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(54) Title: FUNCTIONALIZED PROTEIN-BASED MATERIALS AND THEIR USES

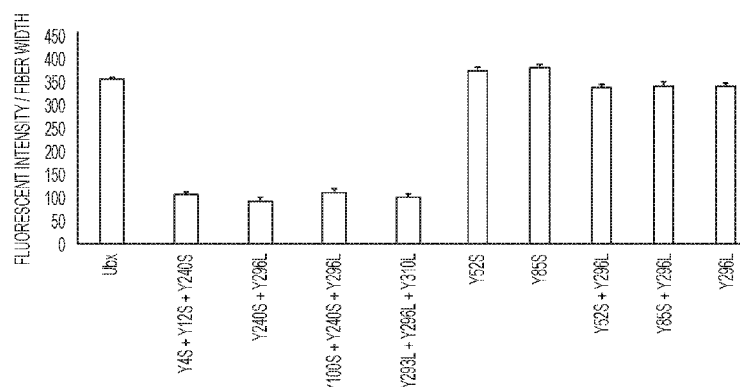


FIG. 1

(57) Abstract: Embodiments of the invention are directed to Ubx-fusion molecules that maintain their mechanical strength and properties even after being fused with Ubx. Ubx fusions with VEGF and other growth factors, cell signaling proteins, and cell binding proteins can be used to induce angiogenesis. Ubx fibers and mesh, embedded within a tissue engineering scaffold, induce formation of vasculature within the scaffold. The presence of vasculature is necessary to provide oxygen and nutrients to other cells growing within the scaffold.





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- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
 - *with information concerning request for restoration of the right of priority in respect of one or more priority claims (Rules 26bis.3 and 48.2(b)(vii))*
 - *with sequence listing part of description (Rule 5.2(a))*

FUNCTIONALIZED PROTEIN-BASED MATERIALS AND THEIR USES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 62/105,132, filed January 19, 2015, which is incorporated
5 herein by reference in its entirety as if fully set forth herein.

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grant No. R01HL095786 awarded by The National Institutes of Health and Grant No. R3E821 awarded
10 by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND

[0003] Proteins implement most of the vital molecular functions of living organisms, including providing structural support, generating energy, sensing biomolecules, and catalyzing, storing, and degrading important biomolecules. Proteins are particularly adept at
15 highly specific molecular recognition, which can be adapted to bind virtually any ligand, ranging from small chemicals to specific types of cells. Such interactions are often regulated by the chemical environment, by binding additional ligands (allostery), or by post-translational modification of the protein.

[0004] Devices that capture proteins in materials have the potential to mimic these
20 functions and be regulated by externally applied factors. However, this approach is technically challenging. The three-dimensional structures of proteins are maintained by non-covalent bonds, which are sensitive to the surrounding medium. Perturbations of this chemical environment can easily result in loss of protein function. Proteins are commonly incorporated into materials either by physically trapping them within the matrix of the
25 materials during assembly or by covalently crosslinking them to the surface of the materials post-assembly. Both of these approaches can result in the loss of functional proteins. Physically trapped proteins can be inactivated by the harsh chemical environment often used to instigate the assembly of the proteins into materials. Furthermore, the functional proteins are free to diffuse out of the materials. Although covalent crosslinking tethers functional

proteins to the materials, the crosslinking agent can also inactivate the appended protein or remain embedded in the materials, rendering them toxic to cells. In addition, materials held together by non-covalent bonds may be too fragile for chemical modifications after assembly. Finally, depending on the specificity of the cross-linking strategy, some portion of the
5 functional protein may be oriented such that the materials structure blocks ligand binding and hence protein function.

[0005] For materials composed of recombinant proteins, the use of protein fusions provides an attractive alternative. By fusing a gene encoding a functional protein to a gene encoding a self-assembling protein, a single polypeptide can be produced that contains the
10 sequences of both proteins. This fusion protein should retain both the functional and self-assembly properties of the parent proteins. This method offers several advantages for incorporating functional proteins: (i) materials assembly and functionalization can be combined into a single step, (ii) stoichiometric levels of functionalization can be achieved, (iii) covalent attachment prevents loss of the functional protein due to diffusion, (iv) toxic by-
15 products (remnants of chemical cross-linking) are not created, and (v) the functional proteins can be patterned within the materials. In addition, all of the appended proteins have a uniform orientation, although there are only two possible points of attachment – the N- and C-termini of the self-assembling protein. The gene fusion approach requires mild conditions for materials assembly that will not perturb the structure of the appended functional protein.

[0006] Not every functional protein is a good candidate for incorporation into
20 materials via gene fusion. Unstable or insoluble functional proteins could hamper expression of the fusion protein, or large/multimeric functional proteins may misposition the self-assembling protein, thus altering the mechanical properties of the materials or even preventing materials assembly. Thus, it is important for any functional protein to maintain its
25 stability and properties.

