(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 February 2013 (14.02.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/021353 A1

(51) International Patent Classification:
A61K 47/48 (2006.01) *A61P 25/28* (2006.01)

(21) International Application Number: PCT/IB2012/054037

(22) International Filing Date: 8 August 2012 (08.08.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/573,033 11 August 2011 (11.08.2011) US

(71) Applicant *(for all designated States except US)*: **BAR-ILAN UNIVERSITY** [IL/IL]; Ramat Gan, 52900 Ramat Gan (IL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **RAHIMIPOUR, Shai** [IL/IL]; Ehad Ha'am Street 5/9, 76261 Rehovot (IL). **RICHMAN, Michal** [IL/IL]; Uzi Narkis 5, 44215 Kfar Saba (IL).

(74) Agents: **A.C. ENTIS-IP LTD** et al.; Raoul Wallenberg 6, 69719 Tel Aviv (IL).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *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 sequence listing part of description (Rule 5.2(a))*

WO 2013/021353 A1

(54) Title: SURFACE MODIFIED PROTEINACEOUS SPHERICAL PARTICLES AND USES THEREOF

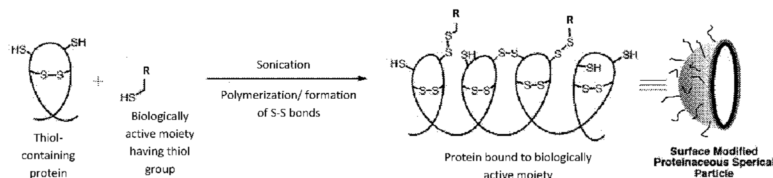

Fig. 1

(57) Abstract: An aspect of embodiments of the invention relates to surface modified proteinaceous spherical particles (SMOP). SMOPs according to an embodiment of the invention may comprise a protein layer and an amyloid-binding moiety bound to the protein. In an embodiment, the protein layer is spherical in shape and comprises proteins linked to each other by disulfide bonds. It is suggested that SMOPs are effective in preventing formation of amyloid and aggregation of Aβ when administered to a patient in need thereof.

SURFACE MODIFIED PROTEINACEOUS SPHERICAL PARTICLES AND USES THEREOF

RELATED APPLICATIONS

[0001] The present application claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application 61/573,033 filed on 11 August 2011, the disclosure of which is incorporated herein by reference.

TECHNICAL FIELD

[0002] Embodiments of the invention relate to surface modified proteinaceous particles, methods of their manufacture, and uses of the particles.

BACKGROUND

[0003] One of the main causes of dementia in elderly patients is Alzheimer's Disease (AD). AD is caused by degeneration in various parts of the brain attributed to loss of brain neurons and synapses. Amyloid fibrils and plaques are commonly found in brains of patients afflicted by AD, and it has been suggested that formation and growth of amyloid plaques are one of the main causes of AD.

[0004] Amyloid plaques are formed primarily by the aggregation and accumulation of naturally occurring proteins. One protein, which causes amyloid formation, is termed Amyloid Beta, also known as Beta Amyloid (Aβ). Aβ is a peptide composed of between 40 and 43 amino acids and it exists in humans in both soluble and insoluble forms. In its soluble form, it circulates in plasma and in brain interstitial and cerebrospinal fluids. In its insoluble form, Aβ forms aggregates comprising amyloid fibrils which can form disease-causing amyloid plaques.

[0005] In addition to Aβ, other proteins such as amylin, alpha-synuclein, Huntingtin and beta-2 microglobulin have been indicated as causes for amyloid formation.

[0006] In addition to AD, other diseases have been associated with accumulation of amyloid plaques including Lewy body dementia, cerebral amyloid angiopathy and inclusion body myositis.

SUMMARY

[0007] An aspect of embodiments of the invention relates to surface modified proteinaceous spherical particles (SMOP). SMOPs according to an embodiment of the invention may comprise a protein layer and an amyloid-binding moiety bound to the protein. In an embodiment, the protein layer is spherical in shape and comprises proteins linked to each other by disulfide bonds.

[0008] It is suggested that SMOPs are effective in preventing formation of amyloid and aggregation of Aβ when administered to a patient in need thereof. Without being bound by theory, the amyloid-binding moiety on the surface of the SMOP binds to an amyloid forming protein, such as Aβ (both soluble and aggregated forms) to form an SMOP- Aβ complex. The SMOP- Aβ complex prevents Aβ aggregation and toxicity. In addition, the SMOP- Aβ is then recognized by cells (including macrophages) which perform phagocytosis, thereby eliminating the SMOP- Aβ complex. As a result, the systemic concentration of Aβ and/or Aβ plaques present in the patient is reduced.

[0009] In addition, SMOPs may be used for diagnostics in the detection of amyloid plaques and/or Aβ- containing plaques present in the patient. SMOPs may be manufactured to further comprise a contrasting agent. Upon administration to a patient, SMOPs may migrate to areas of the patient's body comprising high concentrations of amyloid and/or Aβ plaques. SMOPs comprising a contrasting agent may then be detected using diagnostic methods to analyze for accumulations of SMOPs which may indicate high concentrations of Aβ and/or Aβ plaques in the patient.

[00010] Additional aspects of embodiments of the invention relate to methods of manufacture of SMOPs using sonication. In an embodiment of the invention, a protein having a thiol group, for example, a cysteine residue, is combined with a biologically active agent having a thiol group to form a mixture, and the mixture is sonicated to form SMOPs.

[00011] In the discussion unless otherwise stated, adjectives such as "substantially" and "about" modifying a condition or relationship characteristic of a feature or features of an embodiment of the invention, are understood to mean that the condition or characteristic is defined to within tolerances that are acceptable for operation of the embodiment for an application for which it is intended.

[00012] This summary is provided to introduce a selection of concepts in a simplified form that are further described below in the detailed description. This summary is not intended to identify key features or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter.

BRIEF DESCRIPTION OF FIGURES

[00013] Non-limiting examples of embodiments of the invention are described below with reference to figures attached hereto that are listed following this paragraph. Identical structures, elements or parts that appear in more than one figure are generally labeled with a same numeral in all the figures in which they appear. Dimensions of components and features shown in the figures are chosen for convenience and clarity of presentation and are not necessarily shown to scale.

[00014] Fig. 1 shows a schematic representation of a synthesis of an SMOP, in accordance with an embodiment of the invention;

[00015] Fig. 2A shows a histogram depicting reduction of aggregation of Aβ in an in vitro model using various concentrations of SMOPs comprising Aβ binding moiety KLVFF (SEQ ID NO 1) (designated KLVFF-CM) according to embodiments of the invention as compared to soluble KLVFF-NH_2 (SEQ ID NO 2) peptide and scrambled peptide surface modified protein (scrambled);

[00016] Fig 2B shows a histogram depicting reduction of Aβ induced toxicity in a model involving rat phaeochromocytoma (PC12) cells, in which SMOPs according to embodiments of the invention (designated KLVFF-CM+ $A\beta_{40}$ (4:1)) were effective in reducing toxicity of Aβ to a greater extent than to equivalent amounts of soluble KLVFF-NH_2 + $A\beta_{40}$ (4:1)peptide.

DETAILED DESCRIPTION

[00017] As will be described in the following examples, it has been found that SMOPs comprising an Aβ binding moiety bound to a protein, have been effective in reducing concentration of Aβ and preventing aggregation and toxicity of Aβ. SMOPs according to embodiments of the invention may be used for treatment and diagnosis of Aβ- mediated diseases.

[00018] Example 1A: Synthesis of modified protein particles using sonication

[00019] SMOPs modified with a peptide having the sequence KLVFFC-NH_2 (SEQ ID NO 3) motif were prepared. KLVFF is a short peptide representing residues 16-20 in native Aβ and has been shown to bind homologous Aβ and inhibit its aggregation.

[00020] In a one-step process, peptides having a sequence KLVFFC-NH_2 or its analogs were covalently attached via disulfide bonds to protein molecules, and the protein molecules' tertiary structure re-organized via breaking and formation of disulfide bonds to form SMOP particles. A general depiction of this process is shown in Fig. 1.

[00021] In order to attach the Aβ binding element KLVFF to protein molecules which would form spherical protein particles, an amino acid having a thiol group, cysteine, was introduced on the C-terminal of the KLVFF peptide. Peptides having the sequence KLVFFC-NH_2 were synthesized using solid phase peptide synthesis. A Rink amide MBHA (paramethylbenzhydrylamine) resin was used, with FMOC (Fluorenylmethyloxycarbonyl) protection. Coupling was carried out in N-methyl-2-pyrrolidone (NMP), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as the coupling agent. The peptides were cleaved from resin and deprotected by a mixture of trifluoroacetic acid (TFA) : Triisopropylsilane : H_2O (95 : 2.5 : 2.5) and purified to homogeneity by RP-HPLC (reversed phase high-performance liquid chromatography). The pure peptides were analyzed by mass spectrometry using MALDI-TOF/TOF (Matrix-assisted laser desorption/ionization, time of flight) or ESI (electrospray ionization) mass spectrometer.

