water (as described above) at a concentration of 0.5 mg/mL and allowed to gently shake for 1 hour at room temperature (~22 ºC). Silica-coated red iron oxide pigments (with an average particle size distribution 200 nm) were added to the IBT233.HC263 containing solution to 0.1% final conc. (w/v) and incubated for 90 min under gentle shaking. KCl was added to 50 mM and the mixture was incubated overnight. The samples were spun down at 10,000 rpm for 2 min in a microfuge, and the supernatant discarded. The mixture was resuspended in pH 10 de-ionized water, vortexed, and spun down again. The supernatant was discarded and the samples were resuspended in 1 mL of 10 mM MES buffer, pH 5. Hair tresses (natural white hair bundle of about 2.5 cm length and 0.5 cm width; International Hair Importers and Products, Bellerose, NY) were added to the microfuge tube containing the fusion peptide-pigment adducts. The hair was incubated for 15 minutes with intermittent vortexing. The tresses were removed and washed under running tap water and gentle embrocation. The hair was dried and the color was measured in X-rite SP64 spectrophotometer, measuring L*, a*, b* values according to Commission Internationale d'Eclairage (CIELAB76).

The experiment was carried out in triplicate, along with no-peptide controls, where everything was carried out identically except no peptide was added to the pigment.

Table 4: Color update in the presence of fusion peptide IBT233.HC263

Sample	Color Uptake (ΔE)	Standard Deviation
No peptide (control)	4.9	1.4
IBT233.HC263 (SEQ ID NO: 13)	26.7	2.1

This example shows that the fusion peptide including the inclusion body tag and the hair-binding domain can mediate the binding of a pigment

particle to hair and thus demonstrates the multifunctional role of the inclusion body tag.

EXAMPLE 8

Fusion Peptide IBT255.AuBD Mediates Binding Between Two Target Materials

The purpose of this example is to demonstrate bridging the surfaces of two target materials using a fusion peptide comprising a multi-functional inclusion body tag having affinity for a first target material (glass) and a peptide of interest (POI) having affinity for a second target material (gold particle).

Fusion peptide IBT255.AuBD (SEQ ID NO: 497) was prepared using standard cloning and expression techniques similar to those described in Examples 1-3. IBT255.AuBD contains two engineered structural domains: an inclusion body tag (IBT255; SEQ ID NO: 45) and a POI (AuBD, binding to gold; SEQ ID NO: 496).

The constructs comprising IBT255 and the physio-chemical environment are described in Examples 1-5 for its use as a multi-functional (tri-functional in this case) inclusion body tag. The three functions of IBT255 (when incorporated in a fusion peptide) are:

1. It promotes fusion peptide insolubility in the *E. coli* cells to facilitate inclusion body formation (Example 2);

2. It provides reversible solubility for the fusion peptide (Examples 2, 3, 4 and 5); and

3. It provides surface binding activity for gold (see Brown, S., (1997) *Nat. Biotechnol.* 15:269-172).

This example shows that fusion peptide IBT255.AuBD (SEQ ID NO: 497) can be used to bind gold particles to glass. IBT255.AuBD was dissolved in pH 10 water (as described above) at a concentration of 0.5 mg/mL and allowed to gently shake for 1 hour at room temperature (~22 ºC). 200 microliters of the solution were spotted onto a microscope glass slide and allowed to incubate for 1 hour at room temperature. The solution was decanted and the glass slide rinsed extensively with distilled water. As a control, the peptide comprised of IBT255 (SEQ ID NO: 45) alone was

treated in the same manner as described for the fusion peptide. 200 microliters of a dilute solution (1OD) of 40 nm gold particles in water (Naked Gold, BioAssay Works) were spotted onto the glass slide and incubated for 1 hour at room temperature. The solution was decanted and the glass slide rinsed extensively with distilled water.

Results: On the glass slide incubated with IBT255.AuBD a purple circle (comprised of gold particles of 40 nm size) coinciding with the area of peptide application was visible, indicating the binding of gold particles to the glass. In the case of IBT255 alone, such a purple color was not evident. The experiment shows that IBT255.AuBD is able to anchor gold particles to the glass surface, where IBT255 binds to glass, and the AuBD binds to the gold particles.

EXAMPLE 9

Accumulation of the Fusion Peptide IBT255.HC263 as Inclusion Bodies in *Bacillus megaterium*

The purpose of this example is to describe the production of a fusion peptide (including multi-functional solubility tag IBT255; SEQ ID NO: 45) in the Gram positive bacterial host *Bacillus megaterium* as insoluble inclusion bodies.

