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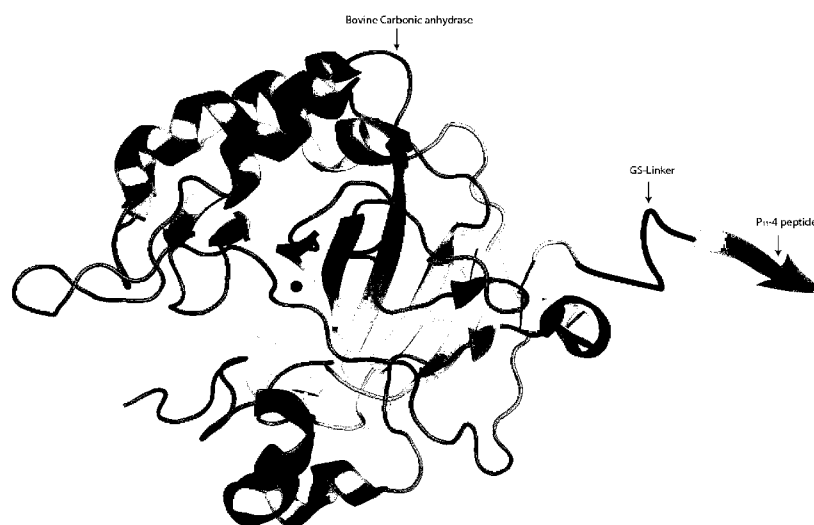
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(54) Title: ENZYMATICALLY ACTIVE SUPRAMOLECULAR ASSEMBLIES

Figure 1



(57) Abstract: The present invention generally relates to enzymatically active supramolecular assemblies and methods of production thereof. In particular, the present invention relates to an enzymatically active supramolecular assembly comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

ENZYMATICALLY ACTIVE SUPRAMOLECULAR ASSEMBLIES

TECHNICAL FIELD

[001] The present invention generally relates to enzymatically active supramolecular assemblies and methods of production thereof. In particular, the present invention relates to an enzymatically active supramolecular assembly comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

BACKGROUND OF THE INVENTION

[002] Enzymes are catalysts bearing some excellent properties (high activity, selectivity and specificity) that permit performing complex chemical processes.

[003] The growing needs of industrial catalysis and the demand for development of sustainable processes are having a profound influence on the progress in the field of biocatalysis.

[004] Historically, enzymatic reactions were carried out such that the enzymes were in their free soluble forms. This is still the preferred mode of biocatalysis for the detergent, food and animal feed industries, because of the low cost of enzymes and the fact that it is acceptable to have residual enzyme at the end of the reaction process. Free enzymes are preferred where high activity is required for a reaction, or if the substrate is in solid form and cannot diffuse.

[005] However, proteins such as enzymes become unstable after purification, undergoing irreversible conformational changes, denaturing, and loss of biochemical activity.

[006] In spite of their excellent catalytic properties, enzyme properties have to be usually improved before their implementation at industrial scale (for example, where many cycles of high yield processes are desired). Generally, soluble enzymes have to be immobilized to be reused for long times in industrial reactors.

[007] Furthermore, if the cost of enzyme production is high, then reuse and recycling of the enzyme is an attractive approach. For this reason, enzymes are attached to various solid supports that allow their easy reuse. Several immobilisation methods like adsorption, entrapment, encapsulation and covalent binding have been investigated to improve the reusability of enzymes.

[008] The choice of immobilisation technique also depends on the conditions required for the process and optimal enzyme activity. For example, immobilisation of enzymes can be used for preventing enzyme residue contamination in the final enzymatically treated product, rather than for cost reduction.

[009] By far the most robust immobilisation method in terms of enzyme retention and stability has been the covalent binding method. This has been further improved using multi-point covalent technique.