[00022] SMOPs were prepared sonochemically by sonicating a reaction mixture of peptide, solvent and protein. In an embodiment of the invention, a solution of bovine serum albumin (BSA) in an amount of 1.5 milligrams (mg) in 30 milliliters (ml) of double-distilled water was combined with canola oil (20 ml) in a cylindrical reaction vessel. KLVFFC-NH_2 was added to the BSA solution in a molar ratio of BSA:peptide 1:500. The tip of a high-intensity ultrasonic probe was then placed at the aqueous-organic interface and the mixture was irradiated at an acoustic power of 150 Watts per square centimeter (W/cm^2) of surface area of probe at 20 kilohertz for three minutes while the reaction vessel was cooled in an ice-water bath. After synthesis, the reaction mixture was left at 4°C (Celsius) for 24-36 hours, and SMOP phase was separated from the unreacted BSA. The SMOPs were washed by centrifuging twice with 25 ml of distilled water at 800 revolutions per minute.

[00023] In alternative embodiments of the invention, other proteins can be used, including human serum albumin or transferrin. Antibodies may also be used in place of proteins. SMOPs

may be formed of any proteins or peptides having amino acids comprising thiol groups, such as cysteine or homocysteine. In addition, SMOP protein layers may be formed from polymers and biopolymers, optionally having thiol groups. These proteins or peptides may form disulfide bonds to form spherical protein or peptide structures, capable of chemically bonding to Aβ binding elements.

[00024] Without being bound by theory, it is suggested that syntheses of SMOP described herein may be explained as follows. Sonication of reaction mixtures comprising an oil, a thiol containing protein, and an amyloid binding moiety comprising a thiol agent, breaks and reforms disulfide bonds between protein molecules and between proteins and amyloid binding moieties. Oil-filled particles having a spherical shape and having a protein coating are formed, and the amyloid binding moieties are bound to external surfaces on the outside of the protein coatings by a thiol exchange reaction.

[00025] Example 1B: Synthesis of labeled SMOPs

[00026] In order to estimate amount of peptide bound on each SMOP, labeled SMOPs were synthesized. The procedure used in Example 1A was followed with the exception that the peptide used, KLVFFC-NH_2, was labeled at its N-terminus by reaction with 4-nitrobenzo-1,2,5-oxadiazole (NBD) chloride after solid state synthesis, before detachment from the resin. The labeled peptide formed, designated NBD-KLVFFC-NH_2 was then sonicated with BSA as in Example 1A to form NBD labeled SMOPs.

[00027] In addition, SMOPs encapsulated with Nile red were synthesized by following the procedure in Example 1A, with the addition of 5 mg Nile red to the 20 ml canola oil used in the reaction mixture. The Nile red- encapsulated SMOPs were then characterized using various methods, including fluorescent and electron microscopy.

[00028] Example 1C: Synthesis of additional SMOPs

[00029] SMOPs were formed with a "scrambled" peptide having the same amino acids as KLVFF in a different sequence, FVKLF. The method for preparation used was similar to that used in Example 1A with the exception of using FVKLFC-NH_2 as a starting material. These SMOPs function as a comparator in *in vitro* studies to KLVFF-containing SMOPs.

[00030] In example 1A, molar ratio of protein to peptide starting material was approximately 1:500. Other SMOPs were prepared using a molar ratio of starting materials ranging from 1:100 to 1:1000.

[00031] SMOPs comprising polyethylene glycol (PEG) were also prepared using the general method as in Example 1A but with the addition of PEG. To prepare PEG-SMOPs 0.6 mg of mercapto-polyethylene glycol monomethyl ether (molecular weight of about 5000 grams/mole (g/mole)) was mixed in the BSA (1.5 mg, 0.023 micromole (μmol)) and KLVFFC-NH_2 (8.6 mg, 11.4 μmol) solutions and combined with a layer of canola oil. The mixture was then subjected to the sonication as described in Example 1A.

[00032] Example 1D: Characterization of SMOP

[00033] The KLVFF-comprising SMOPs were characterized by optical and scanning electron microscopy (SEM), dynamic light scattering (DLS) and fluorescent microscopy. The shape and morphology of the SMOPs were characterized by optical-fluorescence microscopy (Apo-Tome AxioImager.z1 microscope, Zeiss, Germany), scanning electron microscopy (SEM, FEI Quanta™ 200 FEG, Hillsboro, Oregon) and confocal microscopy (Leica-SPE microscope, Mannheim, Germany). For the SEM analyses, a sample (10 microliters (μl)) of SMOP was spotted onto a glass wafer, followed by drying and gold sputtering. The samples were then analyzed by SEM. The size and the size of distribution of the SMOPs were determined by Coulter Laser Diffraction (Coulter LS 100 Q Laser Sizer, Beckman Coulter, Miami, FLA, USA).

[00034] The optical and SEM images show the SMOPs to be spherical with relatively smooth surfaces. The DLS measurements demonstrated that the KLVFF-modified SMOPs have an average size of about 1.5 micrometer (μm) with a narrow size distribution. Using fluorescent microscopy for detection of NBD in NBD- labeled KLVFF-modified SMOPs showed that KLVFF peptide was confined to the outer surface of the SMOPs. Fluorescent microscopy analysis of SMOPs encapsulating oil-soluble Nile red confirmed that the SMOPs were filled with an organic oil-based core which was surrounded by a protein shell. This indicates that SMOPs can be used as encapsulation agents, in particular of oil-soluble reagents, for treatment and/or diagnosis.

[00035] The amount of KLVFF peptide on SMOP's surface was estimated using UV spectroscopy of NBD-labeled SMOPs, at a wavelength of 465 nanometers (nm). The number of SMOPs per volume was calculated using flow-cytometry analysis, (fluorescence-activated cell sorting, (FACS)). The molar ratio of starting materials varied from 1:100 to 1:1000. The amount (number of SMOPs per unit volume), sizes and amounts of peptide present in SMOPs

prepared according to procedures described above are summarized in Table 1. "ND" indicates that value was not determined.

[00036] Table 1: Analysis of various BSA-comprising SMOPs

Peptide	Molar ratio (Protein: Peptide)	Diameter (micrometers)	Amount of SMOPs (millions per ml)	KLVFF amount (nanomoles/ml)
KLVFF	1:1000	1.49±1.36	160.0	128
KLVFF	1:500	1.73±1.37	208	80
KLVFF	1:100	ND	260	38
FVKLF	1:1000	2.40±1.33	260	ND
FVKLF	1:500	2.25±1.30	320	ND

[00037] As seen in table 1, a linear correlation was observed between the amount of NBD-labeled KLVFF on the surface of SMOP and the amount of peptide used as a starting material in each preparation.

[00038] Without being bound by theory, it is suggested that when proteins comprising thiol groups are sonicated with peptides comprising thiol groups in the presence of oil, disulfide bonds are formed between protein molecules and due to hydrophobic/ hydrophilic interactions between proteins and oil, the proteins tend to form spheres which encapsulate the oil, and tend to present the peptides on their outer surfaces.

[00039] Example 1E: Binding of Aβ by SMOP

[00040] Binding of KLVFF-modified SMOP to Aβ was assessed using a fluorescent modified Aβ known as FITC-$A\beta_{40}$ (fluorescein isothiocyanate) in a direct in vitro assay. In order to confirm that the conjugated KLVFF fragments on SMOPs preserved their recognition capability to bind full length Aβ following the sonochemical process and SMOP generation, KLVFF-modified SMOPs were incubated with soluble FITC-$A\beta_{40}$ (25 μl, 0.5 nanomolar, nM) in PBS at 37°C for different periods under constant agitation (750 revolutions per minute (rpm)). At each time point, samples were diluted with cold PBS and the microspheres were washed twice with cold PBS. Ten thousand particles were then analyzed immediately by FACS.

[00041] In addition, an indirect assay (ThT [Thioflavin T] assay) was performed as follows. In the ThT assay, the ThT dye binds to amyloid plaque formations and displays enhanced fluorescence. The fluorescence is measured and correlated to presence of amyloid plaque formations. Agents which bind and inhibit amyloid plaque formation decrease fluorescence of ThT. The inhibitory activity of KLVFF-modified SMOPs on $A\beta_{40}$ aggregation was determined in black 96-well flat-bottom plates by incubating fresh monomerized (non-aggregated) solutions of $A\beta_{40}$ (33 micromolar (μM), 15 μl) with different preparations of the SMOPs (25 μl, Table 1) in PBS. PBS was added to reach a volume of 50 μl, giving a final concentration of 10 μM for $A\beta_{40}$. The inhibitory activity of the KLVFF-modified SMOPs was compared to that of soluble KLVFF-NH_2 peptide alone or to the SMOPs with the scrambled peptide, FVKLFC-NH_2 (SEQ ID NO 4). The plates were sealed with aluminum foil and incubated while shaking at 750 rpm at 37°C for 72 hours to induce aggregation. Following the incubation, a solution of ThT (24 μM, 150 μl) in glycine buffer (50 millimolar (mM), pH 8.5) was added to each well and the fluorescence of amyloid-bound ThT was immediately measured using a plate reader (Infinite M200, Tecan, Switzerland) at excitation and emission wavelengths of 430 and 492 nm, respectively. The ThT solution was freshly prepared by diluting a ThT stock solution (1 mM, in water) with glycine buffer (50 mM, pH 8.5). Experiments were performed in quadruplicate.