The gene for IBT255.HC263 was ordered from DNA2.0 (Menlo Park, CA) codon-optimized for expression in *B. megaterium*. The amino acid sequence of fusion peptide IBT255.HC263 is provided as SEQ ID NO: 499. The gene was cloned into plasmid pP_{T7} (using the kit T7 RNA polymerase expression system for *Bacillus megaterium*' from Mo Bi Tec GmbH, Goettingen, Germany) with restriction sites *Bsr*GI and *Nae*I at the 5' and 3' end of the gene, respectively. The plasmid was transformed into *B. megaterium* protoplasts based on the suppliers instructions (Mo Bi Tec GmbH). Colonies were inoculated into 3 mL cultures LB/Tet (10 µg/mL)/Chloramphenicol (4.5 mg/mL), induced by addition of 0.5% D-xylose (w/v) when the OD_{600} reached about 0.5 and allowed to grow for another 3 hours. To determine soluble versus insoluble cell content, the cells were lysed with 1 µL of READY-LYSE™ (a recombinant lysozyme preparation available from Epicentre, Madison, WI) at 37 °C for 10 min.

Soluble and insoluble fractions were analyzed on an SDS-PAGE gel. The fusion product was all found in the insoluble fraction, showing that the tag IBT255 drives its fusion partner into insoluble inclusion bodies in multiple organisms (*E. coli* and *B. megaterium*).

5

CLAIMS

1. A method comprising:

a) providing a microbial host cell comprising a heterologous nucleic acid molecule encoding an insoluble fusion peptide comprising at least one first portion and at least one second portion, wherein said first portion comprises a multi-functional solubility tag and said second portion comprises a peptide of interest; wherein the multi-functional solubility tag has the general formula of:

i) SEQ ID NO: 1 – *Spacer* – [[SEQ ID NO: 1]-[*Spacer*]$_m$]$_n$ or

ii) SEQ ID NO: 2 - *Spacer*-[[SEQ ID NO: 2]-[*Spacer*]$_m$]$_n$

wherein

SEQ ID NO: 1 is
Xaa1-Gln-[Xaa2]$_p$-[Phe-Xaa3-Xaa4-Xaa5]$_s$-Phe-Xaa6-[Xaa7]$_q$-[Gln]$_r$; and

SEQ ID NO: 2 is
Xaa1-Gln-Xaa8-[Xaa4--Xaa8]$_s$-Phe-[Glu-Gln-Gln]$_r$;

wherein
Xaa1 = Gln or His
Xaa2 = Gln, Arg, His, or Lys;
Xaa3 = Gln, His, Lys, Arg, or Glu;
Xaa4 = Trp or Phe
Xaa5 = Gln, His, Lys, Arg or Glu;
Xaa6 = Glu, Gln, or Arg;
Xaa7 = Gln or Lys;
Xaa8 = Asp, Glu, Gln, His, Lys, or Arg;
p, q, and r are independently 0 or 1;
s is an integer ranging from 1 to 5;
n is an integer ranging from 1 to 10;

m = n-1; and

Spacer = a peptide linker ranging from 1 to 100 amino acids in length.

b) growing the microbial host cell under conditions whereby the insoluble fusion peptide is produced within the microbial host cell in the form of at least one inclusion body;

c) recovering the insoluble fusion peptide from the microbial host cell;

d) subjecting the insoluble fusion peptide to an aqueous medium having a first set of conditions whereby the insoluble fusion peptide becomes a soluble fusion peptide; and

e) contacting the soluble fusion peptide with a first target material having a surface whereby the fusion peptide non-covalently associates with the surface of the first target material; wherein the association between the fusion peptide and the first target material is dependent upon, or enhanced by, the presence of the multi-functional solubility tag.

2. The method of claim 1 wherein at least one condition from said first set of conditions is altered to promote or enhance deposition of the soluble fusion peptide to the suface of said first target material in contacting step (e).

3. The method of claim 2 wherein said at least one condition is altered prior to, or concomitantly with, contacting step (e).

4. The method of claim 2 wherein the at least one condition is altered during or after contacting step (e) to promote or enhance deposition of the soluble fusion peptide on the surface of the first target material.

5. The method of claim 2 wherein the at least one condition that is altered is a change in pH, a change in salt composition, a change in salt concentration, a change in temperature, an addition of an insolubilization agent, an addition of a denaturing agent, an addition of an oxiding agent,

an increase in fusion peptide concentration, and any combination thereof.