[010] However, immobilising enzymes using the covalent method on to solid supports often leads to reduced enzyme activity compared to their free soluble forms. The reasons for this reduced activity are attributed to one of the following; induced changes in structural conformation, enzyme rigidification or deactivation due to agents used in the immobilisation process. Mateo, C. *et al.* (2007) [*Improvement of enzyme activity, stability and selectivity via immobilization techniques*. Enzyme and Microbial Technology, 40(6), pp.1451–1463] reviews the factors governing the stabilisation of immobilised enzymes.

[011] For example, covalent attachment of enzymes often promotes structural rigidity leading to reduction in activity.

[012] In addition to the reduction in catalytic activity caused by structural rigidity, another drawback is that the catalytic efficiency of immobilised enzyme is sometimes limited by the mass-transfer limitations which are controlled by the substrate/product diffusion rates.

[013] Furthermore, enzymes immobilised onto surfaces by non-specific covalent bonding can exist in a large number of possible orientations, for example, with some enzymes oriented such that their binding or active sites are exposed whereas others may be oriented such that their active sites are not exposed, and thus not able to undergo selective catalytic reactions with the substrate of interest.

[014] In addition to orientation, protein density may also be poorly controlled. Proteins are also subject to time-dependent denaturing, denaturing during immobilization, and leaching of the entrapped protein subsequent to immobilization. Furthermore, immobilisation may limit contact between an enzyme and the substrate of interest.

[015] It would therefore be desirable to develop enzymes that can be formed into materials, or incorporated into or onto materials.

[016] It would also be desirable to develop immobilized enzymes that can be formed into materials, or incorporated into or onto materials, without the need to immobilise the enzyme to a support material, such as a solid support.

[017] It would also be desirable to develop immobilized enzymes without reduced catalytic activity compared to the free enzyme.

[018] It would also be desirable to develop immobilized enzymes that can switch between an immobilised and free state.

[019] It would also be desirable to develop immobilized enzymes that can be recovered from their immobilized state, e.g. in solution, and be re-formed into an immobilized state.

SUMMARY OF THE INVENTION

[020] The present invention provides in a first aspect an enzymatically active supramolecular assembly comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme. Advantageously, the present inventors have demonstrated that a self-assembly polypeptide bound to an enzyme allows the formation of a supramolecular assembly where the catalytic activity of the enzyme in the supramolecular assembly is not reduced relative to the free enzyme.

[021] Advantageously, the assembly, components and methods avoid problems associated with conventional enzyme immobilisation systems.

[022] In one embodiment, the plurality of self-assembling enzymatic components are covalently linked.

[023] In another embodiment, the self-assembly polypeptide and the enzyme are covalently linked.

[024] Advantageously, the present inventors have demonstrated that enzymatically active nanoparticles can be formed in the absence of a carrier, a solid support, the need to covalently link the enzyme to a solid support, or to immobilise the enzyme.

[025] Accordingly, the present invention provides a supramolecular assembly which can be formed without the need for immobilisation of the enzyme on a solid support. For example, the supramolecular assembly can be formed without the need to covalently link the enzyme to a solid support.

[026] In one aspect the self-assembly polypeptide is a pH responsive self-assembly polypeptide.

[027] In one aspect the self-assembling enzymatic components are responsive to the concentration of Mg^{2+} .

[028] In one embodiment the assembly is selected from the group consisting of a particle, fibre, sheet or a combination thereof. In a preferred embodiment where the assembly is a particle, the particle is spherical. In one embodiment, the particle has a diameter of from about 20 nm to about 200 nm, and more preferably a diameter of from about 50 to about 100 nm.

[029] In another embodiment, the particle has a diameter of about 20 nm, 30 nm, 40 nm, 100 nm, 200 nm, 400 nm, 600 nm, 1000 nm or about 1500 nm.

[030] In one embodiment, the assembly is a hydrogel or an aggregate.

[031] The present inventors have demonstrated that a supramolecular assembly provided herein can convert carbon dioxide to bicarbonate. Accordingly, in one aspect the present invention provides an assembly as described herein, wherein the enzyme converts carbon dioxide to bicarbonate.