[00042] As controls, proteinaceous SMOP without surface modification (prepared as in example 1A, without the addition of a KLVFF peptide) (naked BSA spheres) were prepared. FVKLF-modified SMOP was also prepared as a control, as in Example 1C.

[00043] Both fluorescent microscopy and FACS analysis showed intense fluorescent signals on the KLVFF- modified SMOPs even after extensive washing steps, indicative of strong affinity of FITC-$A\beta_{40}$ to the KLVFF- modified SMOPs. In contrast, significantly lower amount of FITC-$A\beta_{40}$ was accumulated on the surface of the naked BSA spheres and the FVKLF-modified SMOPs. These results confirm that the selective interaction of KLVFF-modified SMOP with $A\beta_{40}$ is mediated through the KLVFF binding site and that KLVFF-modified SMOP may potentially be used to bind Aβ.

[00044] Example 1F: Comparison of affinity of KLVFF-modified SMOP and KLVFF to Aβ

[00045] The selectivity and relative affinity of the interaction between KLVFF-modified SMOP and $A\beta_{40}$ were further determined by probing the impact of soluble KLVFF-NH_2 (not bound to SMOPs) on the binding of FITC-$A\beta_{40}$ to KLVFF-modified SMOP, having a 1:500 ratio of

BSA to KLVFF. FACS analysis revealed that a large excess of soluble KLVFF-NH_2 (×50 mole excess) used as the competitor only slightly reduced ($P<0.001$) the binding between FITC-$A\beta_{40}$ and KLVFF-modified SMOP, implying a significantly higher affinity of FITC-$A\beta_{40}$ for the KLVFF-modified SMOP than for the free KLVFF-NH_2 and/or the high capacity of the KLVFF-modified SMOP to bind $A\beta_{40}$.

[00046] Example 1G: Inhibition of Aβ aggregation by SMOP

[00047] To evaluate the inhibitory effect of KLVFF-modified SMOPs on Aβ aggregation and kinetics, soluble $A\beta_{40}$ (0.5 nanomol, nmol) was incubated in the absence and presence of different preparations of the SMOPs. Amyloid plaque formation was then monitored by a ThT assay for three days of incubation.

[00048] KLVFF-NH_2 soluble peptide was tested in ThT aggregation assay against equivalent amounts of KLVFF-modified SMOPs prepared using a BSA: KLVFF ratio of 1:500 (SMOPs having equivalent amounts of KLVFF peptide) and scrambled peptide FLVKF-modified SMOPs, having equivalent amounts of peptide. Three experiments were performed in which the molar ratio of Aβ to peptide or peptide equivalent varied from 1:2 to 1:4 to 1:6.5. The KLVFF-modified SMOPs significantly decreased the aggregation of Aβ in a dose-dependent manner as shown in Figure 2A and dramatically slowed the rate of Aβ aggregation. KLVFF-NH_2 soluble peptide had an inhibitory effect on Aβ aggregation, but to a lesser extent than equivalent concentrations of KLVFF-modified SMOP. In contrast, FLKVF-modified SMOP had no or little effect on Aβ aggregation and its kinetics. These results clearly indicate that the effect of the KLVFF-modified SMOP on Aβ aggregation is sequence-specific.

[00049] The finding that KLVFF-modified SMOP had an effect on reducing amyloid aggregation is significant in that previous attempts to inhibit amyloid aggregation using particles bound to amyloid binding agents were not successful. An example of such an attempt is detailed in Kogan et al, in which gold nanoparticles conjugated to an anti-amyloidogenic peptide failed to show anti-aggregation activity, despite the strong and selective binding of the peptides to the Aβ species. The particles only succeeded in inhibiting aggregation when microwave energy was introducted to heat up the gold nanoparticles.

[00050] Transmission Electron Microscopy (TEM) analysis of the samples taken from the ThT assay demonstrated that while $A\beta_{40}$ alone forms long unbranched fibrils following 72 hour incubation, addition of KLVFF-modified SMOP completely arrests the formation of fibrils. In

contrast, naked BSA spheres and FLKVF-modified SMOP had no inhibitory effect on amyloid formation.

[00051] Example 1H: Reduction of Aβ- induced toxicity in PC12 cells

[00052] Having shown that the KLVFF-modified SMOP can effectively and selectively inhibit Aβ aggregation, the toxicity of SMOP was tested in PC12 cells, and the reduction of Aβ-induced toxicity in PC12 cells was tested.

[00053] PC12 cells were maintained in low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum, 2 mM L-glutamine, 100 Units/ml penicillin and 100 mg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. PC12 cells (10,000 cells/well) were plated in 96-well tissue-culture plates in 100 µl of the medium and incubated overnight to attach cells to the plates. The medium was then replaced with 100 µl of fresh medium containing various amounts of the KLVFF-modified SMOP (5-25 µl), and the incubation was continued at 37°C for an additional 24 hours. Cell survival was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

[00054] In the MTT assay, a PBS solution of MTT (10 µl, 5 mg/ml) was added to each well after the medium was replaced with fresh medium and the cells were incubated for an additional 4 hours. The generated formazan crystals were dissolved by adding 100 µl of 50% dimethylformamide containing 20% sodium dodecyl sulfate (pH 4.7) to each well. The plates were then shaken at 37°C overnight and the absorbance of each well was measured at 570 nm (680 nm background) using a plate reader. At the levels tested KLVFF-modified SMOP showed no signs of toxicity in PC12 cells.

[00055] In order to assess effect of KLVFF-modified SMOP on Aβ toxicity, $A\beta_{40}$ (0.5 nmol) in 25 µl of PBS was aged for 48 hours in the absence or presence of either the SMOPs (25 µl, 2 nmol, based on the amount of the conjugated peptide) or the soluble peptide KLVFF-NH_2 (25 µl, 2 or 20 nmol). Prior to the addition of the SMOPs or soluble peptides to the cells, the extent of $A\beta_{40}$ aggregation in each sample was determined by ThT assay as described above. On the day of the experiment, the medium was replaced with fresh medium (50 µl) and the aged samples (50 µl) were then diluted by a factor of two in the medium. Control wells received PBS (50 µl) only. Cells were incubated for an additional 24 hours and cell viability was then determined by the MTT assay as described above. Each experiment was performed in quadruplicate and repeated three times.

[00056] The results are shown in Figure 2B. Levels of cell viability (an indication of lack of toxicity) in the presence of KLVFF-modified SMOP (designated KLVFF-CM) without Aβ and Aβ without any amyloid binding agent are depicted (two leftmost bars) as comparison. KLVFF-modified SMOP (designated KLVFF-CM+ $A\beta_{40}$ (4:1)) significantly reduced ($P<0.003$) the toxicity of the aggregated Aβ as shown in Fig. 2B. In contrast, soluble KLVFF-NH_2 (designated KLVFF-NH_2+ $A\beta_{40}$ (4:1)) exhibited little or no effect on toxicity, suggesting that the activity of the SMOP, which most likely stems from the multivalent presentation of the KLVFF peptides, is greater than the activity of soluble KLVFF on eliminating toxicity associated with amyloid aggregation.

[00057] Example 2A: Administration of SMOP to animals to demonstrate safety

[00058] SMOPs modified with KLVFF (ratio of BSA:KLVFF of 1:500) were used *in vivo* for toxicological studies. Rats weighing about 250 g each were injected intravenously, via their tail vein, with increasing amounts of the SMOP starting from 100 μl and following an up-down protocol based on the outcome of the initial dose. The animals were observed for 2 weeks and were scored for any sign of immediate toxicity. SMOP did not cause immediate toxicity to the rats even at highest amount of administered (200 μL).

[00059] Particles in the size range of SMOPs are known to have high toxicity from animal studies because of self-aggregation and lung damage. SMOPs according to embodiments of the invention were found to be safe even in high doses tested in rats. Without being bound by theory, it is suggested that self-aggregation was not detected in SMOPs according to some embodiments of the invention because SMOPs were highly charged, thereby preventing self-aggregation.

[00060] Example 2B: Macrophage clearance of Aβ bound by SMOP

[00061] As mentioned previously, AD and other diseases may be attributed to lack of clearance of Aβ and other amyloid-forming proteins, leading to amyloid buildup. Since the phagocytic pathway of Aβ has been hypothesized to be defective in AD, it was tested whether the KLVFF-modified SMOP are phagocytosed by the macrophagical/microglial cells through a non-Aβ-mediated pathway. The particle size and high charge density of the SMOP together with their proteinaceous structure may potentially make these particles highly susceptible to phagocytosis, even in patients with defective clearance of Aβ.