SUMMARY

[0007] Embodiments of the invention are directed to Ubx-appended molecules that maintain their mechanical strength and properties even after being fused with Ubx. Ubx
fusions with VEGF and other growth factors, cell signaling proteins, and cell binding proteins
30 can be used to induce angiogenesis. Ubx fibers and mesh, embedded within a tissue engineering scaffold, induce formation of vasculature within the scaffold. The presence of

vasculature is necessary to provide oxygen and nutrients to other cells growing within the scaffold.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] FIG. 1 shows a comparison of the fluorescence intensity of wild-type Ubx and
5 several tyrosine mutants; and

[0009] FIG. 2 shows that EGFP-VEGF-Ubx fibers promote enhanced recruitment and migration of endothelial cells.

DETAILED DESCRIPTION

[00010] In an embodiment of the claimed invention, nanoscale to macroscale
10 materials composed of the Drosophila melanogaster protein Ultrabithorax (Ubx) are generated. These materials are in the form of sheets, fibers, meshes, or fiber/mesh combinations. Ubx materials are biocompatible and non-immunogenic. The materials have mechanical properties appropriate for tissue engineering. The strength of Ubx materials derives from dityrosine covalent bonds that form spontaneously during assembly. Dityrosine
15 bond content can be manipulated by mutagenesis to increase or decrease the strength of the materials. Chemical functions can be easily incorporated into Ubx materials in two ways. First, a gene encoding a protein with a useful function can be fused to the gene encoding Ubx. The resulting fusion gene, when transformed into E. coli, produces a fusion protein encompassing the sequences of both the functional protein and Ubx. Ubx fusion proteins
20 retain the ability to assemble into materials. Furthermore, in the Ubx fusion proteins that have been tested thus far, the appended protein also retains its ability to function. Ubx fusions including the VEGF protein can induce angiogenic behaviors in endothelial cells. Cells can be layered on Ubx fibers (endothelial cells on a fiber, smooth muscle cells on top of the endothelial cells) to mimic the layers of cells in blood vessels. Additionally, fiber/mesh
25 structures in which the morphology resembles that of a vascular bed can also be created. VEGF-Ubx fibers, when placed on top of a chicken embryo, induce the formation of vasculature.

[00011] The Ubx protein contains a DNA binding domain, and this domain remains folded and capable of binding DNA in a sequence-specific manner in Ubx materials.
30 DNA sequences with multiple Ubx binding sites remain bound to Ubx materials for over a

week, even though the Ubx-DNA interaction is non-covalent. A DNA bound to Ubx fibers will orient such that the region with the most Ubx binding sites is facing the fiber.

[00012] Ubx fusions with VEGF and other growth factors, cell signaling proteins, and cell binding proteins can be used to induce angiogenesis. Ubx fibers and mesh, embedded within a tissue engineering scaffold, should induce formation of vasculature within the scaffold. The presence of vasculature is necessary to provide oxygen and nutrients to other cells growing within the scaffold. Because all of the proteins that induce vasculature formation (angiogenic proteins) are only located on Ubx materials, they will not misinstruct cells in other locations in the scaffold (e.g., liver cells will not be instructed to become blood vessels). Because the angiogenic proteins are covalently bound to Ubx materials, they cannot diffuse away, thus losing the instructions or "fuzzing" the pattern.

[00013] Several proteins have been fused to Ubx to examine their impact on Ubx assembly and properties. Ubx self-assembles rapidly in gentle buffers to form films and fibers. Ubx materials are biocompatible, strong, and remarkably extensible. Furthermore, proteins fused to Ubx retain their activity once incorporated into materials. The proteins fused to Ubx were selected to test a range of sizes, structures, stabilities, solubility, and charges. It was found that the appended protein had a large effect on protein production, with the solubility and quaternary structure of the appended protein being the best predictors for success. In contrast, the ability to self-assemble into materials was dominated by Ubx, and the presence or identity of a fused protein had little impact on materials assembly. Although the appended protein can alter the mechanical properties of the materials, these properties, like those of Ubx fibers, (i) can be adjusted by varying fiber diameter and (ii) remain similar to the extracellular matrix protein elastin. In addition, unlike other self-assembling proteins found in nature, Ubx is unique in that it contains a DNA binding domain.

[00014] The presence of dityrosine provides an opportunity to manipulate the properties of Ubx materials by controlling dityrosine bond formation. Controlling dityrosine bond formation makes the Ubx protein easier to manipulate in the context of a fusion protein. Ubx has 15 tyrosine residues. The identity of tyrosines contributing to a single bond may vary, and more than one dityrosine bond may be present in the materials, creating an enormous array of possible bond arrangements. When bound to DNA, Ubx can oligomerize in multiple orientations: side-to-side cooperative interactions when binding to linear DNA, and back-to-back interactions between clusters of cooperatively bound Ubx

proteins to form the stem of a DNA loop. Because Ubx fibers retain the ability to bind DNA, it is possible that interactions used on a small scale to enable cooperative DNA binding and DNA loop formation in vivo may also be applied on a much larger scale to form Ubx materials in vitro: side-to-side interactions to form nanoscale fibrils, and back-to-back interactions to allow the fibrils to interact to form films and fibers.