6. The method of claim 5 wherein pH is increased.

7. The method of claim 5 wherein pH is decreased.

8. The method of claim 5 wherein pH is altered by at least one pH unit.

9. The method of claim 1 wherein the aqueous medium in step (d) comprises a suitable pH whereby the insoluble fusion peptide becomes soluble.

10. The method of claim 9 wherein the aqueous medium comprises a higher pH relative to the pH wherein the fusion peptide is insoluble.

11. The method of claim 9 wherein the aqueous medium comprises a lower pH relative to a pH wherein the fusion peptide is insoluble.

12. The method of claim 1 or claim 2 wherein the second portion of the fusion peptide is characterized by a binding functionality, an antimicrobial functionality or a combination thereof.

13. The method of claim 12 wherein the binding functionality is characterized by a strong affinity for a second target material; wherein the first target material and the second target material are different.

14. The method of claim 1 or claim 2 wherein the first target material is a particle having an average particle size ranging from 100 nm to 10 μm as measured by a light scattering method.

15. The method of claim 1 or claim 2 wherein the first target material is selected from the group consiting of a pigment, a body tissue, a cell surface receptor, hair, skin, nail, teeth, oral cavity tissues, plant tissue, a polymer, polystyrene, polyethylene, polypropylene,

polytetrafluoroethylene, polyester, polyvinyl chloride, poly (methyl methacrylate), polyethersulfone, polyimide, polyamide, aramids, poly *para*-phenylene terephthalamide, poly *meta*-phenylene terephthalamide, poly urethanes, polysaccharides, cellulose, starch, chitin, minerals, silica, silicates, micas, titanium dioxide, alumina, iron oxides, clays, metals, gold, silver, carbon, carbon black, graphite, and carbon nanotubes.

16. The method of claim 15 wherein the second target material is a keratin-containing material selected from the group consisting of skin, nail, and hair.

17. The method of claim 1 or 2 wherein the first portion of the fusion peptide is capable of forming a hydrogel and the second portion has affinity for a human tissue or cell surface receptor.

18. The method of claim 17 wherein the second portion having affinity for a human tissue or cell surface receptor comprises at least one RGD peptide.

19. The method of claim 1 wherein the multi-functional solubility tag comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, and 77.

20. The method of claim 1 where the multi-functional solubility tag comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 37, 40, 42, 43, 44, 45, 46, 47, 49, 50, 51, 62, 63, 64, 65, 66 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, and 77.

21. A multi-functional solubility tag comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 19, 20, 21, 22, 23, 24,

25, 26, 27, 28, 29, 30, 37, 40, 42, 43, 44, 45, 46, 47, 49, 50, 51, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, and 77.

22. A fusion peptide having at least one multi-functional solubility tag of claim 21.

23. A composition comprising a particle comprising the fusion peptide of claim 22; wherein the particle comprises an average particle size of 100 nm to 10 μm as measured by a light scattering method.

24. The composition of claim 23 wherein the particle is partially or completed coated with said fusion peptide.

25. A personal care product comprising the fusion peptide of claim 20 or the composition of claim 23.

26. The personal care product of claim 25 wherein the personal care product is selected from the group consisting of shampoos, hair gels, hair sprays, mousses, hair coloring products, hair bleaching products, hair conditioners, body washes, skin creams, lotions, skin moisturizers, sunscreens, tonics, toothpastes, dental creams, tooth gels, tooth powders, mouth washes, breath fresheners, and dental floss.

27. An affinity media having a solid support comprising an effective amount of a fusion peptide coating, said fusion peptide having a first portion and a second portion, wherein said first portion comprises a multi-functional solubility tag that is bound to the solid support and said second portion of the fusion peptide comprises an affinity for a target material; wherein the multi-functional solubility tag has the general formula:

a) SEQ ID NO: 1 – *Spacer* – [[SEQ ID NO: 1]-[*Spacer*]$_m$]$_n$ or

b) SEQ ID NO: 2 - *Spacer*-[[SEQ ID NO: 2]-[*Spacer*]$_m$]$_n$

wherein

SEQ ID NO: 1 is
Xaa1-Gln-[Xaa2]$_p$-[Phe-Xaa3-Xaa4-Xaa5]$_s$-Phe-Xaa6-
[Xaa7]$_q$-[Gln]$_r$; and

