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(54) Title: ENGINEERED NUCLEOBINDIN1-IMMUNOGLOBULIN FUSION PROTEIN

(57) Abstract: Provided are fusion proteins, methods of making the fusion proteins, pharmaceutical formulations comprising the fusion proteins, and therapeutic methods that include administering the fusion proteins to individuals in need thereof. The fusion proteins include an Ig and nucleobindin1. The fusion protein is referred to as a NUCBody.

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ENGINEERED NUCLEOBINDIN1-IMMUNOGLOBULIN FUSION PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/795,867, filed on January 23, 2019, the disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to nucleobindin1-immunoglobulin fusion proteins, and methods of use thereof.

BACKGROUND OF THE INVENTION

Aging is a time-dependent process of functional decline and is a major risk factor for amyloid related degenerative syndromes. Currently, there are no amyloid-targeting, disease-modifying therapeutics approved by the FDA. There is a huge unmet need for novel approaches to create disease-modifying therapeutics targeting amyloidosis syndromes. Amyloid aggregation and deposition are pathophysiological components of many severe disorders in the CNS [Alzheimer's disease (AD) (Graham WV, Bonito-Oliva A, Sakmar TP. Update on Alzheimer's Disease Therapy and Prevention Strategies. Annu Rev Med. 2017;68:413-430, PMID: 28099083) and Parkinson's disease (PD)] as well as in the periphery [type 2 diabetes mellitus (T2DM), Senile Systemic Amyloidosis (SSA) and Light-chain Amyloidosis (AL)]. Specific proteins have been implicated as the primary amyloidogenic insult in each of these diseases [e.g., Aβ42 and tau for AD, islet amyloid polypeptide (hIAPP) for T2DM, α-Synuclein (α-Syn) for PD, transthyretin (TTR) for SSA, and immunoglobulin light chain for AL]. While mechanisms of disease progression are varied, each disease is associated with protein misfolding and progressive aggregation of soluble amyloid into oligomers, protofibrils, and eventually fibrils that accumulate in tissues. For example, misfolding and aggregation of TTR, a normally functioning transport protein in the serum, can lead to SSA, which is characterized by buildup of amyloid deposits in cardiac tissue causing clinical signs of heart failure with preserved ejection fraction (HFpEF) in the elderly. Not all protein misfolding results in disease. Protein misfolding can occur during normal protein biogenesis due to stochastic folding errors. In most cases, cells promptly activate the so-called Protein Quality Control (PQC) system that efficiently restores the protein homeostasis. Protein aggregates are quickly removed and in some cases protein re-folding can also occur. A key component of the PQC system includes molecular chaperones, which in addition to stabilizing and ensuring the correct folding of proteins, can also help

misfolded proteins to unfold and correctly re-fold. Typically, molecular chaperones recognize exposed hydrophobic domains in unfolded or misfolded proteins, preventing them from self-associating and aggregating. If this early intervention fails, chaperones can collaborate with proteolytic machinery to disaggregate already formed aggregates by activating two main mechanisms, the ubiquitin-proteasome system (UPS) and autophagy. Conditions such as advanced age, excessive stress (oxidative stress, toxic chemicals, heat, inflammation), and genetic mutations may contribute to the impairment in the PQC efficiency that ultimately results in propagation of folding defects and consequent deposits of misfolded proteins. The risk for misfolded proteins to accumulate and cause disease is heightened when amyloidogenic proteins misfold and adopt the characteristic and pathological cross-β-sheet conformation that is highly prone to self-aggregate and deposit in tissue in form of plaques. When expressed at normal levels and folded correctly, amyloidogenic proteins and peptides have important biological functions, but their misfolded structures can lead to the formation of soluble pre-fibrillar aggregates that are highly toxic to cells and tissues even before they go on to form amyloid deposits. Due to their rapid aggregation and structural characteristics, misfolded amyloidogenic proteins are resistant, at least partially, to protective proteolytic mechanisms, resulting in proteinopathies. Notably, the age-related deterioration of PQC efficacy plays a key role in facilitating amyloid aggregation and significantly contributes to the occurrence and morbidity of amyloidosis syndromes in the elderly population. Unfortunately, clinical trials of immunotherapeutics with reactivity toward monomeric amyloidogenic proteins have been met with dramatic failures. On the other hand, accumulating evidence suggests that targeting the pathogenic aggregated form of amyloid might be able to prevent disease progression. In addition, the greater toxicity shown by soluble protofibrils, as compared with mature insoluble fibrils, indicates that “intermediate” amyloid conformational species are the ideal therapeutic target. While appealing, the development of conformation-specific biologics that target the quaternary amyloid structure is extremely challenging due to the transient nature of the protofibril during the process of misfolding and aggregation. Clinical applications of antibodies that target amyloid conformations are primarily limited to AD (Graham WV, Bonito-Oliva A, Sakmar TP. Update on Alzheimer's Disease Therapy and Prevention Strategies. Annu Rev Med. 2017;68:413-430, PMID: 28099083) and the general approach of targeting conformational variants of toxic protofibrils is an understudied approach for amyloidosis syndromes in general.

Although the molecular pathophysiology of amyloid varies depending on the specific disease state, it is a common shared feature that properly folded amylogenic proteins or peptides with important physiological function become highly toxic upon misfolding. Despite a lack of primary structure homology, amyloid proteins share a common cross-β-sheet secondary structure and similar quaternary structure when they misfold and aggregate. For this reason, some chaperone-like amyloid binding proteins (CLABPs) and antibodies can target amyloids from multiple sources and have so-called pan-amyloid activity. But only a small number of pan-amyloid CLABPs and antibodies have been identified to date, and even fewer of these target soluble, early intermediate amyloid aggregates such as oligomers and protofibrils. The molecular interactions between CLABPs and transient protofibrils remain unknown, constituting a major obstacle for the development of high affinity pan-amyloid therapeutics. Thus, there is an ongoing and unmet need for improved compositions and methods to treat a variety of disorders that are related to the deleterious effects of amyloid proteins. The present disclosure is pertinent to these and other needs.

SUMMARY

The present disclosure provides compositions and methods for use in diagnosis and treatment of disorders that are correlated with aggregation of proteins. In embodiments, the disclosure provides fusion proteins that comprise an amino acid sequence of a Nucleobindin-1 (NUCB1) and a segment of an immunoglobulin, such as Ig heavy chain, including but not limited to a human Ig heavy chain.

The compositions and methods are useful for inhibiting the aggregation or any formation of deleterious protein forms, such as amyloid fibrils. The disclosure accordingly provides composition and methods for inhibiting aggregation of proteins that include but are not necessarily limited to islet amyloid polypeptide (IAPP), amyloid-β (Aβ), α-Synuclein, or Transthyretin (TTR). In embodiments, the compositions and methods provide for inhibiting aggregation of Aβ42 or Aβ40, or combinations of any of the foregoing.

In non-limiting embodiments, fusion proteins of this disclosure comprise or consist of a NUCB1 amino acid sequence that is at least 90% identical to amino acids 27-452 of SEQ ID NO:3. In one embodiment, an Ig heavy chain is present in the fusion protein and comprises or consists of a sequence that is at least 90% identical to amino acids 453-680 of SEQ ID NO:3. In embodiments, the fusion comprises or consists of a sequence that is at least 90% identical to amino acids 27-680 of SEQ ID NO:3. In one non-limiting embodiment, the

fusion protein comprises or consists of a sequence that is at least 90% identical to SEQ ID NO:3.

In embodiments, one or more fusion protein is administered to provide a therapeutic or prophylactic benefit to an individual in need thereof. In embodiments, the individual is in need of treatment for any of age-related amyloidosis, Alzheimer's Disease, Parkinson's disease, Huntington disease, Familial Amyloidosis syndrome, Amyotrophic Lateral Sclerosis, post-concussion syndrome, Chronic Traumatic Encephalopathy, Lewy body dementia, inclusion body myositis, cerebral amyloid angiopathy, periphery type 2 diabetes mellitus, Senile Systemic Amyloidosis or Light-chain Amyloidosis. In embodiments, a pharmaceutical formulation comprising a described fusion protein is provided, and is used for prophylaxis and/or treatment of any of the foregoing conditions.

Expression vectors encoding the described fusion proteins are encompassed by the disclosure. Also provided are methods of making the fusion proteins, such as by expressing the protein in a cell culture, and separating the expressed protein from the cell culture.

Diagnostic methods are also provided, and comprise testing a sample with a fusion protein described herein to detect the presence, absence and/or amount of a protein to which the fusion protein binds with specificity. In embodiments, a complex comprising the fusion protein and a protein in a biological sample is detected. In embodiments, a described fusion protein is labeled with a detectable label to facilitate quantification and/or localization of the fusion protein, such as in a biological sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an illustrative schematic representation of pan-amyloid, protofibril capping mtNUCB1 and the engineering of NUCBodies. The amyloid pathological pathway is initiated by monomer misfolding into cross-β sheet species that progressively aggregate to form fibrils and plaques (top). The engineered mtNUCB1 recognizes amyloid protofibrils through a capping mechanism producing a stable, non-toxic complex (bottom). The study of mtNUCB1 biology and the understanding of the molecular interactions involved in its amyloid capping (EXAMPLE 1, 2) will lead to the development of NUCBodies that can be used to as a proof-of-concept for a therapeutic strategy targeting age-related amyloidosis.

Figure 2 shows the sequence of the complete protein product of the NUCBody. There is an IL-2 signal sequence (bold-italic) that is cleaved during protein processing and secretion. The NUCB1 protein sequence is depicted in bold and the human IgG1 FC domain is depicted in italic.

Figure 3 shows supernatant from CHO-K1 cells stably transfected with NUCBody was A) applied to a Protein G column to the NUCBody protein contained in the elution peak (fractions 2 and 3). B) The obtained peak containing the NUCBody protein was then validated by SDS-PAGE to verify the correct protein dimeric size and sample purity.

Figure 4 shows the size distribution of NUCBody imaged by atomic force microscopy. NUCBody was diluted to 30 nM in phosphate buffered saline with Ca2+ and Mg2+ and added to freshly cleaved mica in a total volume of 500 µl and imaged in tapping mode. The height images were analyzed by a custom written FIJI code and their pixel-by-pixel volume calculated. Histograms of volume (nm^3) were then plotted to determine the size distribution of the imaged sample.

Figure 5 shows the effect of NUCBody on the aggregation kinetics of the Alzheimer's disease related Aβ42 protein monitored through the Thioflavin T (ThT) assay. 2.5 µM Aβ42 was incubated alone or in presence of increasing concentrations of NUCBody obtained through Ig fusion and 10 µM ThT. The aggregation was monitored for 20h at 37 °C in quiescent conditions. Data are presented as mean ± SEM. The graphs show that NUCBody concentration-dependently inhibits Aβ42 aggregation.

Figure 6 shows the effect of NUCBody on the aggregation kinetics of the Type 2 diabetes mellitus-associated hIAPP peptide monitored through a Thioflavin T (ThT) fluorescence assay. 2.5 µM hIAPP was incubated alone or in presence of increasing concentrations of NUCBody and 10 µM ThT. The aggregation was monitored for 24h at 25 °C in quiescent conditions. Data are presented as mean ± SEM. The graphs show that NUCBody potently and concentration-dependently inhibits hIAPP aggregation.

Figure 7 shows the effect of NUCBody on the aggregation kinetics of the Parkinson's disease associated α-Syn protein monitored through a Thioflavin T (ThT) fluorescence assay. 60 µM α-Syn was incubated alone or in presence of 5 µM NUCBody and 10 µM ThT. The aggregation was monitored for 96h at 37 °C in shaking conditions (333 rpm). Data are presented as mean ± SEM. The graphs show that NUCBody inhibits α-Syn aggregation.

Figure 8 shows the effect of NUCBody on the aggregation kinetics of the Senile Systemic Amyloidosis -associated TTR peptide monitored through a Thioflavin T (ThT) fluorescence assay. 10 µM TTR was incubated alone or in presence of increasing concentrations of NUCBody and 10 µM ThT. The aggregation was monitored for 24h at 37 °C in quiescent conditions. Data are presented as mean ± SEM. The graphs show that NUCBody potently inhibits TTR aggregation.