[00015] Two dityrosine bonds in Ubx are identified by site-directed mutagenesis followed by measurements of fiber fluorescence intensity. One bond is located between the N-terminus and the homeodomain (Y4/Y296, Y12/Y293), and another bond is formed by Y167 and Y240. The intensity of blue fluorescence corresponds with immunostaining using the antidityrosine primary antibody (confirming that changes in fiber fluorescence directly correspond to alterations in dityrosine content). FIG. 1 shows that the mutants Y4S + Y12S + Y240S, Y240S + Y296L, Y100S + Y240S + Y296L, and Y293L + Y296L + Y310L all show a loss of 250 fluorescence units per micrometer when compared to Ubx, suggesting a loss of more than one tyrosine bond. However, mutation of tyrosines Y52 and Y85, which were not predicted to be involved in dityrosine bonds has no effect either in wild-type Ubx or in the Y296 background.

[00016] The Y167S and Y240S mutations both reduce fluorescence to a similar degree, suggesting that Y167 and Y240 participate in the same dityrosine bond. To test this hypothesis, a Y167S + Y240S double mutant was created. No further reduction in fluorescence was observed for the Y167S + Y240S double mutant (FIG. 1), suggesting that Y167 and Y240 form a single dityrosine bond. The Y167S + Y240S bond is responsible for a significant portion of the observed fluorescence in Ubx fibers (200/360 fluorescence units per micrometer).

[00017] Fiber fluorescence closely correlates with fiber strength, demonstrating that these bonds are intermolecular. The percentage of Ubx molecules harboring both bonds can be decreased or increased by mutagenesis, providing an additional mechanism to control the mechanical properties of Ubx materials. Duplication of tyrosine-containing motifs in Ubx increases dityrosine content in Ubx fibers, suggesting these motifs could be inserted in other self-assembling proteins to strengthen the corresponding materials.

[00018] Since adding an entire region of the Ubx protein might not be feasible for some self-assembling proteins, shorter sequences have been identified to replace the large insertion. The sequences surrounding tyrosines that form dityrosine bonds must contribute to interaction specificity, and thus would need to be transferred to the heterologous system. Based on Ubx sequence conservation, structure/disorder data, and the predicted propensity to engage in protein interactions, the below sequences are recommended for transfer to other proteins to create dityrosine bonds. For protein systems that can accommodate large insertions, the homeodomain (LRRRGRQTYTYQTLELEKE FHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRRMKLKEI; SEQ ID NO: 1) and the N-terminus (MNSYFEQA; SEQ ID NO: 2) are used. As an additional benefit, the solubility and stability of the homeodomain is expected to improve protein production when fused to a self-assembling protein. For protein systems that can only tolerate small insertions or that self-assemble upon exposure to denaturing conditions, the conserved motifs surrounding residues 167 (VRPSACTPDSRVGGYLDTS; SEQ ID NO: 3) and 240 (FYPWMAIA; SEQ ID NO: 4) are used. Thus the specific dityrosine-bond forming motifs in Ubx have the potential to be a useful tool for engineering the fluorescent and mechanical properties of other protein systems.

[00019] In certain embodiments of the claimed invention, the full length Ubx protein is used to create a fusion protein. In other embodiments of the invention, the Ubx protein that is used to form a Ubx fusion protein is a combination of SEQ ID NO: 1 and SEQ ID NO:2, or a combination of SEQ ID NO:3 and SEQ ID NO: 4.

[00020] In certain embodiments, Ubx fusion proteins are used to create materials that induce damaged endothelium in blood vessels to heal. Clogged arteries are typically re-opened by angioplasty, in which a balloon is inflated to open the artery, and a stent placed to hold the artery open. Both the initial damage and deployment of the stent damage the endothelial lining and can cause a clot to form, thus re-blocking the artery. Stents coated in functionalized Ubx materials have been shown to cause a new, healthy endothelial layer to form and prevent clot formation.

[00021] Because 3-dimensional structures can be easily designed and created using DNA, many materials are composed of DNA, or materials decorated with DNA with some engineered function. Since proteins can expand the possible functions or allow creation of more exotic shapes, there is interest in creating protein/DNA composite materials. Current

approaches are to chemically crosslink protein to DNA (a method that is expensive, generates toxic byproducts, and possesses a geometry that is difficult to control), or to append a string of positively charged amino acids to the protein to get DNA to non-specifically bind (a method that has no control over geometry or orientation, and the binding is low affinity). On the other hand, Ubx materials bind DNA in a sequence specific manner, meaning there is control over affinity, patterning, and orientation. In certain embodiments, Ubx materials are coated in DNA sensors. Both the orientation of the DNA and the affinity of Ubx materials for DNA increases the sensitivity of these devices.