SEQ ID NO: 2 is
Xaa1-Gln-Xaa8-[Xaa4--Xaa8]$_s$-Phe-[Glu-Gln-Gln]$_r$;

wherein
Xaa1 = Gln or His
Xaa2 = Gln, Arg, His, or Lys;
Xaa3 = Gln, His, Lys, Arg, or Glu;
Xaa4 = Trp or Phe
Xaa5 = Gln, His, Lys, Arg or Glu;
Xaa6 = Glu, Gln, or Arg;
Xaa7 = Gln or Lys;
Xaa8 = Asp, Glu, Gln, His, Lys, or Arg;
p, q, and r are independently 0 or 1;
s is an integer ranging from 1 to 5;
n is an integer ranging from 1 to 10;
m= n-1; and
Spacer = a peptide linker ranging from 1 to 100 amino acids in length.

28. The affinity media of claim 27 wherein the solid support comprises a material selected from the group consisting of a pigment, a plant tissue, a polymer, polystyrene, polyethylene, polypropylene, polytetrafluoroethylene, polyester, polyvinyl chloride, poly (methyl methacrylate), polyethersulfone, polyimide, polyamide, aramids, poly *para*-phenylene terephthalamide, poly *meta*-phenylene terephthalamide, poly urethane, polysaccharides, cellulose, starch, chitin, minerals, silica, silicates, micas, titanium dioxide, alumina, iron oxides, clays, metals, gold, silver, carbon, carbon black, graphite and carbon nanotubes.

29. The affinity media of claim 28 wherein the solid support is in the form of a resin, a membrane, a filter, a bead, a fiber, a foam, a film or any combination thereof.

30. The affinity media of claim 27 wherein the second portion of the fusion peptide has affinity for a second target a material that may be present in a biological fluid, an aqueous waste stream, a waste stream from mining operations, an environmental sample, a fermentation medium, fermentation biomass.

31. A pharmaceutical, agricultural, or cosmetic composition comprising the fusion peptide of claim 22 or the composition of claim 23.

32. A biomedical or tissue engineering composition comprising the fusion peptide of claim 22 or the composition of claim 23.

33. The biomedical or tissue engineering composition of claim 32 wherein the fusion peptide comprises a multifunctional solubilityt tag having hydrogel forming activity and a second portion having affinity for a cell surface receptor.

34. The biomedical or tissue engineering composition of claim 33 wherein the second portion having affinity for the cell surface receptor comprising at least one RGD peptide.

35. A method comprising:

a) providing an aqueous matrix comprising a target material to be obtained from the aqueous matrix;

b) contacting the aqueous matrix with the affinity media of claim 27 whereby the target material binds to the fusion protein.

36. The method of claim 35 further comprising (c) eluting the bound target material from the affinity media.

37. The method of claim 35 wherein the aqueous matrix is a biological fluid, a waste stream, a waste stream from mining operations, an environmental sample, a fermentation medium, an aqueous sample comprising fermentation biomass.

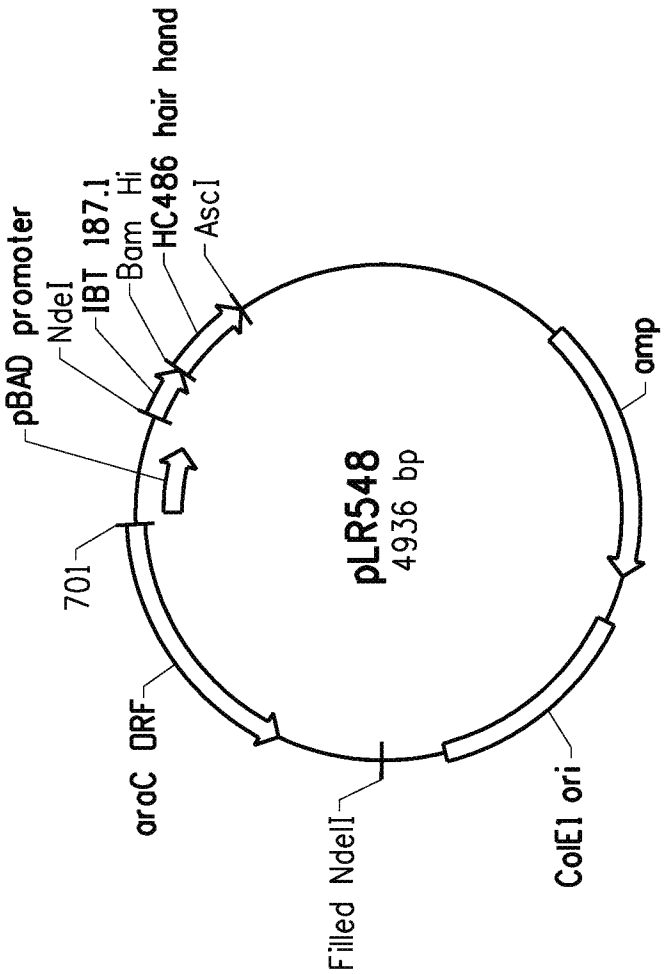
pBAD promoter
NdeI
IBT 187.1
Bam Hi
HC486 hair hand
AscI
pLR548
4936 bp
amp
701
araC ORF
Filled NdeI
ColE1 ori