Figure 9 shows the effect of NUCBody on the aggregation kinetics of Aβ42 and transthyretin (TTR) imaged by electron microscopy (EM) and immunoEM. At the end of the ThT assay, A) Aβ42 2.5 μM and B) TTR 10 μM incubated alone or in presence of NUCBody 5 μM were collected and used for imaging. For the EM negative staining experiment (top panels), the samples were spotted on negatively charged carbon film grids, stained with 1% uranyl acetate. For the immunoEM staining (bottom panels), the samples were incubated in solution with a mouse anti-Aβ42 or a mouse anti-TTR antibody, plated on the grids and successively incubated with an anti-mouse secondary antibody conjugated with a 6nm gold particle (white arrowhead) and an anti-human secondary antibody conjugated with a 12nm gold particle (black arrow), and stained with 1% uranyl acetate. All grids were imaged with a JEOL JEM 1400 Plus Transmission Electron Microscope. Scale bars as indicated. The panels show abundant amyloid aggregates when the amyloid peptides are incubated alone, and their absence when the protein is incubated together with NUCBody. Furthermore, the immunoEM panels show that NUCBody co-localizes on short aggregates of Aβ42 (A, bottom panel) and TTR (B, bottom panel).

Figure 10 shows the thermal unfolding temperature of NUCBody measured by Tycho, and compared to the control antibody 1D4 and the NUCB1 protein.

Figure 11 shows the NUCBody target engagement in pancreatic tissue harvested from a T2DM mouse model. Homozygous hIAPP transgenic mice (FVB/N-Tg (Ins2-IAPP) RHFSoel/J) express hIAPP under the regulatory control of the rat insulin II promoter and have been shown to spontaneously develop symptoms associated with T2D. Pancreas from transgenic or wildtype mice was harvested at 8 weeks of age and frozen in OCT. Histological sections (5 μm) were prepared and stored at -80C until use. Prepared slides were washed with Tris-buffered saline (TBS)/0.1% Tween-20, then blocked with TBS/0.1% Tween 20/5% normal goat serum for 3 hours at room temperature. Pancreatic sections from transgenic and wildtype animals were stained with NUCBody and the nuclear dye DAPI, and subsequently imaged (scale bar indicated).

DETAILED DESCRIPTION

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains.

Unless specified to the contrary, it is intended that every maximum numerical limitation given throughout this description includes every lower numerical limitation, as if

such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

The disclosure includes all amino acid sequences described herein, and all polynucleotides encoding the proteins.

The present disclosure relates in part to fusion proteins that comprise an amino acid sequence of a Nucleobindin-1 (NUCB1), which is a chaperone-like amyloid binding protein (CLABP) with pan-amyloid binding activity. In the present disclosure, we demonstrate a method for making and using certain NUCB1-derived proteins which may be fused to an antibody heavy chain to create Ig-fusion proteins. "Fusion protein" means a single, contiguous polypeptide that comprises at least two amino acid sequences from at least two distinct proteins. Fusion proteins of this disclosure are referred to herein from time to time as a "NUCBody" in the singular and "NUCBodies" in the plural. A representative NUCBody as further described below shows novel and useful activity as an antibody-like, pan-amyloid binding protein with both diagnostic and therapeutic potential. The NUCBody activity has been confirmed using aggregation assays with several amyloid peptides, such as the AD-associated Aβ42, the T2DM-related hIAPP, the Parkinson's disease-associated α-Synuclein (α-Syn) and the SSA-associated TTR. The NUCBody efficiently and potently prevents aggregation of these peptides, as further demonstrated by the following description, and the figures of this disclosure.

In more detail, we recently characterized a novel chaperone-like amyloid binding protein (CLABP) – nucleobindin-1 (NUCB1) – and its ability to interact with age-related amyloid proteins (Gupta R, Kapoor N, Raleigh DP, Sakmar TP. Nucleobindin 1 Caps Human Islet Amyloid Polypeptide Protofibrils to Prevent Amyloid Fibril Formation. Journal of molecular biology. 2012, PMID: 22542527, PMC3398247, the disclosure of which is incorporated herein in its entirety; Bonito-Oliva A, Barbash S, TP, Graham WV. Nucleobindin 1 binds to multiple types of prefibrillar amyloid and inhibits fibrillization. Sci Rep. 2017;7:42880, PMID: 28220836, PMC5318909, the disclosure of which is incorporated herein in its entirety).

NUCB1 appears to be a pan-amyloid, protofibril binding protein, meaning that NUCB1 interacts primarily with soluble protofibril intermediates of at least several different amyloidogenic proteins and peptides that have been tested so far. NUCB1, also known as Calnuc, is a ubiquitously expressed multi-domain secreted DNA binding protein initially cloned in a lupus-prone mouse derived cell line and is highly expressed in humans and other species.

We engineered a soluble variant of the protein (sNUCB1) and characterized its biophysical and biochemical properties (Gupta R, Kapoor N, Raleigh DP, Sakmar TP. Nucleobindin 1 Caps Human Islet Amyloid Polypeptide Protofibrils to Prevent Amyloid Fibril Formation. Journal of molecular biology. 2012, PMID: 22542527, PMC3398247). sNUCB1 contains a signal sequence, a DNA binding motif, a leucine zipper motif, and two EF-hand motifs. The N-terminal signal sequence of NUCB1 targets it to different membrane compartments and its deletion renders sNUCB1 cytosolic. The leucine zipper domain (a.a. residues 347–389) has been postulated to induce NUCB1 dimerization. The EF-hand motifs at the core of the protein sequence comprise an intervening acidic region (a.a. residues 253-316) and seem to be responsible for the calcium binding activity. The sNUCB1 dimer, in the presence of calcium, collapses in radius of gyration as determined by SAXS analysis. The C-terminal region following the leucine zipper domain is predicted to be intrinsically disordered and unstructured. Intriguingly, NUCB1 is strongly conserved from flies to humans 26 and is widely distributed among Golgi (Miura K, Kurosawa Y, Kanai Y. Calcium-binding activity of nucleobindin mediated by an EF hand moiety. Biochem Biophys Res Commun. 1994;199(3):1388-1393, PMID: 8147883) , nucleus, endoplasmic reticulum, and cytoplasm. We have previously shown that sNUCB1 inhibits G protein activation and that Ca2+ binding regulates its interaction with Gαi1 (Gupta R, Kapoor N, Raleigh DP, Sakmar TP. Nucleobindin 1 Caps Human Islet Amyloid Polypeptide Protofibrils to Prevent Amyloid Fibril Formation. Journal of molecular biology. 2012, PMID: 22542527, PMC3398247).

In the Golgi, NUCB1 modulates Ca2+ homeostasis and negatively regulates the unfolded protein response through inhibition of site-1 protease (S1P)-mediated cleavage of ATF6. Up-regulation of the NUCB1 gene has been found in animal models of Lupus and protein levels have been found to be reduced by an average of 50% compared with controls in post-mortem brains of AD patients. NUCB1 interacts with Alzheimer's amyloid precursor protein (APP) in a Ca2+-sensitive manner, and its in vitro over-expression reduces APP levels. Given the dysregulated Ca2+ homeostasis in AD, PD, Huntington disease, Familial

Amyloidosis syndromes and Amyotrophic Lateral Sclerosis, the Ca2+- dependent effect of NUCB1 is particularly interesting.

We have shown that sNUCB1 binds in vitro to protofibrils originating from the T2DM-associated hIAPP amyloid protein, inhibits protofibril cytotoxicity, and prevents its further aggregation into the fibril state (Gupta R, Kapoor N, Raleigh DP, Sakmar TP. Nucleobindin 1 Caps Human Islet Amyloid Polypeptide Protofibrils to Prevent Amyloid Fibril Formation. Journal of molecular biology. 2012, PMID: 22542527, PMC3398247). However, the presence of Ca2+ in these assays prevented the effects, thereby making sNUCB1 an unstable tool for use in in vivo models. We therefore engineered a Ca2+-free mutant variant of sNUCB1 (mtNUCB1) containing the point mutations D253K, E264A, D305K, and E316A. mtNUCB1 is unable to bind Ca2+ while retaining its ability to inhibit hIAPP aggregation (Bonito-Oliva A, Barbash S, TP, Graham WV. Nucleobindin 1 binds to multiple types of prefibrillar amyloid and inhibits fibrillization. Sci Rep. 2017;7:42880, PMID: 28220836, PMC5318909, the disclosure of which is incorporated herein by reference). We employed atomic force microscopy (AFM) techniques to characterize mtNUCB1 and confirmed the presence of both monomers and dimers, with a randomly disordered phenotype with broad volume and morphology distribution.

Because sNUCB1 and mtNUCB1 affects hIAPP aggregation through a mechanism of protofibril stabilization, we tested the hypothesis that the effect might be universal for the protofibril conformation. We evaluated mtNUCB1 effect on Aβ42 protofibrils and observed that the binding occurs with a slow dissociation rate, as measured by Surface plasmon resonance (SPR). Furthermore, Immuno-electron microscopy (ImmunoEM) studies revealed that mtNUCB1 localizes to the ends of Aβ42 protofibrils. Without intending to be bound by any particular theory, it is considered that, similar to its effect on hIAPP, mtNUCB1 “caps” the growing ends of Aβ42 protofibrils, thereby preventing maturation to the fibril state. Only 20 CLABPs have been described in the literature to date. Most of them, including DNAJB6 and BRICHOS, bind exclusively to a single amyloid protein. Specifically, DNAJB6, pro-SP-C Brichos, Bri2-Brichos, and αB-crystallin are CLABPs that bind and disrupt the aggregation of aggregates but only those aggregates derived from Aβ42 (Arosio, P., Michaels, T., Linse, S. et al. Kinetic analysis reveals the diversity of microscopic mechanisms through which molecular chaperones suppress amyloid formation. Nat Commun 7, 10948 (2016) doi:10.1038/ncomms10948). Furthermore, these proteins have been described as affecting amyloid aggregation kinetics, however no other CLABP has been demonstrated to stabilize a protofibril intermediate as has been described for mtNUCB1. mtNUCB1 is a novel and

unique CLABP because it binds to more than one type of amyloid protofibril – including, but not limited to, the age-related amyloid derived from hIAPP, Aβ42, α-Synuclein, and mutant TTR, and prevents their aggregation through a similar "capping" mechanisms. Therefore, it is considered that mtNUCB1 is a pan-amyloid, protofibril capping protein (Bonito-Oliva A, Barbash S, TP, Graham WV. Nucleobindin 1 binds to multiple types of prefibrillar amyloid and inhibits fibrillization. Sci Rep. 2017;7:42880, PMID: 28220836, PMC5318909).

As discussed above, the NUCBodies of this disclosure comprise chimeric antibody-like molecules that have NUCB1 as described herein substituted for Fab domain of antibodies. The engineering of NUCB1 into the Ig-fusion protein NUCBody serves multiples scopes. First, while NUCB1 production was limited by bacteria expression, the Ig heavy chain element allows protein expression into multiple cell lines and facilitates protein purification. Moreover, the presence of the Ig heavy chain makes NUCBody easy to detect with appropriate anti-Ig heavy chain secondary antibodies and therefore suitable for diagnostic purposes *in vitro*, *ex vivo* and eventually *in vivo*. Furthermore, the Ig heavy chain provides the novel protein with the ability of being recognized Fc receptors which are present on the membrane of certain immune cells including B lymphocytes, natural killer cells, macrophages, neutrophils, and mast cells. Because NUCBody has high affinity to soluble amyloid aggregates and forms a stable complex with amyloid protofibrils, the presence of the Ig heavy chain allows detection and clearance by immune cells thus providing an effective therapeutic mechanism.

The disclosure thus relates to NUCB1 and variants thereof. In embodiments, the NUCB1 comprises an amino acid sequence described in U.S. Patent No. 8,703,707, the entire disclosure of which is incorporated herein by reference.

In a non-limiting embodiment, the NUCB1 component of the fusion proteins which further comprise an Ig component comprises or consist of a sequence that is at least 90% identical across its entire length to the following sequence:

PNKEETPATESPDTGLYYHRYLQEVIDVLETDGHFREKLQAANAEDIKSGKLSRELDF
VSHHVRTKLDELKRQEVSRLRMLLKAKMDAEQDPNVQVDHLNLLKQFEHLDPQNQ
HTFEARDLELLIQTATRDLAQYDAAHHEEFKRYEMLKEHERRRYLESLGEEQRKEAE
RKLEEQQRRHREHPKVNVPGSQAQLKEVWEELDGLDPNRFNPKTFFILHKINSDGVL
DEQALEALFTKELEKVYDPKNEEDDMREMEEERLRMREHVMKNVKTNQDRLVTLE
AFLASTQRKEFGDTGEGWETVEMHPAYTEEELRRFEEELAAREAELNAKAQRLSQE
TEALGRSQGRLEAQKRELQQAVLHMEQRKQQQQQQQGHKAPAAHPEGQLKFHPDT
DDVPVPAPAGDQKEVDTSEKKLLERLPEVEVPQ (SEQ ID NO:1).