[00022] Ultrabithorax (Ubx) protein molecules have the ability to self-assemble. Ubx materials can be functionalized 1) more easily and cheaply 2) with defined orientations of the functional molecules and 3) with a greater range of functional molecules than other materials. This characteristic of Ubx proteins makes it possible to create protein composite materials that are not otherwise available.

[00023] It has previously been established that materials with Ubx fusion proteins can be made. However, the proteins used in those studies were small, stable, highly soluble, and monomeric and thus likely to succeed. Since that time, materials with large, less soluble, and/or multimeric proteins have been made, greatly expanding the range of proteins that can be used.

[00024] In certain embodiments of the invention, a Ubx fusion protein with VEGF is created. This fusion is made with either the full length Ubx protein, a combination of SEQ ID NO: 1 and SEQ ID NO:2, or a combination of SEQ ID NO:3 and SEQ ID NO: 4. Studies with VEGF-Ubx (VEGF is a dimer) demonstrate that the fused protein is still active. VEGF-Ubx fibers induce formation of vasculature. Fusing the gene encoding vascular endothelial growth factor (VEGF) with the ultrabithorax (Ubx) gene, to produce fusion proteins capable of self-assembly into materials. VEGF-Ubx materials enhance human endothelial cell migration and prolong cell survival. FIG. 2 shows quantification of the number of cells/100 μ m distance attached to EGFP-Ubx vs. EGFP-VEGF-Ubx fibers. A significantly greater number of cells migrated onto EGFP-VEGF-Ubx fibers when compared to control EGFP-Ubx fibers, indicating that VEGF-Ubx materials stimulated migration of ECs in low serum conditions. This approach provides an inexpensive and facile mechanism to stimulate and pattern angiogenesis.

[00025] Embodiments of the invention can be understood by reference to the following examples:

Creating protein fusions

[00026] The gene of the functional protein, followed by DNA encoding a flexible, water soluble linker, followed by the ubx gene, are placed in tandem without intervening stop codons. The linker DNA encodes alternating glycine and serine residues. Glycine provides flexibility and serine provides water solubility. This fusion gene is cloned into the pET-19b plasmid (it should be noted that other plasmids work equally well). The plasmid is transformed into E. coli. E. coli are fermented until the optical density reaches ~ 0.6 - 0.7, and then production of the fusion protein is induced with 1 mM IPTG. Depending on the solubility and stability of the fusion protein, the E. coli are allowed to produce the fusion protein between 2 and 16 hours.

Coating the surface of materials with DNA

[00027] Fibers are wrapped around a structural support and dipped into a buffer containing DNA. The DNA sequence needs to contain at least one Ubx binding sequence to adhere to Ubx materials.

Embedding DNA in the materials

[00028] Ubx monomers are incubated with DNA prior to materials assembly. Both the Ubx and DNA concentrations need to be higher than 160 pM, the affinity constant.

Embedding Ubx materials in scaffolds

[00029] Ubx fibers are wrapped around a supporting structure (e.g. a plastic inoculation loop) or Ubx film is supported by the loop (think about soapy water in a bubble wand). The Ubx materials are then placed in a container, such as a well in a cell culture plate. The scaffold material (e.g., collagen) is then polymerized around the Ubx materials.

Creating Ubx mesh

[00030] Ubx film is allowed to dry in air. We generally use inoculation loops to support the film, but other supports (e.g. plastic coated paper clips) work as well.

Creating layers of cells on Ubx materials

[00031] Ubx fibers are dipped in the first cell type (endothelial cells) and the cells are allowed to attach. Subsequently, the fibers are moved to a culture containing the second cell type (smooth muscle cells) and these are given the opportunity to attach.

5 Creating Ubx materials in structures that look like vascular beds

[00032] Ubx fibers are wrapped around an inoculation loop or other support. The loop is used to pick up a Ubx film. When the film dries, the structure is complete.