[00062] To induce optimal phagocytic activity, murine microglia (BV-2) cells were pre-stimulated with lipopolysaccharide (LPS) that generates inflammatory responses similar to those seen in AD patients. Phagocytotic activity was then studied by confocal microscopy as well as SEM. SMOPs were pre-loaded with Nile-red as a fluorescent probe to allow their identification within the cells. To ensure that the SMOPs localized to the intracellular space, the outer membrane and the nucleus of the live BV-2 cells were stained with Alexa-red wheat-germ agglutinin (WGA) and Hoechst dye, respectively. The stacked confocal images were then reconstructed to provide three-dimensional pictures of the cells. The images show that SMOPs with a diameter of about 0.7-1 μm were better phagocytosed by the BV-2 cells than SMOPs with larger diameter size. Further support for the phagocytosis of the SMOPs by BV-2 cells was provided by SEM analyses, which demonstrated the formation of hole-like structures (2.5-5 μm) on the membrane of the cells stimulated with LPS and incubated with the SMOPs. The holes most likely occurred on the cell membrane due to the disruption of the SMOPs inside the cells following the sample preparation and conditions under which SEM analyses were performed.

[00063] The uptake of the fluorescent microspheres by BV-2 cells was also confirmed by flow cytometry under conditions similar to those used for the microscopy studies. Cells were exposed for 2 hours to the Nile red-loaded KLVFF-modified SMOPs, washed and analyzed by FACS following detachment from plates. Fluorescent intensity of the cells increased upon incubation with the SMOPs, suggesting the phagocytosis of the particles by the cells. Moreover, pre-treatment of cells with LPS (1 microgram/ml (μg/ml)) for 4 h significantly enhanced the fluorescent intensity as compared to untreated cells. Assuming that microglia and macrophages in AD are exposed to inflammation conditions, these results suggest that SMOPs could clear Aβ in AD even while exposed to inflammation conditions.

[00064] To determine if the phagocytic pathway of the SMOPs is distinct from that of aggregated Aβ, BV-2 cells were incubated with pertussis toxin (PTX) and fucoidan as specific inhibitors of the G-protein receptor and the scavenger receptor, respectively, which have been shown to be essential for phagocytosis of Aβ. Previous studies have shown that inhibition of these receptors reduces the uptake of Aβ by macrophages. LPS-treated cells were incubated with PTX and fucoidan and then exposed for 30 minutes to aggregated FITC-Aβ or Nile-red-loaded KLVFF-modified SMOP. The extent of phagocytosed FITC-Aβ or KLVFF-modified SMOP was then compared by FACS for treated and non-treated cells. Treatment of BV-2 cells

with either PTX or fucoidan reduced the uptake of the aggregated Aβ, as expected. However, these agents had a minimal effect on the phagocytosis of SMOPs, suggesting that the uptake mechanism of the SMOPs is distinct from that of Aβ, and may be effective in patients experiencing defective phagocytosis of Aβ.

[00065] Example 2C: PEG-modified SMOPs

[00066] SMOP modified by PEG and comprising a KLVFF Aβ-binding unit were synthesized as described in Example 1C. SEM showed that the SMOPs were spherical with relatively smooth surfaces. The average size of the SMOP was 373 nm which is smaller than the SMOPs without PEG modification. Confocal microscopy confirmed that the PEG-modified SMOPs encapsulated hydrophobic molecules, for example Nile-red, in their non-aqueous solvent-filled cores.

[00067] When tested in BV-2 cells, the rate of phagocytosis of PEG modified KLVFF-modified SMOPs reached a maximum later and the amount of phagocytosis increased relative to KLVFF- modified SMOPs without PEG.

[00068] Modification of SMOPs with PEG may further enhance the efficacy of SMOPs by allowing their enhanced time of vivo circulation and binding of amyloid to increase before undergoing phagocytosis by microglia or by macrophages.

[00069] Example 2D: Additional modifications of SMOPs to enhance biological characteristics

[00070] In addition to modifications by PEG, SMOPs may also be modified by sugars to modify their biological characteristics. For example, SMOPs may be modified by mannose to modify their interaction with macrophages. Skirtenko et al describes methods of modifying mannose to form thiol-containing mannosyl derivatives. Such mannosyl derivatives may be added to reaction mixtures comprising proteins and sonicated according to embodiments of the invention to provide mannose substituted SMOPs. In addition to mannose, SMOPs may be modified with additional sugars such as glucose to provide enhanced biological characteristics.

[00071] Example 3A: Additional Aβ-binding moieties comprising cyclic D,L-α-peptides

[00072] Cyclic hexapeptides comprising alternating D- and L-amino acids were evaluated for their ability to bind Aβ and to prevent amyloid aggregation. The linear peptides were synthesized using solid-phase peptide synthesis using Fmoc methodology. Following synthesis, the linear peptides were cyclized on solid support.

[00073] **Fmoc-L-Lys-ODmab.** Fmoc-L-Lys(Boc)-OH was esterified by 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester (Dmab-OH) and treated with 50% TFA in CH_2Cl_2 to afford Fmoc-L-Lys-ODmab in 70% overall yield. The product was used for the peptide synthesis without further purification.

[00074] **Synthesis of cyclic D,L-α-peptides.** Cyclic D,L-α-peptides were synthesized on 2-Cl-trityl resin as follows. Fmoc-Lys-ODmab (0.78 g, 1 millimoles (mmol)) and DIEA (2 ml) in CH_2Cl_2 (8 ml) were added to 2-Cl-trityl chloride polystyrene resin (1 g, 1.6 mmol/gr) and the mixture was agitated for 4 hours. The resin was then washed with CH_2Cl_2 (3×) and MeOH and dried. The loading of the resin was then determined by quantification of Fmoc release following treatment of the resin with 20% piperidine in DMF. Peptides were then synthesized on an AAPPTec automated peptide synthesizer (Vantage) with HBTU/DIPEA mediated coupling reactions. Following completion of linear peptides, the resin was treated with 2% hydrazine hydrate in DMF to remove the Dmab protecting group. The resin was then washed with 5% DIEA in DMF and the linear peptide was cyclized, while still on resin, using a NMP solution of PyBOP, HOBT and DIEA (5, 5 and 15 eq.)) for 6 hours. The peptides were cleaved from the resin by treatment with a mixture of 95% TFA: 2.5% triisopropylsilane and 2.5% water for 2 hours. Crude peptides were then purified to homogeneity (>95% purity) by preparative RP-HPLC using a C_{18} column, and identified by MALDI- or ESI-MS.

[00075] A ThT assay was performed as described previously at concentrations of 10 μM Aβ with a ratio of Aβ:cyclic D,L-α-peptide of either 1:1 or 1:1/3. Table 2 summarizes the structures of D,L-α-peptides tested as well as the reduction of amyloidogenic activity in percent at the concentrations tested. The cyclic peptides tested all consisted of 6 amino acids, in alternating D- and L-conformation. The sequences are described using single-letter amino acid code. Upper and lower case letters represent L- and D- amino acid residues, respectively. J and Z represent Norleucine and 2–aminooctanoic acid, respectively. Square brackets refer to cyclic peptides.

[00076] Table 2:

Sequence of cyclic peptide	Inhibition at 1:1	Inhibition at 1:1/3
[lLwHsK]: Cyclo- D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine	60±7	32±9

[lLwHsA]: Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-alanine	67±5	48±6
[lLwHaK]: Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-alanine-L-lysine	56±4	47±10
[lLwAsK]: Cyclo-D-leucine-L-leucine-D-tryptophan-L-alanine-D-serine-L-lysine	73±6	49±8
[lLaHsK]: Cyclo-D-leucine-L-leucine-D-alanine-L-histidine-D-serine-L-lysine	4±4	5±4
[lAwHsK]: Cyclo-D-leucine-L-alanine-D-tryptophan-L-histidine-D-serine-L-lysine	44±9	3±10
[aLwHsK]: Cyclo-D-alanine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine	53±12	12±12
[lJwHsK]: Cyclo-D-leucine-L-noreucine-D-tryptophan-L-histidine-D-serine-L-lysine	69±5	37±10
[lZwHsK]: Cyclo-D-leucine-L-2–aminooctanoic acid -D-tryptophan-L-histidine-D-serine-L-lysine	55±14	42±6

[00077] As seen in the table above, cyclic D,L-α-peptides had an anti-amyloid aggregation effect. The cyclic peptide (designated cyclic peptide-2) having the sequence [lJwHsK], had a drastic effect on amyloid aggregation. However, the linear equivalent of cyclic peptide-2 did not inhibit amyloid formation. Cyclic peptide 2 was also effective in reducing Aβ-induced toxicity in PC12 cells.

[00078] According to embodiments of the invention, cyclic peptides may comprise between 6 or 8 amino acids. According to an embodiment, the cyclic peptide comprises 6 amino acids. In an embodiment, half of the amino acid residues are in the D-formation, and the other half are in the L-formation. In an embodiment of the invention, the amino acids alternate between the D and L- formations.

[00079] Without being bound by theory, it is suggested that cyclic peptides associate with Aβ via hydrogen bonding, π−π and hydrophobic interactions. They then induce conformational changes to the structure of Aβ, thereby inhibiting aggregation and/or fibril formation. The

inventors suggest that the cyclic D,L-α-peptides induce a parallel β-sheet structure in Aβ (as opposed to toxic anti-parallel conformation of aggregated Aβ) and alter its oligomer distribution by stabilizing low-molecular-weight species.