To determine the percent identity of two amino acid sequences, the sequences are aligned and amino acids at corresponding positions are then compared. When a position in the first sequence is occupied by the same amino acid as the corresponding position in the second sequence, then the polypeptides are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=# of identical positions/total # of positions x100). Accordingly, Ig fusion proteins of this disclosure may comprise various amino acid mutations, relative to the express sequences described herein. In embodiments, at least one amino acid is substituted for another amino acid, or one or more amino acids are deleted, including in the NUCB1 component and/or the Ig component of the fusion proteins described herein. Amino acid insertions are also included. Substitution mutations can be made to change an amino acid in the resulting protein in a non-conservative or in a conservative manner. The disclosure includes sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein. Thus, amino acid changes can be made to replace or substitute one or more, one or a few, one or several, one to five, one to ten, or such other number of amino acids in the sequence of the Ig fusion proteins provided herein to generate mutants or variants thereof. Mutants or variants having a change in sequence can be tested using the assays and methods described and exemplified herein, including in the examples.

The NUCB1 component may further comprise additional elements, such as a leader sequence to promote secretion of the Ig fusion protein. Many suitable secretion sequences are known in the art, one non-limiting example of which comprises an interleukin-2 (IL-2) secretion signal. The secretion sequence may be cleavable and/or cleaved during processing of the protein. Thus, Ig fusion proteins of this disclosure may also comprise any suitable cleavage sequence, such as any of a variety of amino acid sequences that are recognized by any suitable protease, so that the leader sequence is appropriately cleaved from the fusion protein, such as prior to secretion.

Thus, in embodiments, in fusion proteins of this disclosure, the NUCB1 domain is present in a fusion protein that comprises at least one Ig fragment crystallizable regions (Fc region). In some implementations the Fc region can be of any Ig isotope. In embodiments, the Ig component comprises an IgG Fc region that is an IgG_1, IgG_2, IgG_3, or IgG_4 isotype. NUCBodies may have a portion of a Fab region, or they may be free from Fab segments, and in embodiments can be completely devoid of any portion of an Fab segment. Further, as is known in the art, the H chain constant domain is considered to comprise CH1-CH2-CH3 for

IgG, as well as for IgA, IgD, and there is a CH4 domain for IgM and IgE. The CH1 domain is located within the F(ab) region, but the other CH domains (CH2-CH3 or CH2-CH4) comprise the Fc fragment. In certain embodiments, NUCBodies of this disclosure comprise an Fc region, and may comprise a CH1 segment of the Fc H chain. In certain embodiments, including CH1 segment provides an additional linker that is relatively stable to proteolysis and thus increases the reach of the NUCBody.

In certain embodiments the Fc region comprises a constant region only, or at least the CH2 and CH3 domains of an IgG heavy chain, and may comprise the hinge region. In an embodiment, the hinge may act as a flexible spacer, and further facilitates formation of disulfide bridges. The disclosure comprises single chain polypeptides, and distinct polypeptides that are covalently linked to one another, such as by a disulfide bond. The NUCBodies may therefore comprise or consist of one or two polypeptide chains, or more polypeptide chains. In embodiments the Fc segment(s)comprise one or more amino acids that have been altered relative to the naturally occurring Fc amino acid sequences, so long as the function of the NUCBody remains adequate for its intended purpose, as described herein. In embodiments, a fusion protein of this disclosure comprises only an Ig heavy chain as the Ig component.

The Fc region of the NUCBodies of this disclosure can comprise or consist of an amino acid sequence that is identical to an Fc region produced by a mammal, such as a human. In various embodiments, the Fc region can thus have between 80% - 100% (inclusive, and including all numbers there between) amino acid sequence similarity to an Fc region produced by a human.

In a non-limiting embodiment, the Ig sequence is a human Ig sequence that comprises or consists of an amino acid sequence that is at least 90% identical across its entire length to the following sequence:

HDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:2)

In a non-limiting embodiment, the disclosure provides a contiguous polypeptide comprising at least an NUCB1 domain and an Ig domain, and comprises or consists of an amino acid sequence that is at least 90% identical across its entire length to the following sequence:

*MYRMQLLSCIALSLALVTNS*ISAMVT**PNKEETPATESPDTGLYYHRYLQEVIDVLE TDGHFREKLQAANAEDIKSGKLSRELDFVSHHVRTKLDELKRQEVSRLRMLLK AKMDAEQDPNVQVDHLNLLKQFEHLDPQNQHTFEARDLELLIQTATRDLAQYD AAHHEEFKRYEMLKEHERRRYLESLGEEQRKEAERKLEEQQRRHREHPKVNV PGSQAQLKEVWEELDGLDPNRFNPKTFFILHKINSDGVLDEQALEALFTKELEK VYDPKNEEDDMREMEEERLRMREHVMKNVKTNQDRLVTLEAFLASTQRKEFG DTGEGWETVEMHPAYTEEELRRFEEELAAREAELNAKAQRLSQETEALGRSQG RLEAQKRELQQAVLHMEQRKQQQQQQQGHKAPAAHPEGQLKFHPDTDDVPV PAPAGDQKEVDTSEKKLLERLPEVEVPQ***HDKTHTCPPCPAPELLGGPSVFLFPPKPK** *DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK* (NUCB1-4x-hIgG1-FC2) (SEQ ID NO:3).

This representative sequence (Figure 2) comprises the following components: An IL-2 signal sequence (bold and italics) that is cleaved during protein processing and secretion. The NUCB1 protein sequence is depicted in bold and the human IgG1 FC domain is depicted in in non-bolded italics at the N-terminus. (SEQ ID NO:3). Thus, fusion proteins of the present disclosure may comprise SEQ ID NO:3, but without the secretory signal, e.g., amino acids 1-26, inclusive, and including all numbers and ranges of numbers there between, of SEQ ID NO:3 may be omitted. In embodiments, amino acids 1-20 may be omitted from the fusion proteins of this disclosure. In embodiments, the ISAMVT (SEQ ID NO:4) segment can omitted, or substituted with another linker sequence.

NUCBodies of this disclosure can comprise one or more linkers that connect segments of a single fusion protein. The term "linker" thus refers to a chemical moiety that connects one segment of a polypeptide to another segment of the same polypeptide, or to another polypeptide, or to another agent. Linkers include amino acids, but other linkers are encompassed as well. Generally speaking, amino acid linkers may be principally composed of relatively small, neutral amino acids, such as Glycine, Serine, and Alanine, and can include multiple copies of a sequence enriched in Glycine and Serine. In embodiments the linker has a coiled-coil topology. The coiled-coil topology can be an extended coiled-coil comprised by, for example, a two-stranded α-helical coiled coil segment. Multiple copies of the same or distinct linkers can be used in a single fusion protein, and may be connected in series or separated from one another.

The orientation of the Ig Fc(s) and the NUCBody domain(s) is not limited to a single configuration. The disclosure accordingly encompasses Fc segments that are either at or near the N-terminus or at or the C-terminus of the polypeptide, a vice versa with respect to the NUCB1 domain.

NUCBodies of this disclosure can be modified to improve certain biological properties, e.g., to improve stability, and/or to enhance certain capabilities. Other modifications may involve alteration of a glycosylation pattern, including deletions of one or more glycosylation sites, or addition of one or more glycosylation sites. NUCBodies can be expressed in engineered cell lines with altered glycosylation pathways to result in increased or decreased effector functions NUCBodies may be provided in a composition, in a complex, or covalent linkage with other moieties, including but not necessarily limited to effector molecules such as cytokines. NUCBodies can be conjugated to other agents for other numerous purposes, such as diagnostic applications. NUCBodies can accordingly be modified to be conjugated to detectable labels, including but not limited to visually detectable labels, such as compounds that can fluoresce or emit other detectable signals, such as radiolabels, and to particles for use in separating binding partners, such particles including but not limited to various substrates, including beads made of any material, including but not limited to glass, polymers, and metals, including magnetic beads.

NUCBodies of this disclosure can be made by adapting conventional molecular biology approaches. For example, DNA sequences encoding any NUCBody can be constructed based on the coding sequence of a NUCBody and any coding sequence of a suitable Ig Fc domain. Thus, the DNA sequences comprise a sequence encoding a fusion protein that contains the NUCBody and the Fc as a contiguous polypeptide. The resulting DNAs can be placed into any suitable expression vector. The expression vector can include any additional features that may or may not be part of the encoded fusion proteins, such as any suitable promoter, restriction enzyme recognition sites, selectable markers, detectable markers, origins of replication, etc. The vectors can encode leader sequences, purification tags, and hinge segments that separate two or more other segments of the encoded protein. In embodiments, at least one hinge segment separates a NUCBody domain from an Fc region. In an embodiment, a tag sequence, such as purification of the protein, such as a poly-Histidine tag, can be included in a fusion protein of this disclosure.

The expression vectors can be introduced into any suitable host cells, which can be prokaryotic and eukaryotic cells, including but not limited to *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, human embryonic kidney 293 cells, or any other

suitable cell types. The NUCBodies can be expressed and separated from cell cultures that produce them using any suitable reagents and approaches, including but not necessarily limited to protein purification methods that use purification tags, including but not limited to histidine tags, and separating the NUCBodies using such tags. Thus, the disclosure includes isolated polynucleotides encoding the NUCBodies of this disclosure, cloning intermediates used to make such polynucleotides, expression vectors comprising the polynucleotides that encode the NUCBodies, cells and cell cultures that comprise the DNA polynucleotides, cells and cell cultures that express the NUCBodies, their progeny, cell culture media and cell lysates that contain the NUCBodies, NUCBodies that are separated from the cells and are optionally purified to any desirable degree of purity, and compositions comprising one or more NUCBodies.

In certain embodiments NUCBodies of this disclosure are provided as components of compositions that comprise a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" as used herein refers to a substantially non-toxic carrier for administration of pharmaceuticals in which the compound will remain stable and bioavailable. Combining a pharmaceutically acceptable carrier in a composition with a NUCBody yields "pharmaceutical compositions." Some suitable examples of pharmaceutically acceptable carriers, as well as excipients and stabilizers can be found in Remington: The Science and Practice of Pharmacy (2005) 21st Edition, Philadelphia, PA. Lippincott Williams & Wilkins.

In an aspect of the disclosure, a composition comprising NUCBodies is administered to an individual in need thereof. The individual can be diagnosed with, suspected of having, or be at risk for any disorder that is positively correlated with the presence of Aβ and/or amyloid fibrils, including but not limited to amyloid protofibrils. The individual may be in need of treatment for any disorder that is correlated with the presence of proteins that include are but not necessarily limited to the age-related amyloid derived from hIAPP, Aβ42, α-Synuclein, and mutant TTR. In embodiments the disorder is Alzheimer's disease, Parkinson's disease, Huntington disease, Familial Amyloidosis syndrome, Amyotrophic Lateral Sclerosis, or is a post-concussion syndrome, or Chronic Traumatic Encephalopathy (CTE), Lewy body dementia, inclusion body myositis, and/or a disorder associated with Aβ aggregates that may coat cerebral blood vessels in, for example, cerebral amyloid angiopathy, periphery type 2 diabetes mellitus (T2DM), Senile Systemic Amyloidosis (SSA) and Light-chain Amyloidosis (AL).

Methods for administering compositions comprise parenteral, intraperitoneal, intrapulmonary, oral, and topical administrations. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, and subcutaneous administration. Intracranial and intra-CNS injections are also included.

The amount of the NUCBodies and any other active agent to be included in a composition and/or to be used in the method can be determined by those skilled in the art, given the benefit of the present disclosure. Thus, in one embodiment, an effective amount of a composition of the invention is administered. An effective amount can be an amount that that alleviates disease symptoms associated amyloid beta, including but not limited to amyloid fibrils, including but not limited to the aforementioned hIAPP, Aβ42, α-Synuclein, and mutant TTR.

An effective amount can vary depending on pharmaceutical formulation methods, administration methods, the patient's age, body weight, sex, diet, administration time, administration route, and other factors that will be apparent to those skilled in the art. Compositions can be administered once, or over a series of administrations. Those skilled in the art will be able to determine or predict the half-life of any particular NUCBody, which can affect administration. In embodiments, the disclosure includes a single dose, or several doses. In embodiments, an amount of NUCBody from 1 microgram/kg to 1000 milligrams/kg, or higher amounts, are administered as necessary.