FIG. 1

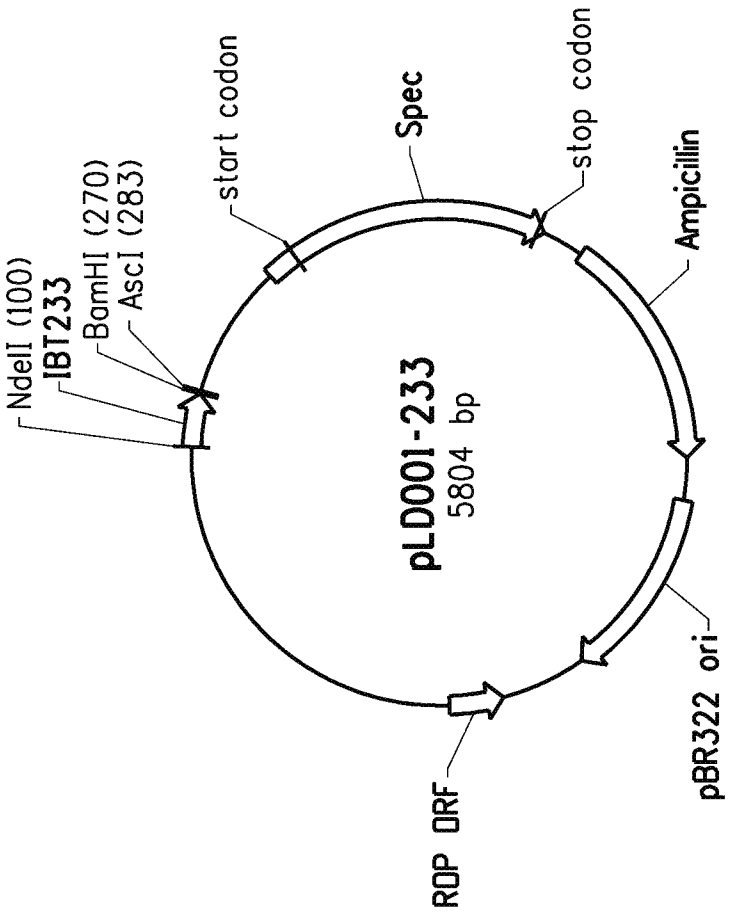
NdeII (100)
IBT233
BamHI (270)
AscI (283)
start codon
Spec
stop codon
Ampicillin
pLD001-233
5804 bp
ROP ORF
pBR322 ori