[00080] Cyclic D,L-α-peptides may be used as independent agents for treatment and/or diagnosis of amyloid-associated diseases according to embodiments of the invention. In addition, cyclic D,L-α-peptides may be modified to comprise at least one thiol group without altering their biological activity and may be conjugated to proteinaceous particles to form SMOPs via methods described herein including sonication. Such cyclic D,L-α-peptide-modified SMOPs may be used for treatment or diagnosis of diseases associated with amyloid accumulation.

[00081] Example 3B: Additional Aβ-binding moieties

[00082] Various agents have been found to inhibit amyloid fibril formation. SMOPs according to embodiments of the invention may be prepared using various amyloid-binding agents. Examples of such amyloid-binding agents include scy6llo-cyclohexanehexol, tramiprosate, 2-Hydroxy-3-ethoxy-benzaldehyde and phenol red. These agents can be modified by a thiol group and sonicated with a protein in order to form SMOPs according to embodiments of the invention.

[00083] Polyphenols are a class of compounds which may be used to form amyloid- binding SMOPs according to an embodiment of the invention. Examples of such polyphenols include: Nordihydroguaiaretic acid, curcumin, dobutamine, rosmarinic acid, congo red, 3,3'-bis(3-hydroxyphenyl)pyridazine-3,6-diamine, resveratrol, myricetin, morin, quercetin, kaempferol, apomorphine, exifone, 2,2'-dihydroxybenzophenon, 2,3,4,2',4'-pentahydroxybenzophenone, baicalein, apigenin, catechin, epicatechin, phenolsulphonaphthalein, epicatechin gallate, epigallocatechin gallate, hypericin and tannic acid. These agents can be modified by a thiol group and sonicated with a protein in order to form SMOPs according to embodiments of the invention

[00084] Short peptide inhibitors of amyloid aggregation as described in Neddenriep et al, may be used to form SMOPs according to embodiments of the invention. For example GVVIA-NH_2 and RVVIA-NH_2 are based on the sequence of Aβ(38-42) and have been shown to inhibit amyloid aggregation and toxicity. (Doig) Other peptides derived from the Aβ sequence may

also be used. Another example is a peptide having the sequence STVIIE with all of its amino acids in the D-configuration.

[00085] As to amino acid sequences, including peptides derived from the Aβ sequence, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "substantially identical derivative" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[00086] The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M)

[00087] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Sequences are "substantially identical" if they have a percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, optionally about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence. The identity can exist over a region that is at least about 50 amino acids or nucleotides in length, or over a region that is 75-100 amino acids or nucleotides in length, or, where not specified, across the entire sequence or a polynucleotide or polypeptide.

[00088] Example 4: Administration of SMOP to humans for treatment of disease

[00089] SMOP according to embodiments of the invention may be combined with a pharmaceutically acceptable excipient and administered to a patient in need of amyloid modifying treatment. In an embodiment, the patient suffers from a disease associated with amyloid formation selected from the group consisting of Alzheimer’s disease, Lewy body dementia, cerebral amyloid angiopathy and inclusion body myositis. In an embodiment, the

patient suffers from a disease selected from the group consisting of Diabetes mellitus type 2, Parkinson's disease, transmissible spongiform encephalopathy, Huntington's disease, medullary carcinoma of the thyroid, isolated atrial amyloidosis, atherosclerosis, rheumatoid arthritis, prolactinomas, familial amyloid polyneuropathy, amyloidosis, lattice corneal dystrophy, systemic AL amyloidosis and inclusion body myositis.

[00090] Of the mentioned diseases associated with amyloid formation, different types of amyloids are involved in the pathology of the disease. In an embodiment of the invention, methods of treatment using specific amyloid-binding moieties are conjugated to SMOPs for treatment of specific diseases associated with specific amyloids.

[00091] In an embodiment, a pharmaceutical composition is provided comprising a plurality of SMOP particles and at least one pharmaceutically acceptable excipient.

[00092] In an embodiment of the invention, provided is a method for treating a patient, the method comprising parenteral administration of a pharmaceutical composition, comprising a plurality of SMOP particles. In an embodiment, the parenteral administration is through the intravenous, subcutaneous or intramuscular route.

[00093] In an embodiment, the amount of SMOP administered to the patient ranges from 0.01 mg per day to about 1 g per day. In an embodiment, the treatment is administered between 1 and 4 times daily. In an embodiment, the treatment is administered once weekly, or once a month.

[00094] Example 5: Administration of SMOP to humans for diagnosis of disease

[00095] In order to diagnose a patient to determine if he or she suffers from a disease associated with amyloid aggregation, SMOPs according to embodiments of the invention may be used. In an embodiment, an SMOP comprising a contrasting agent is administered. In an embodiment, the contrasting agent is dissolved within a non-aqueous layer of solvent enclosed by the protein layer of the SMOP. In an embodiment, the contrasting agent is chemically bound to the surface of the SMOP by binding to a protein or to an Aβ binding moiety.

[00096] In an embodiment of the invention, a method of diagnosis of a patient is provided comprising administering to a patient an SMOP comprising an Aβ binding moiety and at least one contrasting agent; subjecting the patient to a diagnostic test to determine presence of an amyloid plaque; and treating the patient with a treatment to treat a disease associated with an amyloid plaque. An exemplary contrasting agent which may be used is magnetite. SMOPs may

be manufactured with magnetite in an oil layer, surrounded by a protein, and may be administered to allow binding of an amyloid plaque. A patient may then undergo magnetic resonance imaging (MRI) diagnosis to assess for quantities and locations of magnetite, thereby indicating presence and locations of amyloid plaques.

[00097] In addition, other diagnostic agents such as radioactive agents may be used for diagnosis. For example, radioactive Technetium-99 (^{99}Tc) may be incorporated into SMOP according to embodiments of the invention. The SMOPs may then be administered and the patient may undergo computed tomography (CT) scanning to locate potential amyloid plaques.

[00098] Example 6: Synthetic procedures for manufacture of SMOP

[00099] In addition to manufacturing SMOPs modified by Aβ-binding moieties, additional embodiments of the invention relate to manufacturing SMOPs modified (on their outer surfaces) by other biologically active components, such as peptides.

[000100] Methods according to embodiments of the invention comprise sonicating mixtures of thiol-containing proteins and biologically active components to form SMOPs modified by biologically active components. In an embodiment of the invention, the mixtures further comprise an organic solvent. In an embodiment of the invention, the organic solvent is an oil. In an embodiment, the organic solvent is n-dodecane. In an embodiment, the mixture further comprises a fluorescent agent. In an embodiment, the fluorescent agent comprises curcumin or Nile red.

[000101] In an embodiment of the invention, SMOP further comprises a contrasting agent. SMOPs comprising, for example a metal-comprising contrasting agent such as magnetite, may be detectable via MRI. SMOPs comprising magnetite may be synthesized by addition of iron pentacarbonyl to the reaction mixture to form magnetite-comprising SMOPs. In addition, magnetite may be added to the reaction solution and incorporated into the microspheres.

[000102] In an embodiment, the oil is a pharmaceutically acceptable oil. In an embodiment, the pharmaceutically acceptable oil is selected from the group consisting of: a naturally-derived liquid oil, such as canola oil, corn oil, coconut oil, sunflower seed oil, vegetable oil, cottonseed oil, mineral oil, peanut oil, sesame oil, soybean oil, and/or olive oil.

[000103] In an embodiment, the ratio of thiol containing protein to biologically active peptide in the reaction mixture is 1:1 to 1:1000.

[000104] In an embodiment the acoustic power applied to the reaction mixture is between 100 and 500 W/cm^2. In an embodiment, the acoustic power is about 150 W/cm^2.

[000105] In an embodiment, the mixture is sonicated for between about 1 and about 30 minutes. In an embodiment the mixture is sonicated for about 3 minutes.

[000106] In an embodiment, the reaction provides particles having an average particle size, as determined by DLS, between about 0.2 and about 2 μm. In an embodiment, the average particle size is between about 0.4 and about 1 μm. In an embodiment, the average particle size is between about 0.7 and about 1 μm.

[000107] There is further provided in accordance with an embodiment of the invention, a particle comprising: a spherical protein layer comprising a protein, a polypeptide, a biopolymer or a polymer, and an amyloid-binding moiety conjugated to the protein layer. Optionally, the diameter of the particle is between about 0.2 and about 2 μm, between about 0.4 and about 1 μm, between about 0.7 to about 1 μm. Optionally, the amyloid-binding moiety is a peptide which optionally binds Aβ. Optionally, the peptide is derived from Aβ. Optionally, the peptide comprises the amino acid sequence KLVFF. Optionally, the amyloid-binding moiety comprises a cyclic peptide having an even number of amino acids. Optionally, the cyclic peptide comprises 6 or 8 amino acids, optionally alternating L- and D-amino acids. Optionally, the cyclic peptide is composed of 6 amino acids, 3 of the amino acids in L-configuration and 3 of the amino acids in D-configuration. Optionally, the cyclic peptide is selected from the group consisting of cyclic peptides having the sequence: D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine; D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-alanine; D-leucine-L-leucine-D-tryptophan-L-histidine-D-alanine-L-lysine; D-leucine-L-leucine-D-tryptophan-L-alanine-D-serine-L-lysine; D-leucine-L-leucine-D-alanine-L-histidine-D-serine-L-lysine; D-leucine-L-alanine-D-tryptophan-L-histidine-D-serine-L-lysine; D-alanine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine; D-leucine-L-noreucine-D-tryptophan-L-histidine-D-serine-L-lysine and D-leucine-L-2–aminooctanoic acid -D-tryptophan-L-histidine-D-serine-L-lysine. Optionally, the amyloid-binding moiety is bound to the protein through a disulfide bond. Optionally, the protein comprises an albumin protein. Optionally, the protein comprises human serum albumin or BSA. Optionally, the particle comprises an inner solvent layer, surrounded by the protein layer. Optionally, the solvent layer comprises an oil, optionally a pharmaceutically acceptable oil, optionally, canola oil. Optionally, the particle

further comprising PEG attached to the surface of a particle. Optionally, the particle comprises a contrasting agent.