In certain embodiments a method of the disclosure is implemented using an expression vector, such as a plasmid encoding a suitable NUCBody to form a type of DNA vaccine. For example, a composition comprising such an expression vector can be administered instead of, or in addition to, the NUCBodies themselves. The expression vector would facilitate expression, correct folding, glycosylation and secretion when introduced into mammalian cells in an individual. In an embodiment, cells modified to express a NUCBody are introduced into a an individual in need thereof.

In embodiments the disclosure comprises NUCBodies that have been reversibly or irreversibly attached to a substrate, and thus may be used in a variety of assays, and kits. The NUCBodies that have been reversibly or irreversibly attached to a substrate may be in physical association with one or more other proteins, such as Aβ. The substrate may be a component of a diagnostic device. Thus, in certain aspects the invention provides for detecting the presence or absence amyloid proteins using any of a variety of approaches for detecting proteins that include NUCBodies as detection agents, such as immunodetection

methods, including but not limited to Western blotting, multi-well assay plates adapted for detection of proteins, beads adapted for detection of proteins, a lateral flow device or strip that is adapted for detection of proteins, ELISA assays, or any other modification of an immunodetection or other assay type that is suitable for detecting proteins. Those skilled in the art will recognize that, given the benefit of the present disclosure, these and other detection methods can include use of one or more NUCBodies as binding partners in diagnostic detection assays. In various embodiments, one or more NUCBodies partners can be reversibly or irreversibly attached to a substrate, such as by being covalently, ionically, or physically bound to a solid-phase immunoadsorbent using methods such as covalent bonding via an amide or ester linkage, ionic attraction, or by adsorption. The substrate can be any suitable substrate onto which a NUCBody can be attached. Examples include substrates typically used in immunodetection assays, lateral flow devices, bead-based assays, microfluidic devices, etc. Thus, the solid substrate can be a porous solid substrate that allows the flow of liquid through the substrate. The liquid can flow through the porous substrate via any suitable means, such as by capillary action, microfluidics, etc. The substrate can also be a non-porous solid substrate, such as beads formed from glass or other non-porous materials. The immune assay can include any form of direct detection, or any form of ELISA assay.

In one aspect, the present disclosure comprises obtaining and testing any suitable biological sample from an individual who is suspected of having or is at risk for developing a disease or other condition associated with amyloid beta, as described herein. In embodiments, the individual from whom the sample is obtained is more than 50 years old. In embodiments, the sample comprises whole blood or plasma. In embodiments, the sample comprises a solid tissue, such as a biopsy or other section of a tissue or organ. The biological sample can be used directly, or it can be subjected to a processing step before being tested. In embodiments, the individual has been diagnosed with, is suspected of having, or is at risk for developing AD, Lewy body dementia, inclusion body myositis, and/or Ab aggregates that may coat cerebral blood vessels in, for example, cerebral amyloid angiopathy.

The amount of Aβ or any other amyloid protein described herein detected can be compared to any suitable reference, examples of which include but are not limited to samples obtained from confirmed AD patient plasma, or non-demented control plasma, or a standardized curve(s), and/or experimentally designed controls such as a known amyloid protein amount used to normalize experimental data for qualitative or quantitative determination, or a cutoff value, and to normalize for mass, molarity, concentration and the

like. The reference level may also be depicted as an area on a graph. In certain embodiments, determining amyloid protein as described herein facilitates staging the degree and/or severity of AD, and/or can be used to monitor the progress of an AD therapeutic approach, including but not necessarily limited to medicinal, nutritional and behavioral AD therapies designed to improve cognitive function or to slow its deterioration.

In embodiments, fusion proteins of this disclosure bind to and/or inhibit the formation of proteins involved in a protein aggregation process. In embodiments, fusion proteins of this disclosure bind to proteins that are known to comprise intermediate Aβ morphologies, such as protofibrils. The structure and composition of protofibrils of the type associated with, for example, AD, are well known in the art.

The present disclosure also provides articles of manufacture, including but not necessarily limited to kits. In embodiments, the articles of manufacture contain one or more NUCBodies provided in one or more sealed containers, one non-limiting example of which is a sealable glass or plastic vial. The antibodies or antigen binding fragments may be unlabeled, or detectably labeled. The articles of manufacture can include any suitable packaging material, such as a box or envelope or tube to hold the containers. The packaging can include printed material, such as on the packaging or containers themselves, or on a label, or on a paper insert. The printed material can provide a description of using the NUCBodies in an assay described herein for the purpose of diagnosing or aiding in the diagnosis of a disease, or for determining the amounts of Ab in a sample. The articles can also include, for example, reagents for performing an immunodetection assay. Non-limiting examples of such reagents include one or more buffers, such as buffers that are suitable for diluting plasma, and/or for performing steps of an immunodetection assay. In embodiments, the article comprises a kit which includes a buffer for diluting human plasma, such as a buffered saline solution, one example of which is phosphate buffered saline (PBS). The kit may also include a blocking buffer, such as PBS+0.1% Tween-20+1% BSA+0.02% NaN3, and may further comprise a wash buffer, such as Phosphate Buffered Saline Tween-20 (PBS-T), and a reaction stop solution, such as an acid solution, and any suitable diluent solutions for performing an immunodetection assay. Antibody detection reagents may also be included, including but not necessarily limited to enzymes, enzyme substrates, and various conjugates thereof, for producing detectable signal, all of which are well known to those skilled in the art and include but are not necessarily limited to avidin, streptavidin, biotin, phosphatases, peroxidases, fluorescein, such as FITC, and fluorogenic sensors, etc. may be included.

Isotype antibody controls can also be included. In embodiments, article of manufacture includes printed information providing information on how to use the diagnostic components of the kit.

In embodiments, a result based on a determination of amyloid protein(s) can be fixed in a tangible medium of expression, such as a digital file saved on a portable memory device, or on a hard drive. The determination can be communicated to a health care provider for aiding in the diagnosis of a disease such as AD, or for monitoring or modifying a therapeutic or prophylactic approach aimed at reducing the severity or symptoms of a disease, such as AD. In embodiments the disclosure comprises providing a diagnosis of AD and subsequently administering a drug to the individual to alleviate one or more sign or symptom of AD. In embodiments, the disclosure comprises selecting a patient to receive a drug intended to alleviate AD based on the result of an assay described herein, and/or administering to the individual such a drug based on receiving the result of such as assay.

EXAMPLES

The following Examples are intended to illustrate but not limit the disclosure.

As discussed above, the present disclosure makes use of a novel protein with unique qualities in order to 1) determine the key characteristics that yield efficient amyloid protofibril binding, 2) define those molecular interactions involving pan-amyloid binding activities, and 3) engineer an Ig-fusion protein to demonstrate a therapeutic strategy targeting age-related amyloidosis.

EXAMPLE 1

With NUCB1 identified as the functional pan-amyloid, protofibril capping unit, structural interaction with amyloid protofibrils are analyzed. Aβ is, perhaps, the best studied of the age-related amyloids regarding its structural properties. The disclosure includes structural studies using Aβ as a model system. In order to characterize the NUCB1 mutant proteins, a series of standard biophysical assays to determine its native parameters is performed. We have previously shown in a series of SEC experiments that sNUCB1 gradually increases in average molecular weight with increasing concentrations of the protein (Gupta R, Kapoor N, Raleigh DP, Sakmar TP. Nucleobindin 1 Caps Human Islet Amyloid Polypeptide Protofibrils to Prevent Amyloid Fibril Formation. Journal of molecular biology. 2012, PMID: 22542527, PMC3398247). Therefore, whether the sNUCB1 mutant forms a dimer can be determined using the thermodynamics of the dimerization using SEC-multi-

angle light scattering (MALS) analysis, or other suitable approaches. Interestingly, the far-UV CD spectrum of sNUCB1 shows a smooth structural transition from an α-helical secondary structure at lower concentrations to a predominant β-sheet structure at higher concentrations. Because it is expected that the mutant sNUCB1 protein is intrinsically disordered, SAXS and solution nuclear magnetic resonance (NMR) can be used as an effective combination to analyze the structure. Furthermore, this approach can be used to determine structural elements of the biomolecular complex between the mutant sNUCB1 protein and Aβ. Isotopically labelling either mutant sNUCB1 or Aβ can be performed. From this approach, molecular interactions between the mutant sNUCB1 protein and Aβ protofibrils can be determined. Our earlier attempts to crystallize sNUCB1 or a complex of sNUCB1 with Gαi1 have remained unsuccessful, most likely due to its intrinsically disordered elements. However, crystallization trials can be carried out with truncated mutant versions of NUCB1 that retain functional activity, provided that their intrinsic flexibility is decreased. Based on the molecular interactions determined using structural techniques, alanine scanning in combination with SPR can be performed and the results compared with the slow off rate showed by sNUCB1-Aβ protofibrils binding. Mutant NUCB1 protein with alanine substitutions rationally selected are encompassed by the disclosure.

EXAMPLE 2

In embodiments, the disclosure comprises a mtNUCB1 functional unit that replicates the whole protein activity. We previously engineered mutants of NUCB1, the soluble sNUCB1 and the Ca2+-free mtNUCB1 (containing the point mutations D253K, E264A, D305K, and E316A, and S44C, and confirmed that these changes did not affect pan-amyloid, protofibril capping activity. The disclosure includes the mtNUCB1 mutant that, together with the sNUCB1 protein, are expressed recombinantly and purified using affinity chromatography. After removing the affinity tag, the engineered proteins can be further purified by size-exclusion chromatography (SEC) and the proper proteins folding may be confirmed by Circular dichroism (CD). To evaluate if the recombinant mtNUCB1 mutant retains the inhibition effect on protofibrils, the disclosure includes coincubating mtNUCB1 described herein with Aβ42, hIAPP, α-Synuclein, and TTR and monitoring the kinetics of fibril growth with a conventional thioflavin-T (ThT) fluorescence binding assay. We showed that mtNUCB1 prevents aggregation of Aβ42, hIAPP, α-Synuclein, and TTR at sub-stoichiometric concentrations. Moreover, by using defined microscopic rate constants (Arosio P, Michaels TC, Linse S, et al. Kinetic analysis reveals the diversity of microscopic

mechanisms through which molecular chaperones suppress amyloid formation. Nat Commun. 2016;7:10948, PMID: 27009901; Cohen SI, Linse S, Luheshi LM, et al. Proliferation of amyloid-beta42 aggregates occurs through a secondary nucleation mechanism. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(24):9758-9763, PMID: 23703910) we have characterized the inhibitory effect of mtNUCB1 on Aβ aggregation and showed that mtNUCB1 inhibits elongation and secondary nucleation events. The present disclosure extends studies to the NUCB1 mutants and provides an understanding of the inhibitory mechanisms on the microscopic rate constants of amyloid peptide aggregation. Successively, the inhibition of amyloid aggregation exerted by the NUCB1 mutants is tested by transmission electron microscopy (TEM) to visualize the occurrence of fibril formation in their presence. We observed that mtNUCB1 prevents fibril formation of Aβ42, hIAPP, α-Synuclein, and TTR by binding to the protofibrils ends, hence causing an increase of protofibril species (Bonito-Oliva A, Barbash S, TP, Graham WV. Nucleobindin 1 binds to multiple types of prefibrillar amyloid and inhibits fibrillization. Sci Rep. 2017;7:42880, PMID: 28220836, PMC5318909). Similar studies may be used to show the NUCB1 mutants display a matching pattern of activity. Protofibril-specific binding activity of NUCB1 mutants can be confirmed by sandwich ELISA. Results of this disclosure indicate that mtNUCB1 and fusion proteins described herein are novel and efficient amyloid protofibril capture agents since, when coated on an ELISA plate, the agents specifically bind to amyloid protofibrils and not to the unstructured monomers. These data are in line with the present disclosure describing mNUCB1 as a pan-amyloid, protofibril capping protein.