[032] In one embodiment, the enzyme is selected from the group consisting of a formate dehydrogenase, a carbonic anhydrase, a RuBisCO and combinations thereof.

[033] In another embodiment, the enzyme is selected from the group consisting of a cutinase, a tyrosinase and an aminotransferase.

[034] Advantageously, the present inventors have demonstrated that enzymatically active nanoparticles can be formed in the absence of a carrier or other components. Accordingly, in one aspect the invention provides a supramolecular assembly wherein the assembly self assembles in the absence of a carrier. In another embodiment, the present invention provides an assembly wherein the assembly consists of the plurality of self-assembling enzymatic components.

[035] The present inventors have also demonstrated that an assembly as described herein is stable across a range of temperatures for a period of months. Accordingly, in one aspect the present invention provides an assembly as described herein wherein the assembly is stable between 25 to 50°C. In another aspect, the assembly is stable for at least 2 months.

[036] In one embodiment, the catalytic activity of an enzyme of the assembly is at least 80% of the catalytic activity of the free enzyme. Preferably, the catalytic activity of the enzyme is at least 98% of the catalytic activity of the free enzyme.

[037] In another aspect the assembly is switchable between the supramolecular assembly state and an unassembled state. In one embodiment, the assembly is pH responsive.

[038] In one embodiment, the self-assembly polypeptide is a P₁₁ family polypeptide. In another embodiment, the self-assembly polypeptide is P₁₁-4.

[039] In another aspect, a linker binds the self-assembly polypeptide to the enzyme of the self-assembling enzymatic component. In one embodiment, the linker covalently binds the self-assembly polypeptide to the enzyme. In another

embodiment, the linker comprises a polypeptide. In a further embodiment, at least 90% of the amino acids in the linked polypeptide are glycine or serine or a combination thereof. For example, in one embodiment, the linker is a glycine-serine (GS) linker, and in another embodiment the GS linker comprises SEQ ID NO: 2.

[040] The present inventors have demonstrated that a supramolecular assembly provided herein can convert carbon dioxide to bicarbonate. Accordingly, the present invention provides a process for sequestering carbon dioxide from a carbon dioxide containing fluid or gas, wherein the method comprises the steps of: providing an enzymatically active supramolecular assembly described herein, wherein the enzyme is carbonic anhydrase; contacting the assembly with the carbon dioxide containing fluid or gas; and converting carbon dioxide in the carbon dioxide containing fluid or gas to bicarbonate. In one embodiment the enzymatically active supramolecular assembly is immobilised. In another embodiment the assembly is immobilised on a film or membrane. In a further embodiment, the fluid or gas is a fluid or gas stream.

[041] The present inventors have demonstrated that a supramolecular assembly provided herein can convert carbon dioxide to bicarbonate.

[042] In another aspect the present invention provides an enzymatically active supramolecular assembly comprising a first self-assembling enzymatic component and a second self-assembling enzymatic component, wherein the first self-assembling enzymatic component comprises a self-assembly polypeptide bound to a first enzyme, and wherein the second self-assembling enzymatic component comprises a self-assembly polypeptide bound to a second enzyme.

[043] In another aspect the present invention provides a self-assembling enzymatic component wherein the self-assembling enzymatic component

comprises a self-assembly polypeptide bound to an enzyme, and wherein the self-assembling enzymatic component is capable of forming an enzymatically active supramolecular assembly in the presence of at least one further self-assembling enzymatic component comprising a self-assembly polypeptide bound to an enzyme.

[044] The present inventors have demonstrated that a supramolecular assemblies can be formed using tyrosinase, a cutinase and a carbonic anhydrase, and can produce reaction products of these enzymes.

[045] Accordingly, in one embodiment the present invention provides a process for producing a tyrosinase reaction product from a solution comprising a substrate of tryosinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is a tyrosinase;
- contacting the assembly with solution comprising a substrate of the tyrosinase; and
- converting a substrate in the solution comprising the substrate of the tyrosinase to a reaction product of the tryosinase.