[000108] There is further provided in accordance with an embodiment of the invention, a method for the manufacture of a surface modified proteinaceous particle comprising: combining a biologically active moiety having a thiol group and a protein having a thiol group with an organic solvent to form a reaction mixture, and sonicating the reaction mixture to form a surface modified proteinaceous particle. Optionally, the organic solvent is pharmaceutically acceptable oil. Optionally, the method further comprises separating the surface modified proteinaceous particle from the reaction mixture. Optionally, the surface modified proteinaceous particle is formed from the protein and the biologically active moiety in a one-pot reaction. Optionally, the biologically active moiety comprises an amyloid-binding moiety. Optionally, the protein comprises an albumin. Optionally, the reaction mixture further comprises a contrasting agent.

[000109] There is further provided in accordance with an embodiment of the invention, a cyclic peptide having the sequence selected from the group consisting of: Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine; Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-alanine; Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-alanine-L-lysine; D-leucine-L-leucine-D-tryptophan-L-alanine-D-serine-L-lysine; Cyclo-D-leucine-L-leucine-D-alanine-L-histidine-D-serine-L-lysine; Cyclo-D-leucine-L-alanine-D-tryptophan-L-histidine-D-serine-L-lysine; Cyclo-D-alanine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine; Cyclo-D-leucine-L-noreucine-D-tryptophan-L-histidine-D-serine-L-lysine and Cyclo-D-leucine-L-2–aminooctanoic acid -D-tryptophan-L-histidine-D-serine-L-lysine.

[000110] There is further provided in accordance with an embodiment of the invention, a pharmaceutical composition for treatment or diagnosis of a disease comprising a plurality of particles according to embodiments of the invention. Optionally, the average diameter of the plurality of particles is between about 0.2 and about 2 µm, between about 0.4 and about 1 µm or between about 0.7 to about 1 µm.

[000111] There is further provided in accordance with an embodiment of the invention, a method of treating a disease associated with amyloid formation comprising administering to a patient in need thereof a pharmaceutical composition according to an embodiment of the invention. Optionally, the disease is selected from the group consisting Alzheimer's disease, Lewy body

dementia, cerebral amyloid angiopathy and inclusion body myositis, diabetes mellitus type 2, Parkinson's disease, transmissible spongiform encephalopathy, Huntington's disease, medullary carcinoma of the thyroid, isolated atrial amyloidosis, atherosclerosis, rheumatoid arthritis, prolactinomas, familial amyloid polyneuropathy, amyloidosis, lattice corneal dystrophy, systemic AL amyloidosis and inclusion body myositis.

[000112] There is further provided in accordance with an embodiment of the invention, a method for treating a disease associated with amyloid formation comprising: administering to a patient a particle according to an embodiment of the invention, analyzing the patient to determine presence of particles associated with amyloid in the patient and determining if the patient has a disease and treating the patient if the patient was determined to have a disease associated with amyloid. Optionally, the disease is selected from the group consisting of Alzheimer's disease, Lewy body dementia, cerebral amyloid angiopathy and inclusion body myositis, diabetes mellitus type 2, Parkinson's disease, transmissible spongiform encephalopathy, Huntington's disease, medullary carcinoma of the thyroid, isolated atrial amyloidosis, atherosclerosis, rheumatoid arthritis, prolactinomas, familial amyloid polyneuropathy, amyloidosis, lattice corneal dystrophy, systemic AL amyloidosis and inclusion body myositis.

[000113] In the description and claims of the present application, each of the verbs, "comprise," "include" and "have," and conjugates thereof, are used to indicate that the object or objects of the verb are not necessarily a complete listing of components, elements or parts of the subject or subjects of the verb.

[000114] Descriptions of embodiments of the invention in the present application are provided by way of example and are not intended to limit the scope of the invention. The described embodiments comprise different features, not all of which are required in all embodiments of the invention. Some embodiments utilize only some of the features or possible combinations of the features. Variations of embodiments of the invention that are described, and embodiments of the invention comprising different combinations of features noted in the described embodiments, will occur to persons of the art. The scope of the invention is limited only by the claims.

Works Cited:

A. J. Doig. *Current Opinion in Drug Discovery & Development* **2007**, *10*, 533-539.

M. J. Kogan, N. G. Bastus, R. Amigo, D. Grillo-Bosch, E. Araya, A. Turiel, A. Labarta, E. Giralt and V. F. Puntes. *Nano Letters* **2006**, *6*, 110-115.

B. Neddenriep, A. Calciano, D. Conti, E. Sauve, M. Paterson, E. Bruno and D.A. Moffet. *The Open Biotechnology Journal*, **2011**, 5, 39-46.

N. Skiertenko, M. Richman, Y. Nitzan, A. Gedanken and S Rahimipour. *Chem. Commun.*, **2011**, 47, 12277-12279.

CLAIMS

1. A particle comprising:
a spherical protein layer comprising a protein, a polypeptide, a biopolymer or a polymer, and
an amyloid-binding moiety conjugated to the protein layer.
2. The particle according to claim 1 wherein the diameter of the particle is between about 0.2 and about 2 micrometers.
3. The particle according to claim 1 or 2 wherein the diameter of the particle is between about 0.4 and about 1 micrometer.
4. The particle according to any one of the previous claims wherein the diameter of the particle is about 0.7 to about 1 micrometer.
5. The particle according to any one of the previous claims wherein the amyloid-binding moiety is a peptide.
6. The particle according to any one of the previous claims wherein the amyloid-binding moiety binds Aβ.
7. The particle according to claim 5 wherein the peptide is derived from Aβ.
8. The particle according to claim 5 wherein the peptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3.
9. The particle according to any one of the previous claims wherein the amyloid-binding moiety comprises a cyclic peptide having and even number of amino acids.
10. The particle according to claim 9 wherein the cyclic peptide comprises 6 or 8 amino acids.
11. The particle according to claim 9 wherein the cyclic peptide comprises alternating L- and D-amino acids.
12. The particle according to claim 9 wherein the cyclic peptide is composed of 6 amino acids, 3 of the amino acids in L-configuration and 3 of the amino acids in D-configuration.
13. The particle according to claim 9 wherein the cyclic peptide is selected from the group consisting of cyclic peptides having the sequence:
Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine;
Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-alanine;
Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-alanine-L-lysine;

Cyclo-D-leucine-L-leucine-D-tryptophan-L-alanine-D-serine-L-lysine;
Cyclo-D-leucine-L-leucine-D-alanine-L-histidine-D-serine-L-lysine;
Cyclo-D-leucine-L-alanine-D-tryptophan-L-histidine-D-serine-L-lysine;
Cyclo-D-alanine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine;
Cyclo-D-leucine-L-noreucine-D-tryptophan-L-histidine-D-serine-L-lysine and
Cyclo-D-leucine-L-2–aminooctanoic acid -D-tryptophan-L-histidine-D-serine-L-lysine.

14. The particle according to any one of the previous claims wherein the amyloid-binding moiety is bound to the protein layer through a disulfide bond.
15. The particle according to any one of the previous claims wherein the protein comprises an albumin protein.
16. The particle according to any one of the previous claims wherein the protein comprises human serum albumin or bovine serum albumin.
17. The particle according to any one of the previous claims wherein the particle comprises an inner organic solvent layer, surrounded by the protein layer.
18. The particle according to claim 17 wherein the organic solvent layer comprises an oil
19. The particle according to claim 18 wherein the oil comprises a pharmaceutically acceptable oil.
20. The particle according to claim 17 wherein the oil comprises canola oil.
21. The particle according to any one of the previous claims further comprising polyethylene glycol attached to the surface of a particle.
22. The particle according to any one of the previous claims wherein a particle comprises a contrasting agent.
23. A method for the manufacture of a surface modified proteinaceous particle comprising:
combining a biologically active moiety having a thiol group and a protein or peptide having a thiol group with an organic solvent to form a reaction mixture; and
sonicating the reaction mixture to form a surface modified proteinaceous particle.
24. The method according to claim 23 wherein the organic solvent is pharmaceutically acceptable oil.
25. The method according to claim 23 or 24 further comprising separating the surface modified proteinaceous particle from the reaction mixture.