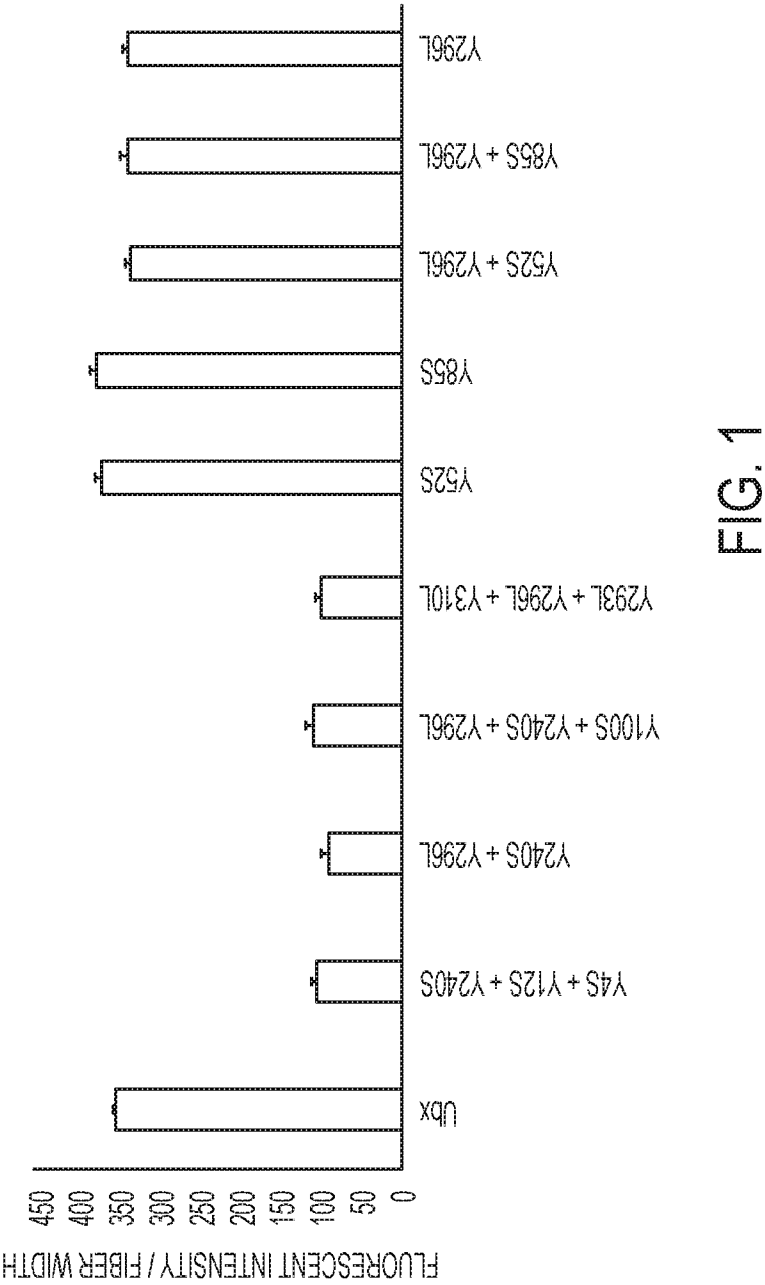
[00033] While the invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art
10 that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

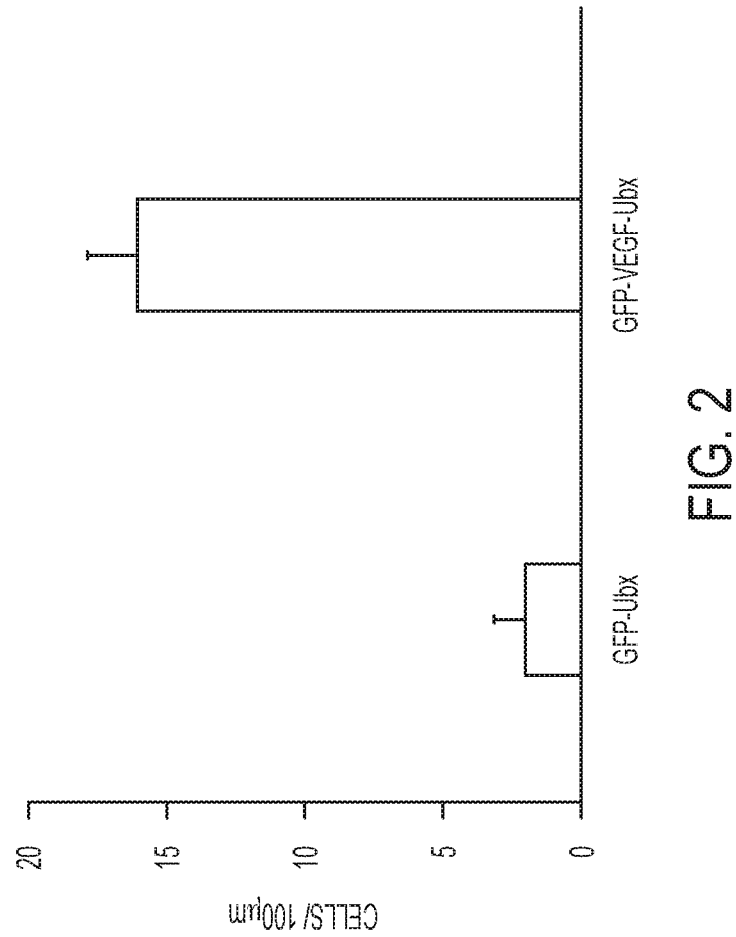
CLAIMS:

What is claimed is:

1. A composition comprising at least one Ubx protein coupled with a first biomolecule.
2. The composition of claim 1, wherein the first biomolecule is coupled with the Ubx
5 protein with a linker.
3. The composition of claim 1, wherein the first biomolecule is a monomeric protein.
4. The composition of claim 1, wherein the first biomolecule is a multimeric protein.
5. The composition of claim 1, further comprising a second biomolecule.
6. The composition of claim 5, wherein the second biomolecule is a highly soluble
10 protein or an additional functional protein.
7. The composition of claim 1, wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bonds.
8. The composition of claim 1, wherein the Ubx protein contains mutations within tyrosine residues that are not involved in the formation of di-tyrosine bonds.
- 15 9. The composition of claim 1, wherein the dityrosine content is lower than in the wild type protein.
10. The composition of claim 1, wherein the dityrosine content is greater than in the wild type protein.
11. The composition of claim 1, wherein the first biomolecule is VEGF, FGF or SDF-1.
- 20 12. The composition of claim 1, wherein the composition is incorporated into a structure.
13. The composition of claim 12, wherein the structure is a mesh, film, fiber or a combination thereof.
14. The composition of claim 12, wherein the structure is coated with a protein.
15. The composition of claim 12, wherein the structure is coupled with DNA.

16. The composition of claim 14, wherein the protein promotes angiogenesis.
17. The composition of claim 14, wherein the protein activates a signaling pathway.
18. The composition of claim 17, wherein the signaling pathway is the VEGF signaling pathway.
- 5 19. The composition of claim 1, wherein the Ubx protein is selected from the group consisting of the full length protein, SEQ ID NO: 1, SEQ ID NO:2, SEQ IS NO:3 and SEQ ID NO: 4.
20. The composition of claim 1, wherein the Ubx protein is selected from the group consisting of the combination of SEQ ID NO: 1 and SEQ ID NO: 2 and the combination of
10 the SEQ ID NO:3 and SEQ ID NO:4.





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/13977

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/18; A61K 9/00; A61F 13/00; A61K 48/00; C07K 14/00 (2016.01)

CPC - A61K 38/1866; C07K 14/475; A61K 8/0241; A61K 48/00; C07K 2319/00

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)

IPC(8) - A61K 38/18; A61K 9/00; A61F 13/00; A61K 48/00; C07K 14/00 (2016.01); USPC - 514/8.1; 424/400,443; 514/44R; 530/350

CPC - A61K 38/1866; C07K 14/475; A61K 8/0241; A61K 48/00; C07K 2319/00

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

IPC(8) - A61K 38/18; A61K 9/00; A61F 13/00; A61K 48/00; C07K 14/00 (2016.01); USPC - 514/8.1; 424/400,443; 514/44R; 530/350

CPC - A61K 38/1866; C07K 14/475; A61K 8/0241; A61K 48/00; C07K 2319/00 - see keyword below

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

PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google; Ultrabithorax, Ubx, Fusion, conjugate, Protein, bityrosine, tyrosine, di-tyrosine, mutation, mutant, mesh, film, fiber, VEGF, FGF, SDF1, angiogenesis, signaling, pathway, targeting, activate, carrier, coated, surface, soluble, nanoparticle, structure, monomer, multimer, dimer, linker, composi

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X		1, 3, 5-6, 12-13, 19
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Y	US 2010/0143436 A1 (BONDOS et al.) 10 June 2010 (10.06.2010), Abstract, para [0006], [0007], [0009], [0021], [0024], [0027], [0029], [0030], [0031], [0035], [0070], [0097], [0112], and SEQ ID NO: 2, the regions between amino acid residues 1-8 and 251-311	2, 4, 7, 9, 11, 14, 16-18
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A		20
Y	US 2003/0027751 A1 (KOVESDI et al.) 06 February 2003 (06.02.2003), para [0005], [0006], [0049], [0050], [0086], [0089], [0091], [0101], [0103], [0104], [0128], and SEQ ID NO: 66	2, 4, 11
Y	US 2013/0302400 A1 (MANEVAL et al.) 14 November 2013 (14.11.2013), para [0013], [0014], [0019], [0032], [0119], and [0352]	14, 16-18
Y	ENDRIZZI et al., Specific covalent immobilization of proteins through dityrosine cross-links. Langmuir. 2006, Vol. 22(26), p. 11305-10. Abstract; pg 11036, Fig 1; and pg 11038, col 2, para 2, and Fig 4	7, 9
Y	PASSNER et al., Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. Nature. 1999, Vol. 397(6721), p. 714-9. pg 714, col 2, top para and last para, and Fig 1b	7, 9
Y	US 2004/0029280 A1 (SOSNOWSKI et al.) 12 February 2004 (12.02.2004), Abstract, para [0012], [0017], [0058], [0090], [0157], [0162], [0167], and [0535]	16-18
A	PIR_B27867, homeotic protein Ultrabithorax - fruit fly (Drosophila melanogaster) (fragment). NCBI Protein Accession Number B27867. 23 February 1997 [online]. [Retrieved on 2016.03.24]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/protein/B27867?report=genpept > Definition, and Origin	1-7, 9, 11-14, 16-18, 19-20

☒ Further documents are listed in the continuation of Box C.