EXAMPLE 3

Based on the unique NUCB1 properties and its occurrence as a CLABP, the disclosure includes using the identified NUCB1 protofibril and fusion proteins described herein as capping units as a proof-of-concept novel therapeutic. As discussed above, the components responsible for pan-amyloid protofibril capping comprise an engineered immunoglobulin (Ig)-fusion protein where the Fc region (CH2 and CH3 domains) is derived from either a human or mouse IgG heavy chain and hinge region. The hinge region serves as a flexible linker between the NUCB1 derived unit and the heavy chain, allowing each protein domain independent function. Each fusion protein can be expressed in a mammalian cell line and purified using standard techniques (Figure 3), as further described above. Resulting fusion proteins, termed NUCBodies as discussed above, are analyzed in biochemical assays to determine their molecular properties. As in EXAMPLE 1, the ability of NUCBodies to

inhibit fibril growth of Aβ42, hIAPP, α-Synuclein, and TTR in ThT kinetic assays can be performed, representative demonstrations of which are provided in the figures (Figures 5-8). The effect of NUCBodies on the microscopic rate constants of Aβ aggregation can be compared with those obtained using the mtNUCB1 and NUCB1 mutant. TEM, ImmunoEM and AFM studies can be used to determine the localization of NUCBodies on amyloid protofibrils, as illustrated in the figures of the present disclosure (Figure 9). This facilitates localization of the NUCBody in these assays because of the presence of the engineered heavy chain portion. NUCBodies may be used as the capture agent in both sandwich ELISAs and SPR to determine binding specificity and off rates of protofibrils from multiple amyloid sources. Finally, NUCBodies can be used to detect amyloid aggregates in situ as demonstrated by the detection of hIAPP protofibril aggregates in the pancreas of a transgenic model of amyloid-induced T2DM but not wildtype control pancreas (Figure 11).

In summary (Figure 1), the described invention extends our previous observations of the extraordinary properties of the naturally occurring CLABP, NUCB1. To date, this is the only CLABP that has pan-amyloid, protofibril capping activity. This proof-of concept molecule has potentially impactful uses as a tool for detecting protofibrils in situ, or ex vivo, and is expected to be useful as a biotherapeutic in models of age-related amyloidosis syndromes, and for treating the same in humans.

MATERIAL AND METHODS

Cloning and vector construction

A cDNA clone for human NUCB1 corresponding to residues 31–461 and point mutations D253K, E264A, D305K, and E316A was cloned into the pFuse-hIgG1-FC2 expression vector (Invivogen) in frame with both an IL2 signal sequence and human IgG1 heavy chain and the hinge region. The clone, termed NUCBody, was confirmed by DNA sequencing.

Cell culture and transfection

CHO-K1 cells were cultured in DMEM containing 10% FBS media using T-75 disposable culture flasks (Thermo Fisher Scientific). Transfections were performed using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The pFuse-hIgG1-FC2 parent vector contains a Zeocin resistance gene to allow for stable cell line selection. CHO-K1 cells transfected with NUCBody DNA were treated with 250μg/ml Zeocin to select a pool of stably transfected cells. NUCBody-CHO stable clones were isolated using limiting dilutions.

Heterologous expression and purification of NUCBody

The NUCBody-CHO cell line was cultured in DMEM containing 10% FBS media containing 250μg/ml Zeocin. Supernatants were collected and NUCBody was purified using a HiTrap Protein G HP column (GE Healthcare) with a running buffer of 20 mmol/L sodium phosphate, pH 7.0, and eluted with 100 mmol/L glycine-HCl, pH 2.7 into faction tubes containing 1 M Tris, pH 9.0. Antibody containing fractions were pooled, dialyzed with 1x DPBS (Life Technologies), concentrated with 30 kDa MWCO filters and stored in 1x DPBS, 10% glycerol, 0.02% NaN3. Purified NUCBody was separated by SDS–PAGE and stained with Coomassie Blue to confirm purity and size.

Peptides preparation

Aβ42 synthetic peptide (American Peptide) was solubilized in HFIP at 1 mg/ml, dried and stored at − 80 °C. On the day of the experiment, the peptide was reconstituted in 2 mM NaOH to 1 mg/ml, dried and diluted in 20 mM sodium phosphate buffer, pH 8.0. The hIAPP (Phoenix Pharmaceutics) was solubilized in HFIP at 1 mg/ml, dried and stored at − 80 °C. On the day of the experiment, the peptide was solubilized in 20 mM sodium phosphate buffer, pH 7.6. α-Synuclein (Bioneer) was solubilized in PBS; the transthyretin (TTR) V30M mutant (Arvys Proteins) was diluted in 10 mM sodium phosphate, pH 7.6, 100 mM KCl, 1 mM EDTA.

Atomic force microscopy

To image NUCBody and measure its size distribution, a 500 uL drop of phosphate buffered saline with Ca2+ and Mg2+ was added to freshly cleaved mica, and 1 uL of 16μM protein (final concentration of 30nM) was added to this drop before immediately washing with ddH2O and blowing dry with dry nitrogen gas. The sample was imaged in 1 x 1 um scans sampling at 512 x 512 pixels in tapping mode on an Asylum Research MFP-3D-BIO with Olympus AC240 probes. Height images were then exported into a custom written FIJI code that identified the mica substrate, selects particles above the surface, and calculates their pixel-by-pixel volume. Histograms of volume were then plotted to determine the size distribution of the imaged sample.

Thioflavin T assay

The effect of NUCBody on the aggregation kinetics of four different amyloid proteins was monitored through the Thioflavin T (ThT) fluorescent assay, as previously described (Bonito-Oliva et al. Scientific Reports, 2017). Aggregation of 2.5 μM Amyloid-β (Aβ42), 2.5 μM human islet polypeptide (hIAPP), 60 μM α-Synuclein (α-Syn) and 10 μM transthyretin (TTR) was monitored in presence of increasing concentrations of NUCBody (0, 0.5, 1, 2.5, 5

μM) and 10 μM ThT (Fisher Scientific). A volume of 50 μL per well (n = 4/group) was added to each well of a pre-chilled (4 °C) Corning 96 well half area black with clear flat bottom polystyrene with non-binding surface and covered with clear self-adhesive topseal. Aβ42, TTR and hIAPP aggregation were monitored for up to 24 h in quiescent conditions and a constant temperature of 37 °C (Aβ42, TTR) or 25 °C (hIAPP). α-Syn aggregation was monitored for 4 days in shaking conditions (333 rpm) and a constant temperature of 37 °C. Fluorescence measurements were performed on a Flexstation II (Molecular Devices) using an excitation wavelength of 450 nm and an emission wavelength of 485 nm. The obtained fluorescence measures were normalized to the relative fluorescence expressed after 30 min of incubation.

Transmission electron microscopy

The effect of NUCBody on the aggregation of Aβ42 and TTR was imaged by Transmission electron microscopy (EM) through negative staining as well as immunoEM experiments. For the EM negative staining experiment, 2.5 μM Aβ42 was incubated at 37 °C for 24 h together with 5 μM NUCBody or a negative control antibody. Subsequently, each sample was placed in a volume of 5 μl onto a negatively charged carbon film 200-mesh copper grids, rinsed with ddH2O and counterstained with 1% aqueous uranyl acetate solution. For the immunoEM experiment, the samples were incubated in solution with a mouse anti-Aβ42 (6E10, BioLegend) or a mouse anti-TTR antibody (LFMA0174 ThermoFisher) for 20 min at room temperature, plated on the grids in a volume of 5 μl, blocked with 3% BSA for 3 min. Subsequently, the grids were incubated with a 6 nm gold-conjugated secondary anti-mouse antibody (Jackson Laboratories, 1:20) and a 12 nm gold-conjugated secondary anti-human antibody (Jackson Laboratories, 1:20) for 20 min at room temperature and stained with 1% uranyl acetate. All grids were imaged with a JEOL JEM 1400 Plus Transmission Electron Microscope.

Thermal unfolding temperature

The stability of NUCBody was assessed by testing its thermal unfolding temperature by the TYCHO Nanotemper instrument. The IgG1 1D4 and NUCB1 were used as controls. The proteins were diluted to 0.6μg/μl and loaded into the glass capillaries. Three replicates were averaged.

Immunohistochemistry (IHC)

The *ex vivo* target engagement of NUCBody was tested in a T2DM mouse model. Homozygous hIAPP transgenic mice (FVB/N-Tg (Ins2-IAPP) RHFSoel/J) express hIAPP under the regulatory control of the rat insulin II promoter and have been shown to

spontaneously develop symptoms associated with T2D. Transgenic and wildtype mice were sacrificed at 8 weeks and pancreata were harvested and frozen in OCT. Histological sections (5 μm) were prepared and stored at -80C until use. Prepared slides were washed with Tris-buffered saline (TBS)/0.1% Tween-20, then blocked with TBS/0.1% Tween 20/5% normal goat serum for 3 hours at room temperature. Pancreatic sections from transgenic and wildtype animals were stained with NUCBody and the nuclear dye DAPI, and subsequently imaged.

While the disclosure has been described through specific embodiments, routine modifications will be apparent to those skilled in the art and such modifications are intended to be within the scope of the present disclosure.

We claim:

1. A fusion protein comprising a NUCB1 amino acid sequence and the amino acid sequence of an immunoglobulin (Ig) heavy chain.

2. The fusion protein of claim 1, wherein the NUCB1 amino acid sequence comprises a sequence that is at least 90% identical to the sequence of amino acids 27-452 of SEQ ID NO:3.

3. The fusion protein of claim 1, wherein the Ig heavy chain is a human Ig heavy chain.

4. The fusion protein of claim 1, wherein the amino acid of the Ig heavy chain comprises a sequence that is at least 90% identical to the sequence of amino acids 453-680 of SEQ ID NO:3.

5. The fusion protein of claim 1 comprising a sequence that is at least 90% identical to amino acids 27-680 of SEQ ID NO:3.

6. The fusion protein of claim 5, wherein the fusion protein comprises a sequence that is at least 90% identical to SEQ ID NO:3.

7. An expression vector encoding a fusion protein of any one of claims 1–6.

8 A method comprising expressing the expression vector of claim 7 in a population of cells such that the fusion protein is expressed by the cells, and separating the fusion protein from the cells.

9. A method for inhibiting aggregation of proteins, the method comprising contacting the proteins with a fusion protein of any one of claims 1–6, such that aggregation of the proteins is inhibited.

10. The method of claim 9, wherein inhibiting aggregation of the proteins comprises inhibiting formation of amyloid fibrils and/or binding of the fusion protein to amyloid protofibrils.

11. The method of claim 9, wherein inhibiting aggregation of the proteins comprises inhibiting aggregation of at least one protein that is islet amyloid polypeptide (IAPP), amyloid-β (Aβ), α-Synuclein, or Transthyretin (TTR).

12. The method of claim 11, wherein inhibiting aggregation of the proteins comprises inhibiting aggregation of Aβ42 or Aβ40, or a combination thereof.

13. The method of claim 12, wherein inhibiting aggregation of the proteins comprises inhibiting aggregation of the Aβ42.

14. The method of claim 9, wherein the contacting the proteins with the fusion protein comprises administering the fusion protein to an individual in need thereof.

15. The method of claim 14, wherein the individual in need thereof has been diagnosed with, is suspected of having, or is at risk for developing at least one of: age-related amyloidosis, Alzheimer's Disease, Parkinson's disease, Huntington disease, Familial Amyloidosis syndrome, Amyotrophic Lateral Sclerosis, post-concussion syndrome, Chronic Traumatic Encephalopathy, Lewy body dementia, inclusion body myositis, cerebral amyloid angiopathy, periphery type 2 diabetes mellitus, Senile Systemic Amyloidosis and Light-chain Amyloidosis.

16. A method comprising contacting a sample with a fusion protein of any one of claims 1–6, and detecting a complex comprising the fusion protein and a protein.

17. The method of claim 16, wherein the fusion proteins are labeled with a detectable label.

18. The method of claim 17, wherein the sample is obtained from a human individual who at risk for developing, is suspected of having, or has been diagnosed with a disorder associated with the presence of one or more amyloid proteins.

19. The method of claim 18, wherein the sample is obtained from a human individual who at risk for developing, is suspected of having, or has been diagnosed developing an age-related amyloidosis, and/or Alzheimer's Disease.