26. The method according to any one of claims 23-25 wherein the surface modified proteinaceous particle is formed from the protein or peptide and the biologically active moiety in a one-pot reaction.
27. The method according to any one of claims 23-26 wherein the biologically active moiety comprises an amyloid-binding moiety.
28. The method according to any one of claims 23-27 wherein the protein comprises an albumin.
29. The method according to any one of claims 23-28 wherein the reaction mixture further comprises a contrasting agent.
30. A cyclic peptide having the sequence selected from the group consisting of:
Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine;
Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-serine-L-alanine;
Cyclo-D-leucine-L-leucine-D-tryptophan-L-histidine-D-alanine-L-lysine;
Cyclo-D-leucine-L-leucine-D-tryptophan-L-alanine-D-serine-L-lysine;
Cyclo-D-leucine-L-leucine-D-alanine-L-histidine-D-serine-L-lysine;
Cyclo-D-leucine-L-alanine-D-tryptophan-L-histidine-D-serine-L-lysine;
Cyclo-D-alanine-L-leucine-D-tryptophan-L-histidine-D-serine-L-lysine;
Cyclo-D-leucine-L-noreucine-D-tryptophan-L-histidine-D-serine-L-lysine and
Cyclo-D-leucine-L-2–aminooctanoic acid -D-tryptophan-L-histidine-D-serine-L-lysine.
31. A pharmaceutical composition for treatment or diagnosis of a disease comprising a plurality of particles according to any one of claims 1 to 22.
32. The pharmaceutical composition according to claim 31 wherein the average diameter of the plurality of particles is between about 0.2 and about 2 micrometers.
33. The pharmaceutical composition according to claim 31 wherein the average diameter of the plurality of particles is between about 0.4 and about 1 micrometer.
34. The pharmaceutical composition according to claim 31 wherein the average diameter of the plurality of particles is between about 0.7 to about 1 micrometer.
35. A method of treating a disease associated with amyloid formation comprising administering to a patient in need thereof a pharmaceutical composition according to any one of claims 31 to 34 or a cyclic peptide according to claim 30.
36. The method according to claim 35 wherein the disease is selected from the group consisting of Alzheimer's disease, Lewy body dementia, cerebral amyloid

angiopathy and inclusion body myositis, diabetes mellitus type 2, Parkinson's disease, transmissible spongiform encephalopathy, Huntington's disease, medullary carcinoma of the thyroid, isolated atrial amyloidosis, atherosclerosis, rheumatoid arthritis, prolactinomas, familial amyloid polyneuropathy, amyloidosis, lattice corneal dystrophy, systemic AL amyloidosis and inclusion body myositis.

37. A method for treating a disease associated with amyloid formation comprising: administering to a patient a particle according to claim 22;
analyzing the patient to determine presence of particles associated with amyloid in the patient and determining if the patient has a disease ; and
treating the patient if the patient was determined to have a disease associated with amyloid.

38. The method according to claim 37 wherein the disease is selected from the group consisting of Alzheimer's disease, Lewy body dementia, cerebral amyloid angiopathy and inclusion body myositis, diabetes mellitus type 2, Parkinson's disease, transmissible spongiform encephalopathy, Huntington's disease, medullary carcinoma of the thyroid, isolated atrial amyloidosis, atherosclerosis, rheumatoid arthritis, prolactinomas, familial amyloid polyneuropathy, amyloidosis, lattice corneal dystrophy, systemic AL amyloidosis and inclusion body myositis.

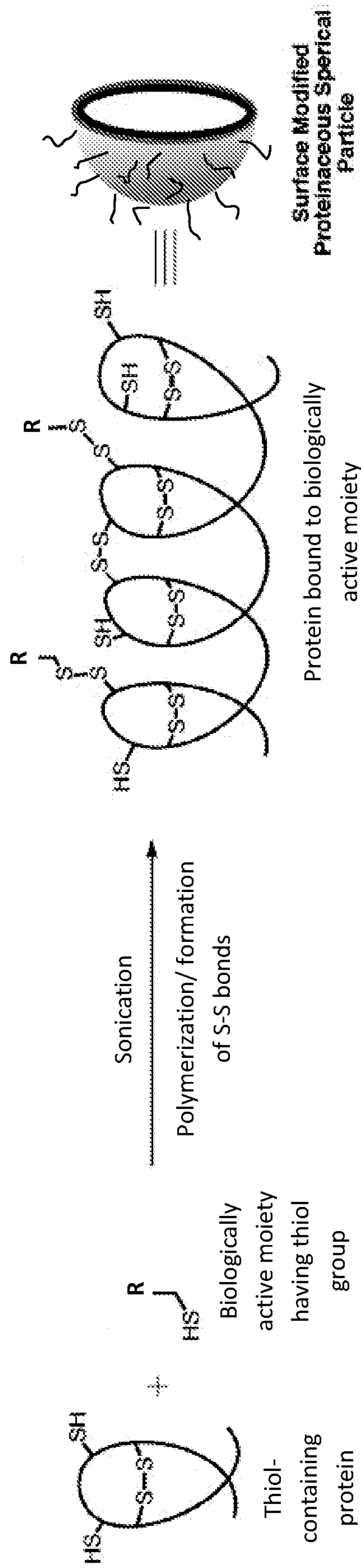

Fig. 1

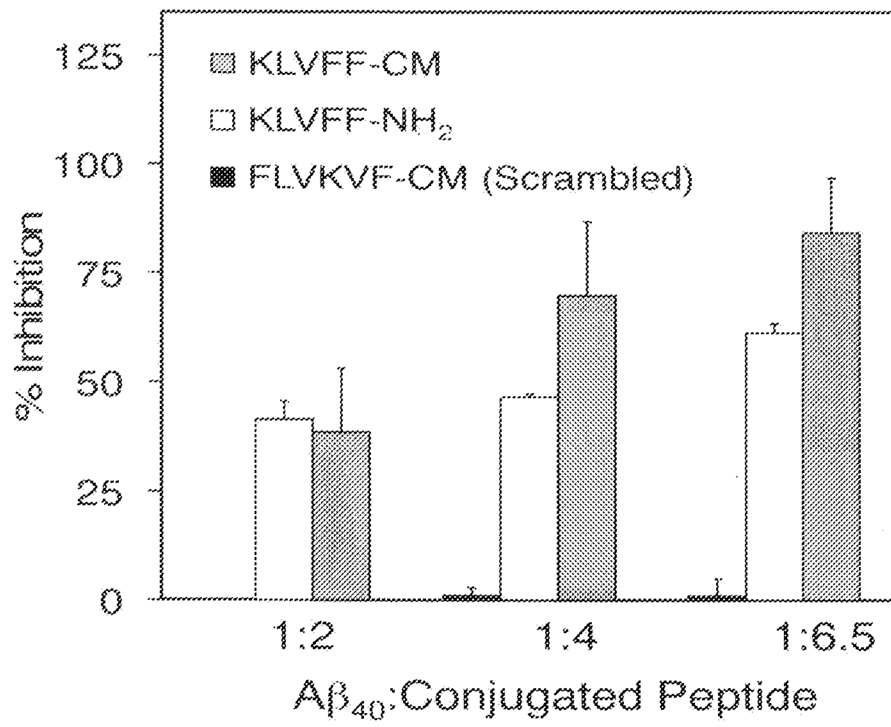

Fig 2A

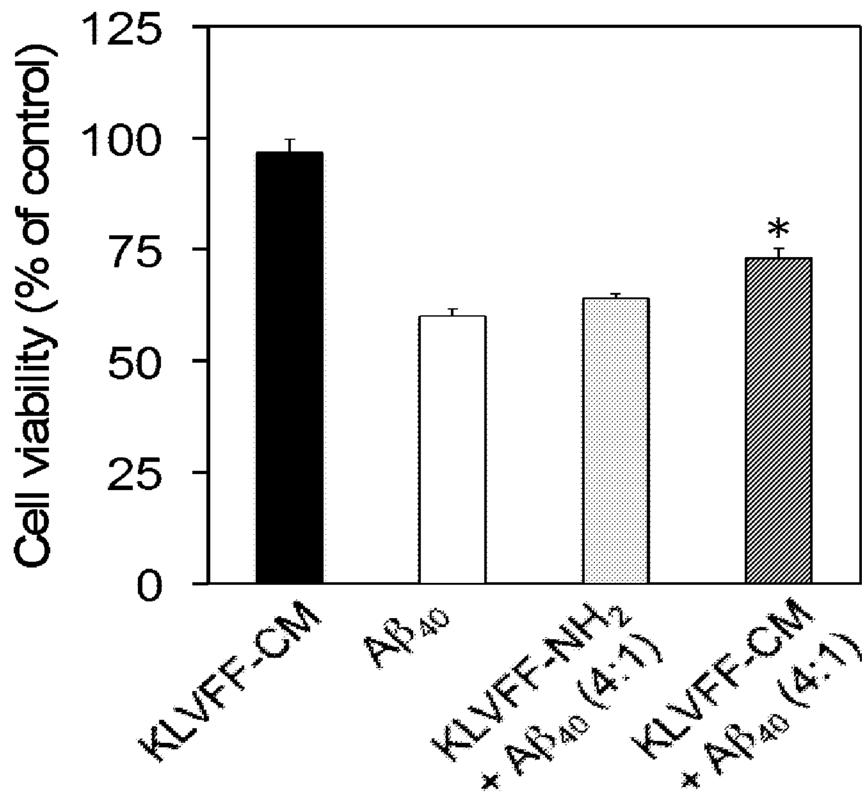

Fig 2B

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2012/054037

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48
ADD. A61P25/28

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)
A61K

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data, DISSERTATION ABS, PASCAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 200359 Thomson Scientific, London, GB; AN 2003-620628 XP002690096, -& JP 2002 265382 A (DOKURITSU GYOSEI HOJIN SANGYO GIJUTSU SO) 18 September 2002 (2002-09-18) abstract -----	1-38
Y	WO 97/21728 A1 (KAROLINSKA INNOVATIONS AB [SE]; NORDSTEDT CHRISTER [SE]; NAESLUND JAN) 19 June 1997 (1997-06-19) examples claims -----	1-38