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"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

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"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

24 March 2016

Date of mailing of the international search report

19 MAY 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/13977

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UniProtKB_Q964V4, Ubx protein. UniProtKB Accession Number Q964V4, 28 November 2006. [online]. [Retrieved on 2016.03.24]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/protein/Q964V4 > Definition, and Origin, the region between amino acid residues 13-73	1-7, 9, 11-14, 16-18, 19-20
A	CROSS et al., FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci. 2001, Vol. 22(4), p. 201-7. Entire documentation, especially Abstract	1-7, 9, 11-14, 16-18, 19-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/13977

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

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

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

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-20, directed to a composition comprising at least one Ubx protein coupled with a first biomolecule. The Ubx protein will be searched to the extent that wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bonds or/and wherein the di-tyrosine content is lower than in the wild type protein; wherein the composition is incorporated into a structure will be searched to the extent that wherein the structure is coated with a protein; and wherein Ubx protein sequence will be searched to the extent that Ubx protein sequence encompasses SEQ ID NO: 1 and/or SEQ ID NO: 2. It is believed that claims 1-7, 9, 11-14, 16-18, 19-20 (in part) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bonds or/and wherein the di-tyrosine content is lower than in the wild type protein; wherein the composition is incorporated into a structure and wherein the structure is coated with a protein; and wherein Ubx protein is selected from the group consisting of the full length protein, SEQ ID NO: 1, and SEQ ID NO:2. *****Continued in the extra sheet*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-7, 9, 11-14, 16-18, 19-20 (in part), limited to SEQ ID NO: 1 and SEQ ID NO: 2

Remark on Protest

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

Continuation of: Box No III (unity of invention is lacking)

Additional the Ubx protein contains mutations, the structure is coated, and/or Ubx protein sequence will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected the Ubx protein contains mutations, the structure is coated, and/or Ubx protein sequence. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be wherein the Ubx protein contains mutations within tyrosine residues that are not involved in the formation of di-tyrosine bonds, and/or wherein the di-tyrosine content is greater than in the wild type protein; wherein the structure is coupled with DNA, and/or wherein Ubx protein is selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4 [claims (1-6), 8, 10, (11-13), 15, 19-20 (in part)].

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature

Among Groups I+, the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bonds or/and wherein the di-tyrosine content is lower than in the wild type protein results in a mutant Ubx protein that structurally different from the Ubx protein contains mutations within tyrosine residues that are not involved in the formation of di-tyrosine bonds, and/or wherein the di-tyrosine content is greater than in the wild type protein; wherein the structure is coated with a protein is different from wherein the structure is coupled with DNA; and each SEQ ID NO represents a structurally different Ubx protein.

Common Technical Features

The inventions of Groups I+ share the technical feature of a composition comprising at least one Ubx protein coupled with a first biomolecule (claim 1);

-- wherein the Ubx protein contains mutations within tyrosine residues (involved or not involved) with in the formation of di-tyrosine bonds (claims 7-10);

--wherein the composition is incorporated into a structure (claim 12); and

-- wherein the structure is coated with a molecule (claim 14: protein; claim 15: DNA).

However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2010/0143436 A1 to BONDOS et al. (hereinafter 'Bondos'); or being obvious over Bondos, in view of an article entitled 'Specific covalent immobilization of proteins through dityrosine cross-links' by ENDRIZZI et al. (hereinafter 'Endrizzi'; Langmuir. 2006, Vol. 22(26), p. 11305-10), and further in view of an article entitled 'Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex' by PASSNER et al., (hereinafter 'Passner'; Nature. 1999, Vol. 397(6721), p. 714-9) as follows:

Bondos discloses a composition comprising at least one Ultrabithorax (Ubx) protein coupled with a first biomolecule (para [0030] - 'a biomaterial comprises a Ubx fusion protein in which a Ubx protein is fused with at least one other protein'; Abstract - 'a biomaterial may comprise one or more polypeptides and/or proteins. A biomaterial may comprise... two or more self-assembled Ultrabithorax (Ubx)'; para [0006] - 'A composition of biomaterials ...capable of forming higher-order structure shapes for various biomedical uses').

Bondos further discloses wherein the composition is incorporated into a structure (para [0007] - 'A biomaterial structure may comprise, ... at least two different Ubx fusion proteins ... a biomaterial composition wherein the structure is a bundle that comprises at least two different Ubx fusion protein fibers'; para [0035] - 'A biomaterial (e.g., a Ubx biomaterial) may be used...to form a porous three dimensional structure (e.g., mesh, lattice, and/or filter)'; and wherein the structure is coated with gold or an antibody-conjugated nanoparticles (para [0070] - 'Ubx sheets and ropes were transferred using a needle, whereas Ubx film was carefully lifted using plastic coated wire bent into a stem-loop shape. Samples were sputter coated with gold'; para [0097] - 'One may pattern materials using multiple Ubx variants (e.g., EGFP-Ubx and m-Cherry-Ubx). ...building complex macroscale structures. ... A surface of a biomaterial may be coated, for example, with antibody-conjugated nanoparticles').