20. A pharmaceutical formulation comprising a fusion protein of any one of claims 1–6.

Figure 1

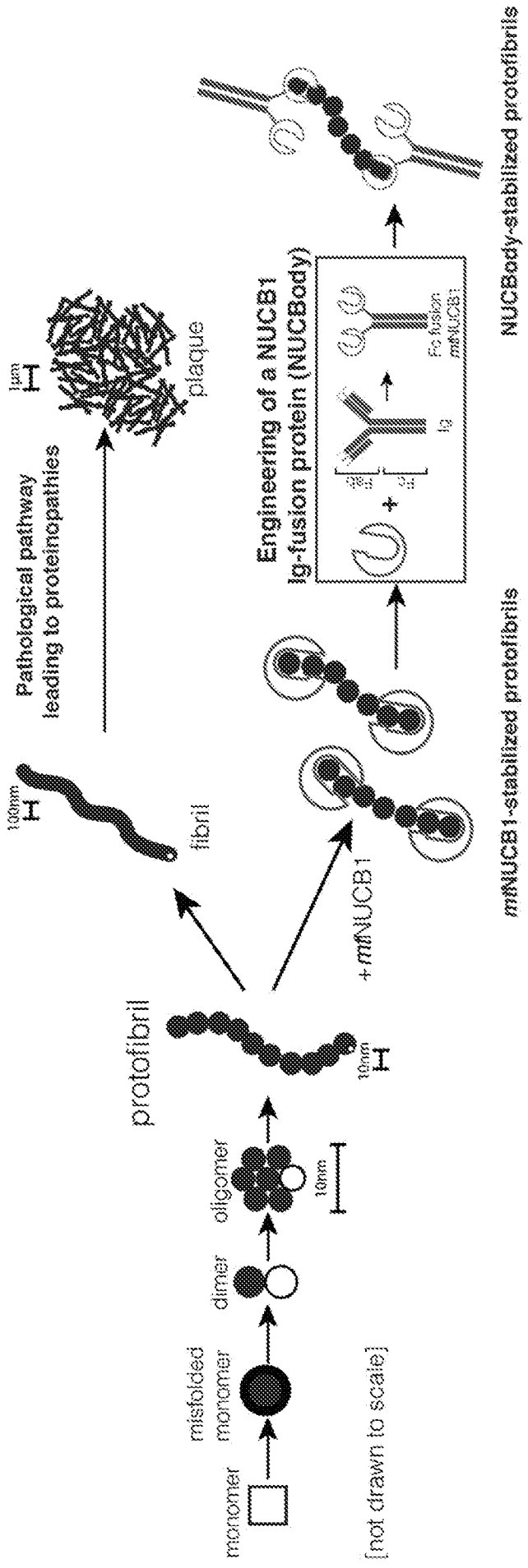

monomer
misfolded monomer
dimer
oligomer
10nm
protofibril
10nm
[not drawn to scale]
+ mfNUCB1
mfNUCB1-stabilized protofibrils
Engineering of a NUCB1 Ig-fusion protein (NUCBody)
Fc fusion mfNUCB1
Ig
NUCBody-stabilized protofibrils
Pathological pathway leading to proteinopathies
1μm
plaque
100nm
fibril

Figure 2

NUCB1-4x-hIgG1-FC2
MYRMQLLSCIALSLALVTNSISAMVT**PNKEETPATESPD**
TGLYYHRYLQEVIDVLETDGHFREKLQAANAEDIKSGKL
SRELDFVSHHVRTKLDELKRQEVSRLRMLLKAKMDAE
QDPNVQVDHLNLLKQFEHLDPQNQHTFEARDLELLIQ
TATRDLAQYDAAHHEEFKRYEMLKEHERRRYLESLGEE
QRKEAERKLEEQQRRHREHPKVNVPGSQAQLKEVWE
ELDGLDPNRFNPKTFFILHKINSDGVLDEQALEALFTKE
LEKVYDPKNEEDDMREMEEERLRMREHVMKNVKTN
QDRLVTLEAFLASTQRKEFGDTGEGWETVEMHPAYTE
EELRRFEEELAAREAELNAKAQRLSQETEALGRSQGRL
EAQKRELQQAVLHMEQRKQQQQQQQQGHKAPAAHP
EGQLKFHPDTDDVPVPAPAGDQKEVDTSEKKLLERLPE
VEVPQ*HDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI*
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL
VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY
SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
*GK** (SEQ ID NO:3)

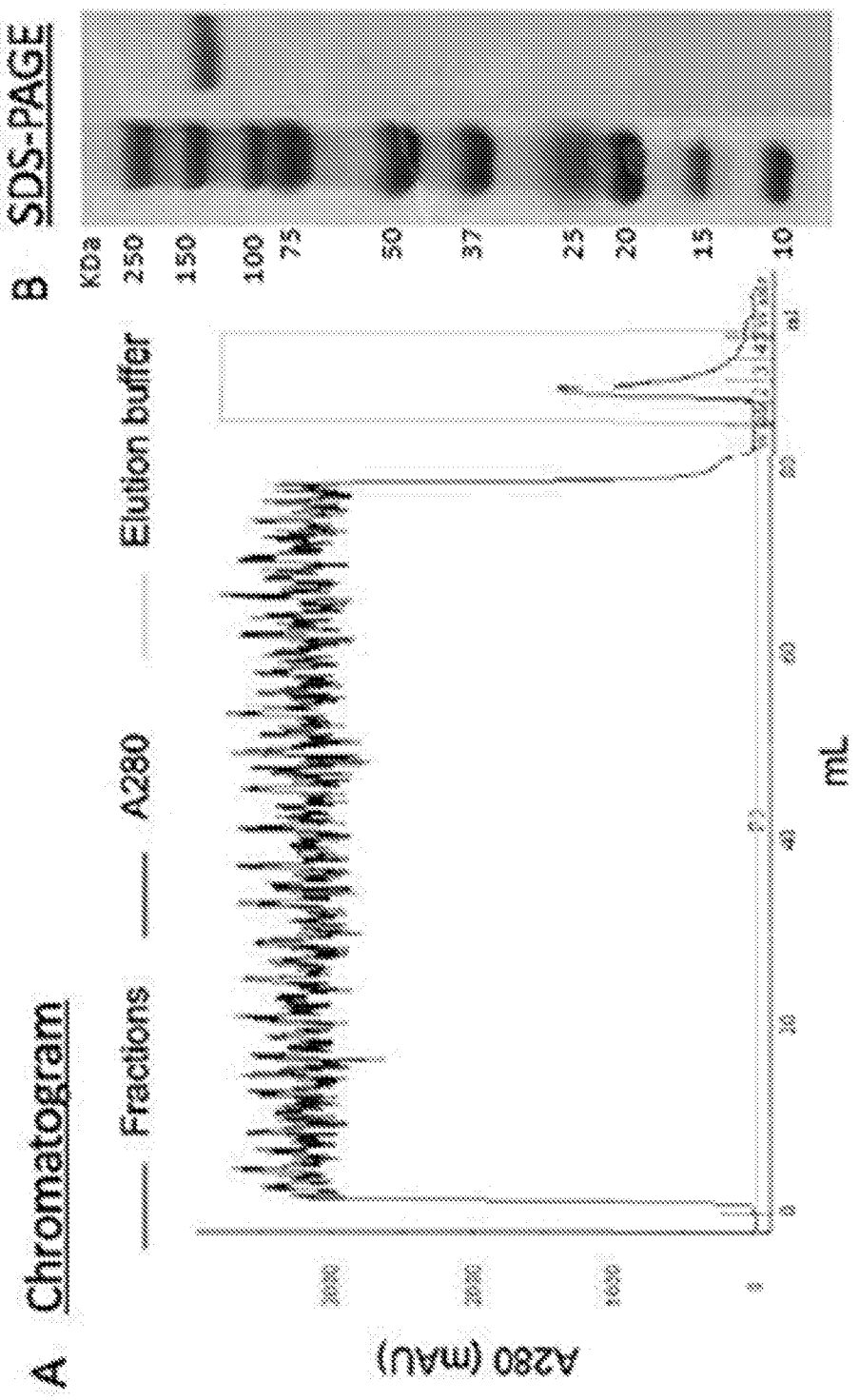

A Chromatogram
Fractions
A280
Elution buffer
A280 (mAU)
mL
B SDS-PAGE
KDa
250
150
100
75
50
37
25
20
15
10

Figure 3

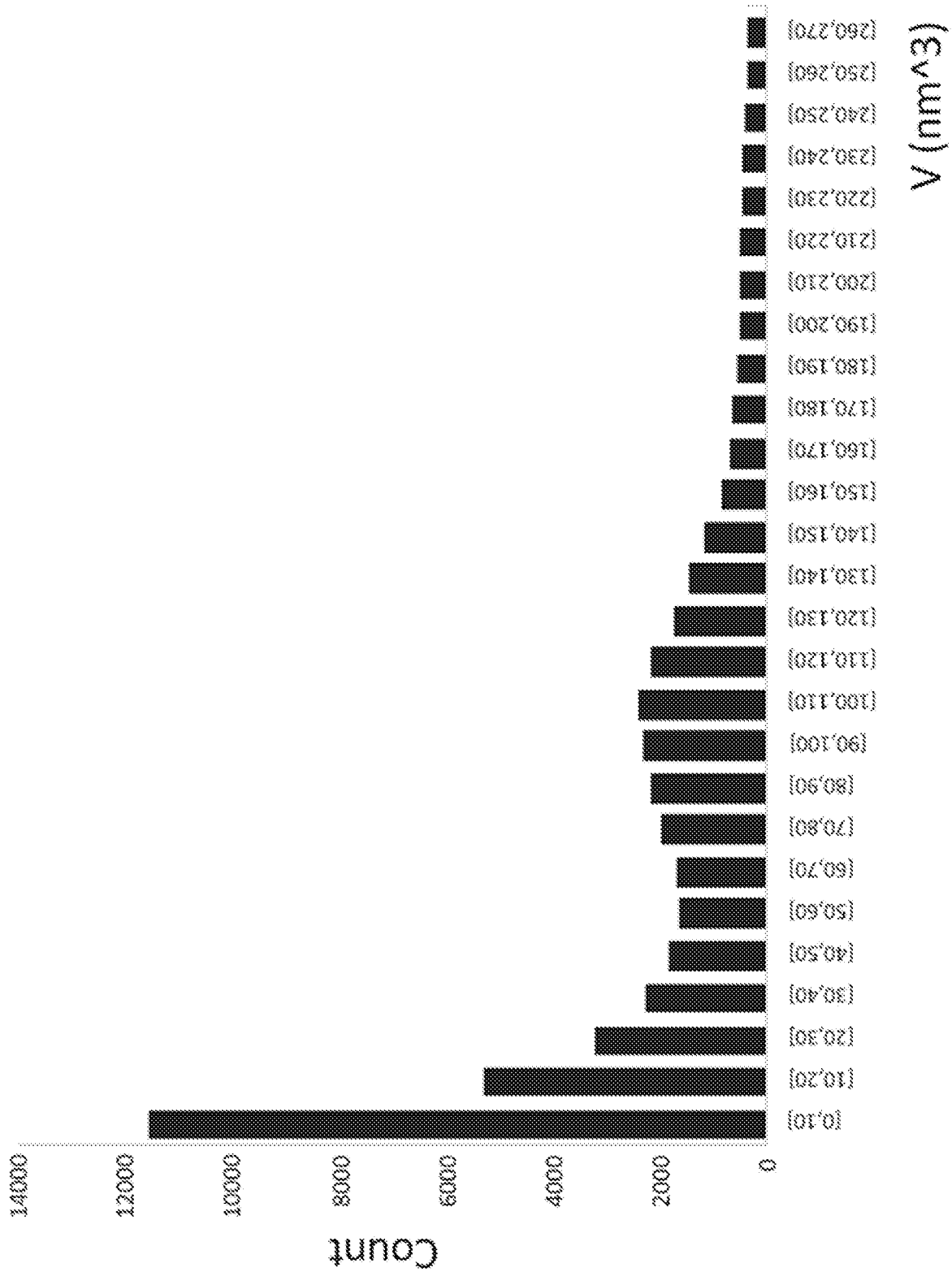

Count
14000
12000
10000
8000
6000
4000
2000
0
V (nm^3)
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[20,30]
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Figure 4