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 2013	23/01/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dullaart, Anwyn

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Week 200281 Thomson Scientific, London, GB; AN 2002-744799 XP002690097, -& JP 2002 241302 A (DOKURITSU GYOSEI HOJIN SANGYO GIJUTSU SO) 28 August 2002 (2002-08-28) abstract -----	1-38
Y	WO 2005/058941 A2 (ELAN PHARM INC [US]; WYETH CORP [US]; ARUMUGHAM RASAPPA G [US]; PRASAD) 30 June 2005 (2005-06-30) examples 10-12 -----	1-38
Y	WO 2007/015107 A2 (HAEMOSTATIX LTD [GB]; GOODALL ALISON HELENA [GB]; TAYLOR SARAH MARGARE) 8 February 2007 (2007-02-08) examples 1-2,5 -----	1-38
Y	WO 2005/023096 A2 (POINT BIOMEDICAL CORP [US]; GABE JEFFREY D [US]; OTTOBONI THOMAS B [US) 17 March 2005 (2005-03-17) example 1 -----	1-38
Y	WO 94/18954 A1 (CLOVER CONS LTD [CH]; GRINSTAFF MARK W [US]; SOON SHIONG PATRICK [US];) 1 September 1994 (1994-09-01) examples 1,11,12 -----	1-38
Y	US 2004/258762 A1 (BOPPART STEPHEN A [US] ET AL) 23 December 2004 (2004-12-23) examples 2,6 -----	1-38
X	WO 2011/053901 A2 (UNIV NORTHWESTERN [US]; DRAVID VINAYAK P [US]; SHARMA SAURABH [US]; TO) 5 May 2011 (2011-05-05)	1-6,14
Y	page 12 - page 13 -----	1-38
Y	BASTUS ET AL: "Gold nanoparticles for selective and remote heating of beta-amyloid protein aggregates", MATERIALS SCIENCE AND ENGINEERING C, vol. 27, no. 5-8, 8 August 2007 (2007-08-08), pages 1236-1240, XP022190479, ISSN: 0928-4931, DOI: 10.1016/J.MSEC.2006.08.003 abstract page 1237 -----	1-38

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IVONNE OLMEDO ET AL: "How Changes in the Sequence of the Peptide CLPFFD-NH 2 Can Modify the Conjugation and Stability of Gold Nanoparticles and Their Affinity for [beta]-Amyloid Fibrils", BIOCONJUGATE CHEMISTRY, vol. 19, no. 6, 1 June 2008 (2008-06-01), pages 1154-1163, XP055048828, ISSN: 1043-1802, DOI: 10.1021/bc800016y abstract page 1155 -----	1-38
Y	EP 2 258 398 A1 (ARACLON BIOTECH S L [ES]) 8 December 2010 (2010-12-08) page 15 - page 16 claims -----	1-38
Y	MAJZIK A ET AL: "Functionalization of gold nanoparticles with amino acid, beta-amyloid peptides and fragment", COLLOIDS AND SURFACES. B, BIOINTERFACES, vol. 81, no. 1, 1 November 2010 (2010-11-01), pages 235-241, XP027435198, ISSN: 0927-7765, DOI: 10.1016/J.COLSURFB.2010.07.011 [retrieved on 2010-07-13] abstract page 237, right-hand column, paragraph 3.2 - page 240 -----	1-38
Y	EL MOUEDDEN M ET AL: "Development of a specific ELISA for the quantitative study of amino-terminally truncated beta-amyloid peptides", JOURNAL OF NEUROSCIENCE METHODS, vol. 145, no. 1-2, 30 June 2005 (2005-06-30), pages 97-105, XP027670294, ISSN: 0165-0270 [retrieved on 2005-06-30] abstract figure 1 -----	1-38
Y,P	BRADLEY NEDDENRIEP ET AL: "Short Peptides as Inhibitors of Amyloid Aggregation", THE OPEN BIOTECHNOLOGY JOURNAL, vol. 5, no. 1, 23 December 2011 (2011-12-23), pages 39-46, XP055049166, ISSN: 1874-0707, DOI: 10.2174/1874070701105010039 cited in the application	1-38
L	the whole document -----	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2002265382 A	18-09-2002	JP 3735705 B2	18-01-2006
		JP 2002265382 A	18-09-2002
WO 9721728 A1	19-06-1997	AU 1072897 A	03-07-1997
		EP 0866805 A1	30-09-1998
		US 6331440 B1	18-12-2001
		US 2002094957 A1	18-07-2002
		US 2004157781 A1	12-08-2004
		WO 9721728 A1	19-06-1997
JP 2002241302 A	28-08-2002	NONE	
WO 2005058941 A2	30-06-2005	AR 047062 A1	04-01-2006
		AU 2004299512 A1	30-06-2005
		AU 2011200785 A1	17-03-2011
		BR PI0417689 A	03-04-2007
		CA 2549552 A1	30-06-2005
		CN 1934127 A	21-03-2007
		CR 8445 A	20-02-2008
		EA 200601168 A1	29-12-2006
		EA 200702209 A1	28-02-2008
		EC SP066646 A	20-12-2006
		EP 1699810 A2	13-09-2006
		EP 2336147 A2	22-06-2011
		EP 2460813 A1	06-06-2012
		EP 2479184 A2	25-07-2012
		IL 176250 A	30-11-2011
		JP 4696079 B2	08-06-2011
		JP 2007534650 A	29-11-2007
		KR 20070026363 A	08-03-2007
		KR 20110117210 A	26-10-2011
		MX PA06006821 A	23-08-2006
		MY 144231 A	15-08-2011
		SG 149039 A1	29-01-2009
		TW I357414 B	01-02-2012
		TW 201206956 A	16-02-2012
		US 2007161088 A1	12-07-2007
		US 2008145373 A1	19-06-2008
		US 2008299074 A1	04-12-2008
		US 2011287042 A1	24-11-2011
		US 2012207706 A1	16-08-2012
		WO 2005058941 A2	30-06-2005
WO 2007015107 A2	08-02-2007	AU 2006274685 A1	08-02-2007
		CA 2625917 A1	08-02-2007
		CN 101267843 A	17-09-2008
		EP 1919508 A2	14-05-2008
		EP 2335737 A2	22-06-2011
		GB 2429153 A	21-02-2007
		JP 2009505964 A	12-02-2009
		US 2009203619 A1	13-08-2009
		WO 2007015107 A2	08-02-2007
WO 2005023096 A2	17-03-2005	EP 1673108 A2	28-06-2006
		US 2005106105 A1	19-05-2005
		US 2009162293 A1	25-06-2009
		WO 2005023096 A2	17-03-2005

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2012/054037

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9418954	A1	01-09-1994	AT	264671	T	15-05-2004
			AU	673057	B2	24-10-1996
			BR	9405798	A	12-12-1995
			CA	2155947	A1	01-09-1994
			CN	1118136	A	06-03-1996
			DE	69433723	D1	27-05-2004
			DE	69433723	T2	24-02-2005
			DK	0693924	T3	09-08-2004
			EP	0693924	A1	31-01-1996
			ES	2219646	T3	01-12-2004
			HK	1097449	A1	09-12-2011
			JP	3746293	B2	15-02-2006
			JP	H08507075	A	30-07-1996
			NO	953278	A	13-10-1995
			NZ	262679	A	22-08-1997
			PT	693924	E	30-09-2004
			US	5498421	A	12-03-1996
			US	5635207	A	03-06-1997
			US	5639473	A	17-06-1997
			US	2009048331	A1	19-02-2009
			WO	9418954	A1	01-09-1994
US 2004258762	A1	23-12-2004	NONE			
WO 2011053901	A2	05-05-2011	US	2012308657	A1	06-12-2012
			WO	2011053901	A2	05-05-2011
EP 2258398	A1	08-12-2010	AR	076892	A1	13-07-2011
			AU	2010251961	A1	22-12-2011
			CA	2763569	A1	02-12-2010
			CN	102458478	A	16-05-2012
			EP	2258398	A1	08-12-2010
			EP	2435093	A1	04-04-2012
			JP	2012528111	A	12-11-2012
			KR	20120052906	A	24-05-2012
			TW	201109033	A	16-03-2011
			US	2012130049	A1	24-05-2012
			WO	2010136487	A1	02-12-2010