Furthermore, Bondos discloses wherein the composition comprising one or more Ubx proteins (para [0030] - 'a biomaterial comprises a Ubx fusion protein in which a Ubx protein is fused with at least one other protein'; Abstract - 'a biomaterial may comprise one or more polypeptides and/or proteins. A biomaterial may comprise... two or more self-assembled Ultrabithorax (Ubx)'), and wherein Ubx protein can comprising a point mutation for obtaining a desired structure (para [0028] - 'point mutations may be introduced to the Ubx protein to alter one or more structural and/or mechanical properties of Ubx protein fibers and superstructures'), for different purposes including drug delivery (para [0006] - 'A composition of biomaterials ...capable of forming higher-order structure shapes for various biomedical uses'; para [0024] - 'protein-based materials may be customized for a variety of advanced applications, including drug delivery').

Bondos does not specifically teach wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bond; or wherein Ubx protein contains di-tyrosine and wherein the di-tyrosine content is lower than in the wild type protein. Endrizzi discloses a method for coupling a protein by forming di-tyrosine bond by using tyrosine residues in a protein and associated coupling peptide for coupling (Abstract - 'Green fluorescent protein (GFP), as a model protein, was genetically tagged with a tyrosine-modified His6 peptide on its carboxy terminus. GFP-YGH6, specifically associated with the NTA-Ni(II) groups, was covalently coupled to the bead surface through dityrosine bond'; pg 11306, Fig 1), based on the naturally occurring di-tyrosine bond in natural proteins (Abstract - 'Dityrosine cross-links are widely observed in nature in structural proteins'). Endrizzi further discloses wherein the coupling of dityrosine bond is monitored by fluorescence emission (pg 11308, col 2, para 2 - 'Dityrosine has a characteristic fluorescence emission at 420 nm which was monitored during the oxidative cross-linking reaction. Dityrosine fluorescence rose concomitantly with the increase in covalently bound GFP'; pg 11306, Fig 1; pg 11308, Fig 4).

*****Continued in the next extra sheet*****

Continuation of: Box No III (unity of invention is lacking)

Endrizzi also does not specifically teach wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bond; or wherein Ubx protein contains di-tyrosine and wherein the di-tyrosine content is lower than in the wild type protein. Passner discloses Ubx protein forms homodimer through the motif comprising tyrosine (pg 714, col 2, top para - 'Ultrabithorax (Ubx)'; pg 714, col 2, top para - 'YPWM amino-acid motif of Ultrabithorax-located amino-terminally to its homeodomain', wherein 'Y' is Tyrosine, and wherein homeodomain that contain the YPWM motif indicating 'Y' in the 'YPWM motif is potentially the tyrosine residue involved in the formation of di-tyrosine bond; pg 714, Fig 1, Ubx sequence containing 'YPWM'), as well as discloses the homodomain region of Ubx sequence comprising tyrosines at different positions (pg 714, Fig 1b).

Although Passner also does not specifically teach wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bond, one of the ordinary skill in the art at the time the invention was made would have known to do so, based on the combination of Passner, Endrizzi, and Bondos, in order to remove a naturally di-tyrosine bond formation by mutating a tyrosine within tyrosine residues involved in the formation of naturally di-tyrosine bond when it is undesired for facilitating developing a composition comprising at least one Ubx protein coupled with different proteins by forming a di-tyrosine bond for different purposes with an expected success and without undue experimentation. Although the said combination also does not specifically teach wherein the di-tyrosine content is lower than in the wild type protein, this limitation is determined by the inherent property of mutating tyrosine within tyrosine residues involved in the formation of naturally di-tyrosine bond.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Bondos, Endrizzi, and Passner, to obtain a composition comprising at least one Ubx protein coupled with a first biomolecule, as taught by Bondos, and further wherein the Ubx protein contains mutations within tyrosine residues involved in the formation of di-tyrosine bond, or/and wherein the di-tyrosine content is lower than in the wild type protein, based on the combination of Endrizzi, Passner, and Bondos, as well as the inherent property of mutating tyrosine within tyrosine residues involved in the formation of naturally di-tyrosine bond, in order to combine the technologies and knowledge available in the art for facilitating developing a composition comprising at least one Ubx protein coupled with different proteins for different purposes with an expected success and without undue experimentation.

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note:

I) Claim 1 is objected as lacking a definition for the first appeared abbreviation "Ubx" limitation in claims. For the purposes of this ISR, "Ubx " in claim 1 is rewritten as "Ultrabithorax (Ubx)" according to the Specification (Specification: para [00010] - 'Ultrabithorax (Ubx)').

II) Claims 9-10 are objected as lacking a proper antecedent basis for the "the di-tyrosine content" limitation. For the purposes of this ISR, claims 9-10 are construed as follows:

9. The composition of claim 1, wherein Ubx protein contains di-tyrosine and wherein the di-tyrosine content is lower than in the wild type protein.

10. The composition of claim 1, wherein Ubx protein contains di-tyrosine and wherein the di-tyrosine content is greater than in the wild type protein.

III) This application contains multiple biomarkers. However there is no full name for each biomarker disclosed.