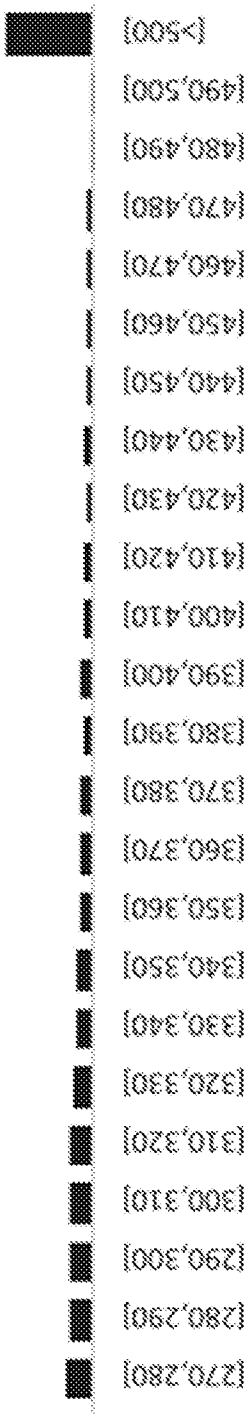


Figure 4, continued

Figure 5

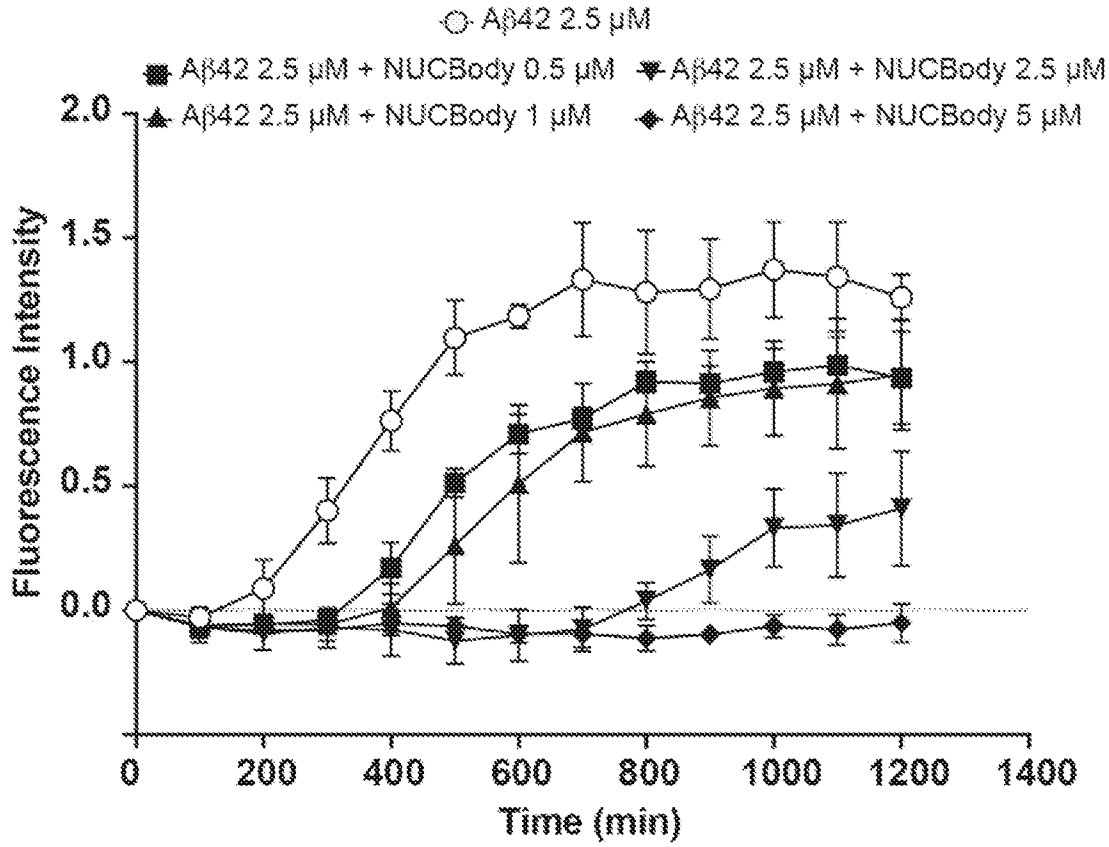


Figure 6

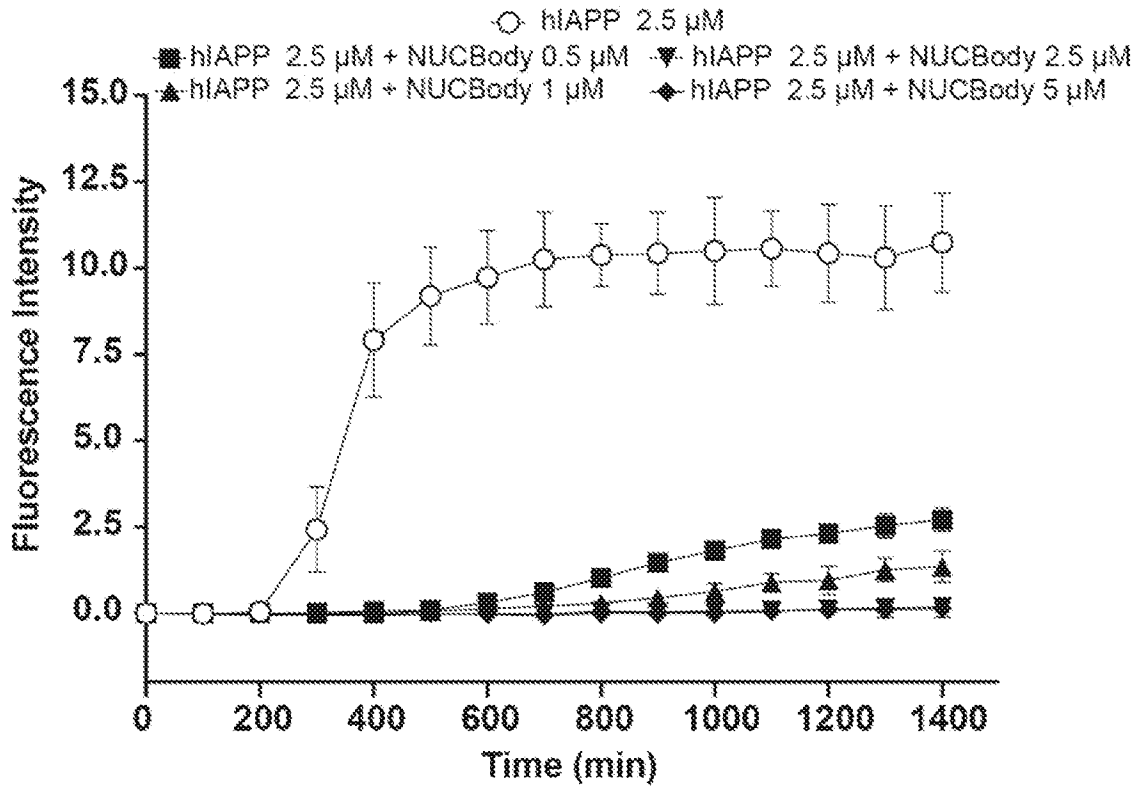


Figure 7

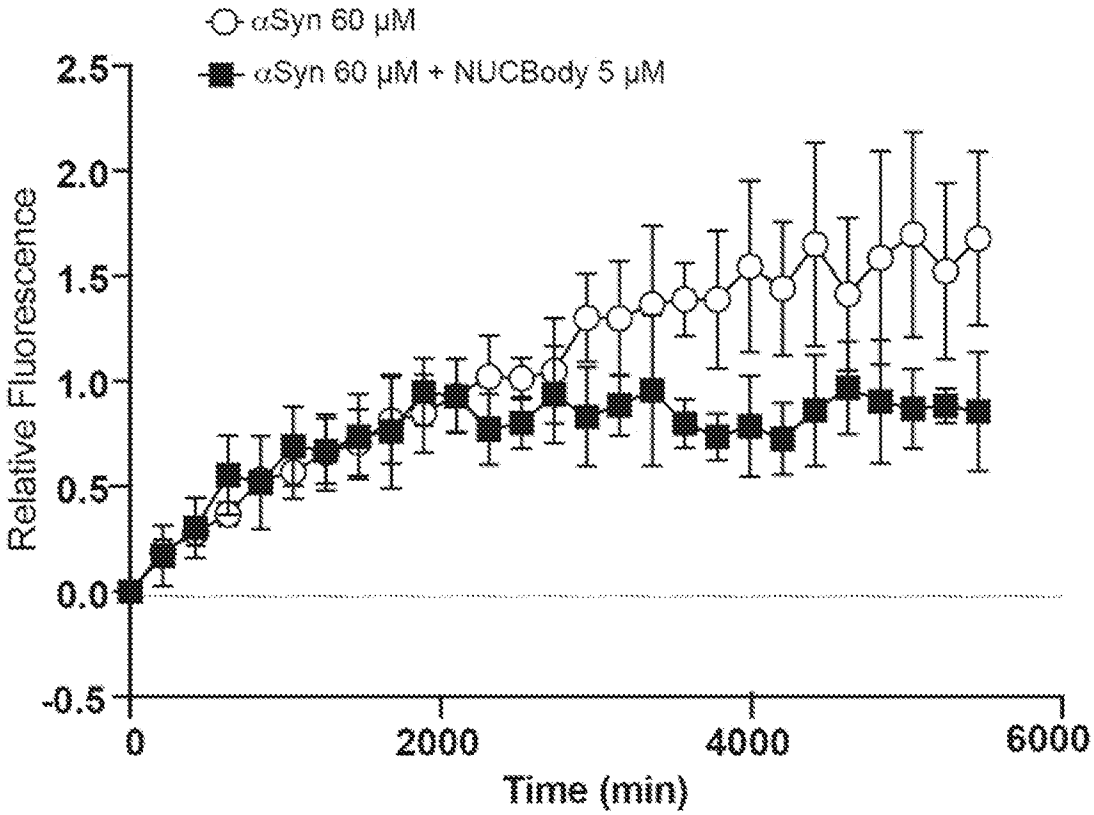
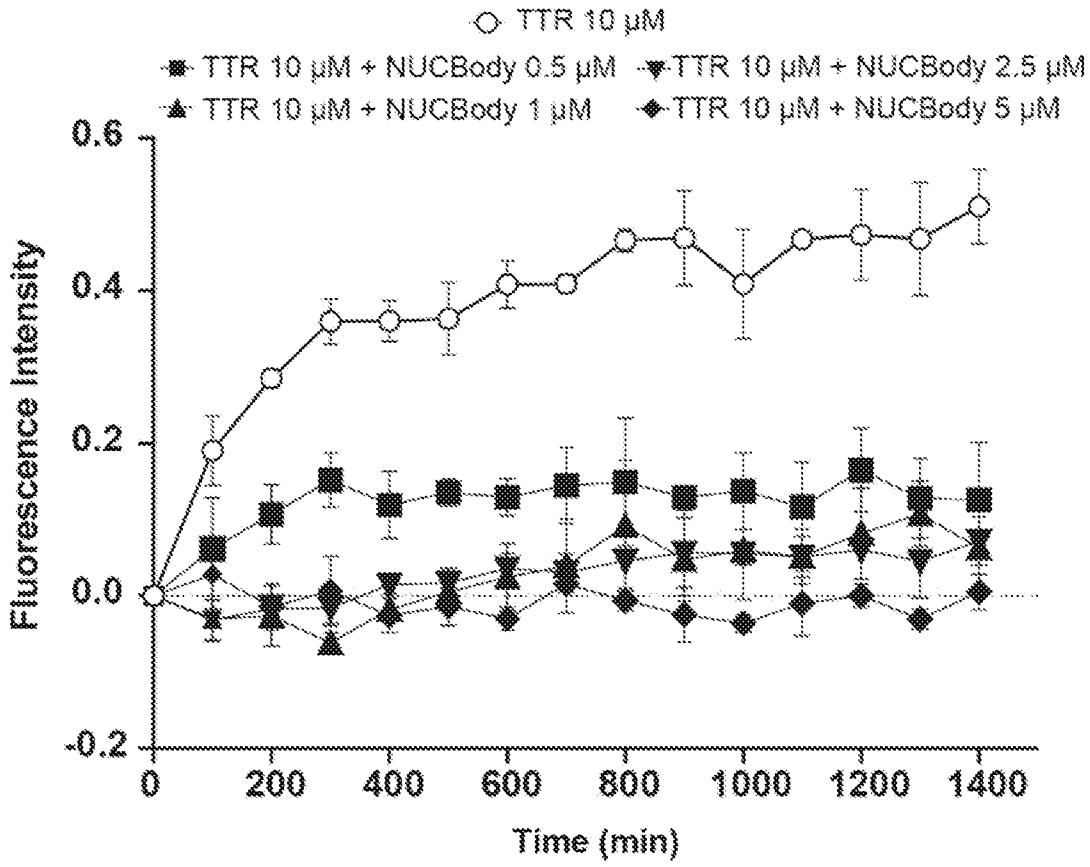
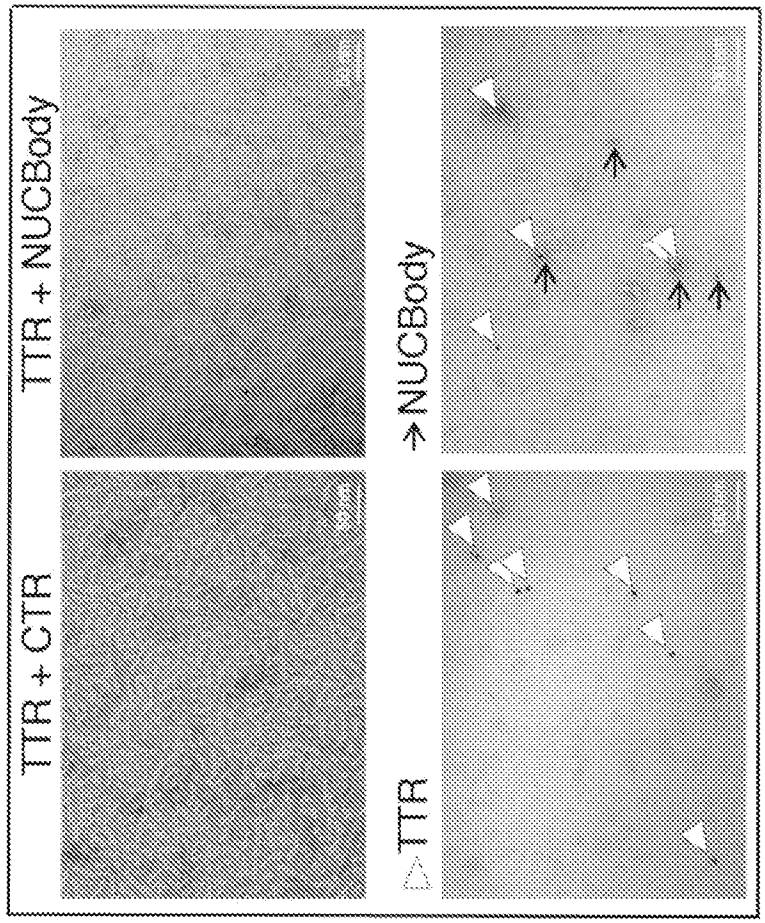