FIG. 2

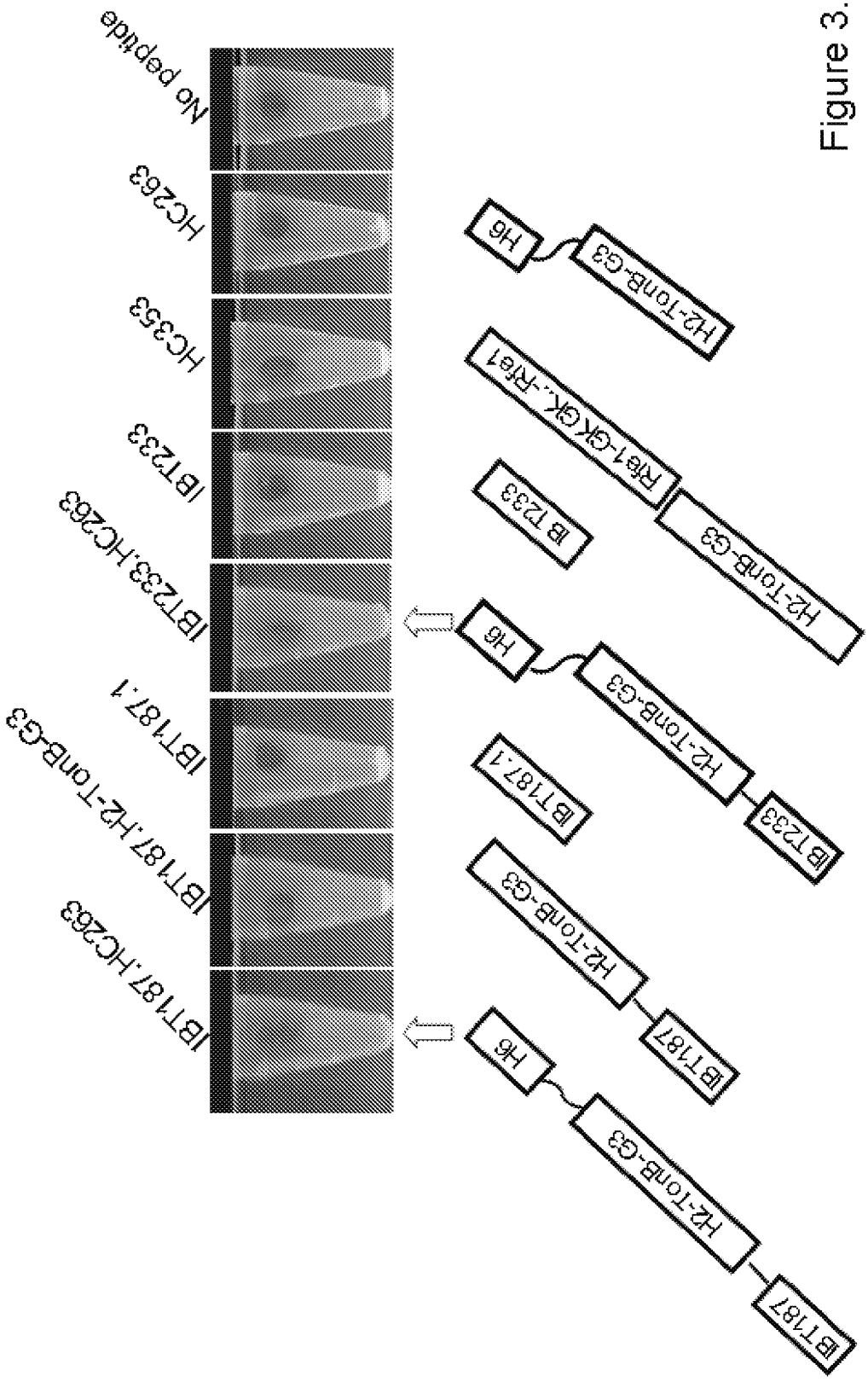
No peptide
HC263
HC353
IBT233
IBT233,HC263
IBT187.1
IBT187,H2-TomB-G3
IBT187,HC263
IBT187
H2-TomB-G3
H6
IBT187.1
IBT233
H2-TomB-G3
H6
IBT187
H2-TomB-G3
Rfe1-GK GK...Rfe1
H2-TomB-G3
H6
IBT233

Figure 3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/043537

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/63(2006.01)i, C07K 19/00(2006.01)i, A61K 8/64(2006.01)i, A61Q 5/02(2006.01)i, A61Q 11/00(2006.01)i, A61Q 19/00(2006.01)i

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)

C12N 15/63; C12P 21/00; C12N; C12P 21/04; C07K 7/08; C07H 21/00; C07K 7/10; C12N 1/20; C12P 21/06

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

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: multi-functional solubility tag, inclusion body tag, fusion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	US 2010-0136621 A1 (QIONG CHENG et al.) 03 June 2010 See Abstract, Claims 1, 15, 21, Paragraphs 0134, 0183.	27-30 1-26,31-37
A	US 2009-0029420 A1 (LINDA JANE DECAROLIS et al.) 29 January 2009 See Abstract, Claim 5.	1-37
A	US 7662587 B1 (QIONG CHENG et al.) 16 February 2010 See Abstract, Claim 6.	1-37
A	US 05215896 A (PETER C. KECK et al.) 01 June 1993 See Abstract.	1-37
A	WO 03-100022 A2 (RESTORAGEN INC.) 04 December 2003 See Abstract, Claim 28.	1-37

☐ 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 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	Date of mailing of the international search report
30 OCTOBER 2012 (30.10.2012)	**30 OCTOBER 2012 (30.10.2012)**

Name and mailing address of the ISA/KR

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Facsimile No. 82-42-472-7140

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Telephone No. 82-42-481-8407

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2012/043537

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