Figure 8




TTR + CTR
TTR + NUCBody
→NUCBody
△TTR

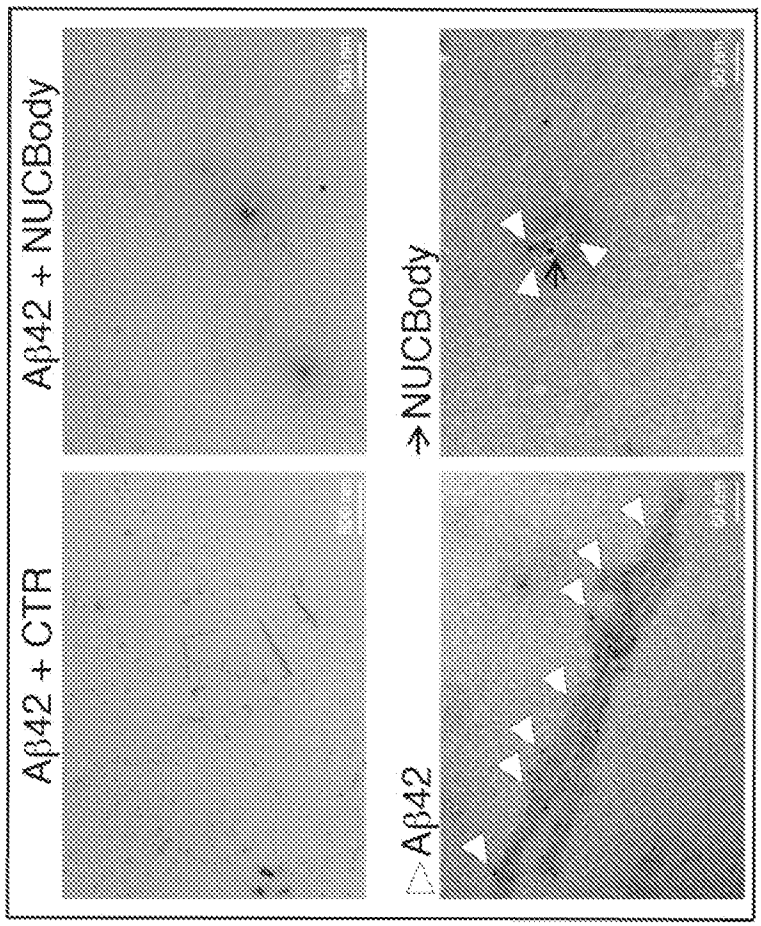

Aβ42 + CTR
Aβ42 + NUCBody
→NUCBody
△Aβ42

Figure 9

Figure 10

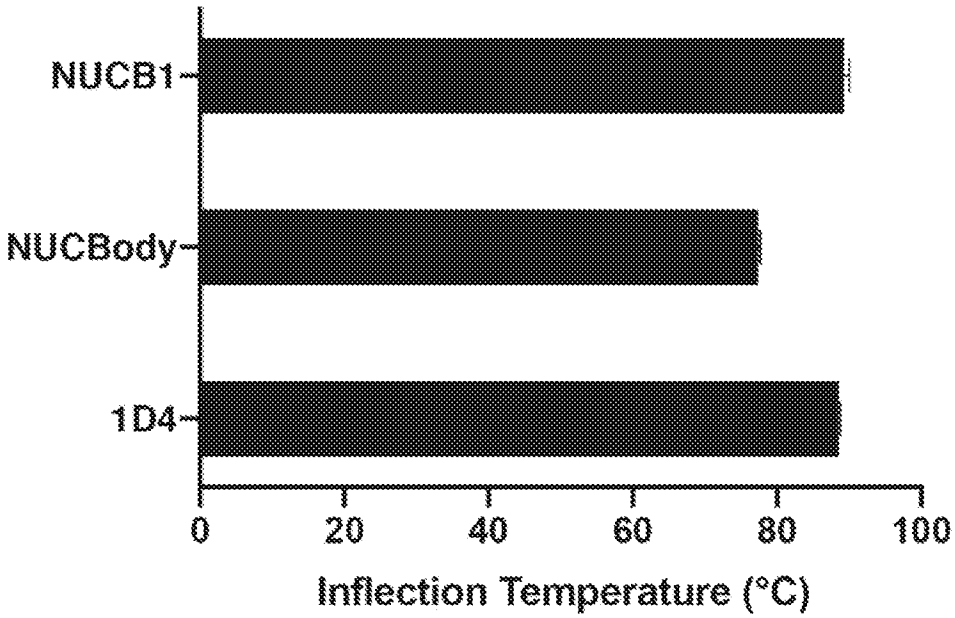
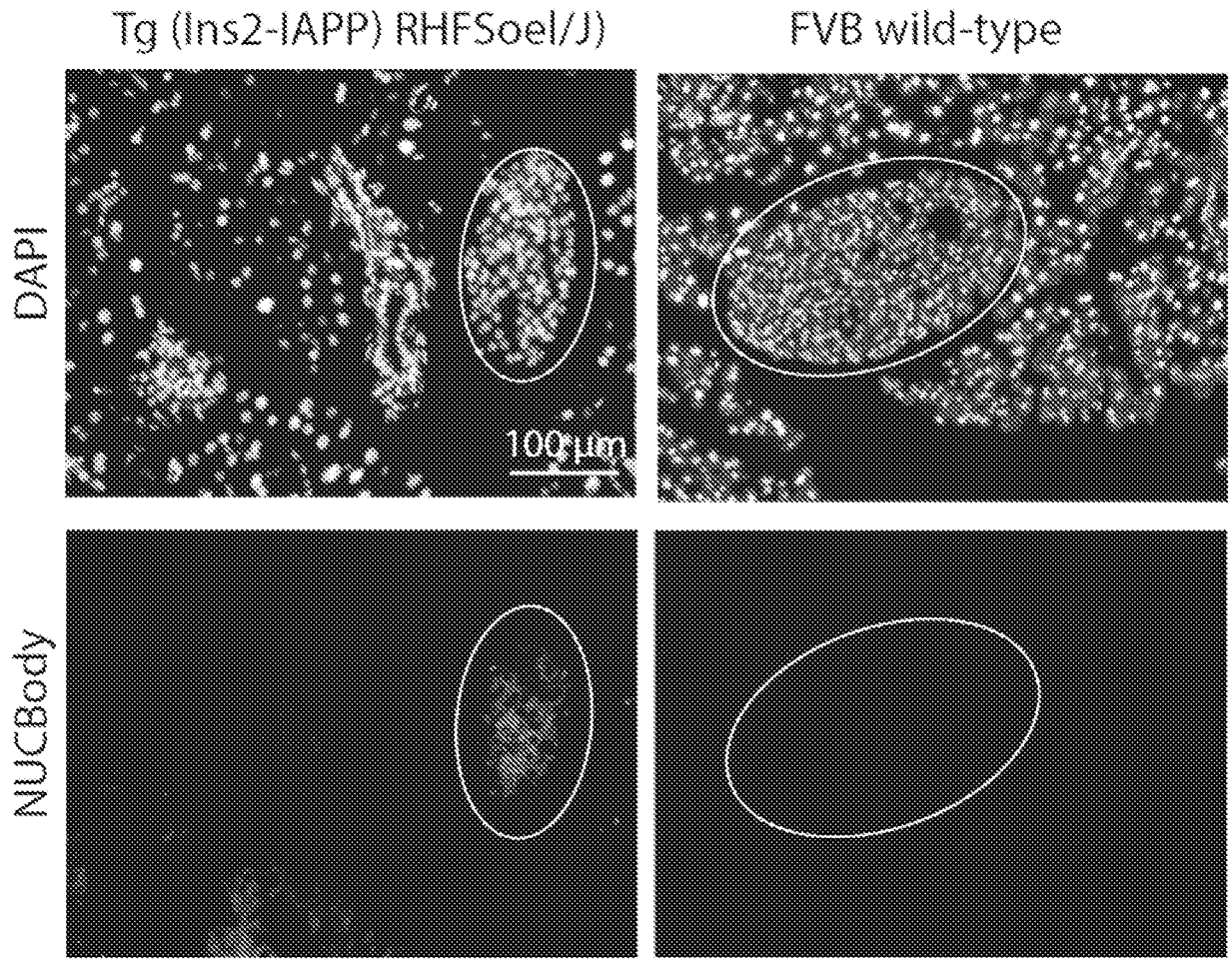


Figure 11



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/14551

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/16, C07K 14/47, C12N 9/48 (2020.01)

CPC - C07K 14/471, A61P 13/12, A61P 1/16

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2011/0224143 A1 (Sakmar et al.) 15 September 2011 (15.09.2011) para [0011]-[0013]; [0024]; [0126]-[0129]; [0139]-[0143]; SEQ ID NO: 6	1, 2, 20/(1,2) --- 3-6, 20/(3-6)
Y	US 2018/0162927 A1 (Janssen Biotech, Inc.) 14 June 2018 (14.06.2018) para [0091]; [0095]; [0131]; Table 3; SEQ ID NO: 36	3-6, 20/(3-6)

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2020	27 MAY 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/14551

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 searched, the appropriate additional search fees must be paid.

Group I, claims 1-6 and 20, directed to a fusion protein or a pharmaceutical formulation comprising the fusion protein.

Group II, claims 7 and 8, directed to an expression vector or a method comprising expressing the expression vector.

Group III, claims 9-19, directed to a method comprising contacting a fusion protein with proteins or a sample.

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

--continued on first 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.:
1-6, 20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 20/14551

--continued from Box No. III Observations where unity of invention is lacking--

Special technical features:

Group I has the special technical feature of a composition comprising or consisting of a fusion protein, that is not required by Group II, III.

Group II has the special technical feature of an expression vector, that is not required by Group I, III.

Group III has the special technical feature of contacting a sample or proteins with a fusion protein, that is not required by Group I, II.

Common technical features:

Groups I-III share the common technical feature of a NUCB1 fusion protein.

However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is previously disclosed by US 2011/0224143 A1 to Sakmar et al., (hereinafter 'Sakmar').

Sakmar teaches a fusion protein comprising a NUCB1 amino acid sequence (para [0126]-[0129] "the isolated nucleic acids that encode NUCB1 protein variants can further comprise an operably linked nucleic acid that encodes a purification tag. A purification tag can be operably linked to either the N- or C-terminus of the NUCB1 protein variant. A purification tag is any peptide sequence that provides for facilitated purification of the linked NUCB1 protein variant. Purification tags include, but are not limited to, a histidine tag, a heavy chain of protein C tag...the isolated nucleic acids that encode NUCB1 protein variants can further comprise an operably linked nucleic acid that encodes a protease cleavage site...nucleic acids that encode NUCB1 protein variants can further comprise an operably linked nucleic acids that provide for expression in a host cell."; [0139] "N-terminal fusions to the NUCB1 protein variant"; [0143]).

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-III inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.