[046] In another embodiment the present invention provides a process for producing a tyrosinase reaction product from a solution comprising a substrate of tryosinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is a tyrosinase;
- contacting the assembly with a solution comprising a substrate selected from the group consisting of tyrosine, DOPA, Tyrosol, Phenols and Catechols, Sericin, Phenolic species, Monophenols, diphenols, and α -lactalbumin
- converting a substrate in the solution comprising the substrate of the tyrosinase to a reaction product of the tryosinase.

[047] In another embodiment provides a process for producing an aminotransferase reaction product from a solution comprising a substrate of aminotrasferase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is a aminotransferase;
- contacting the assembly with a solution comprising a substrate of the aminotransferase; and
- converting a substrate in the solution comprising the substrate of the aminotransferase to a reaction product of the aminotransferase.

[048] In another embodiment the present invention provides a process for producing an aminotransferase reaction product from a solution comprising a substrate of aminotrasferase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is an aminotransferase;
- contacting the assembly with solution comprising at least one substrate selected from the group consisting of amine enantiomers, pyruvate, alanine, ketones, δ -keto acid ester, acetophenone, 1-phenylethylamine, ketone and isopropylamine; and
- converting a substrate in the solution comprising the substrate of the aminotransferase to a reaction product of the aminotransferase.

[049] In another embodiment the present invention a process for producing chiral amines from a solution comprising amino acids, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is an aminotransferase;
- contacting the assembly with a solution comprising amino acids; and

- converting the amino acids in the solution comprising amino acids to chiral amino acids. In one embodiment aminotransferase is ω -aminotransferase.

[050] In another embodiment the present invention provides a cutinase reaction product from a solution comprising a substrate of cutinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is a cutinase;
- contacting the assembly with a solution comprising a substrate of the cutinase; and
- converting a substrate in the solution comprising the substrate of the cutinase to a reaction product of the cutinase.

[051] In another embodiment the present invention provides a process for producing a cutinase reaction product from a solution comprising a substrate of cutinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly as described herein, wherein the enzyme is a cutinase;

[052] contacting the assembly with a solution comprising a substrate selected from the group consisting of apple peel, tomato peel, orange peel, p-nitrophenyl ester, triolein, tricaprillin, fatty acids, geranyl diphosphate, butyl acetate, hexanol organophosphate malathion, Polycaprolone, and Dihexylphtalate; and

- converting a substrate in the solution comprising the substrate of the cutinase to a reaction product of the cutinase.

[053] In another embodiment the present invention provides a method of forming an enzymatically active supramolecular assembly, the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii)

contacting the solution containing a plurality of self-assembling enzymatic components with a buffer to form an enzymatically active supramolecular assembly.

[054] In one embodiment, the assembly is a particle. In another embodiment, the particle is spherical.

[055] In one embodiment, the particle has a diameter of about 100 nm, and wherein the buffer comprises about 50mM Tris-HCl at about pH 6.8. In another embodiment, the particle has a diameter of about 400 nm, and wherein the buffer comprises about 50mM Tris-HCl at about pH 6.5. In another embodiment, the particle has a diameter of about 1500 nm, and wherein the buffer comprises about 50mM Tris-HCl at about pH 5.6. In another embodiment, wherein the particle has a diameter of about 1500 nm, and wherein the buffer comprises about 10 mM MgCl_2 at about pH 6.1. In another embodiment, wherein the particle has a diameter of about 1000 nm, and wherein the buffer comprises about 25 mM MgCl_2 in about 10mM Tris at about pH 8.0. In another embodiment, wherein the particle has a diameter of about 200 nm, and wherein the buffer comprises about 5 mM MgCl_2 in about 10mM Tris at about pH 8.0. In another embodiment, the particle has a diameter of about 400 nm, and wherein the buffer comprises about 50 mM MgCl_2 in about 10mM Tris at about pH 8.0. In another embodiment, the particle has a diameter of about 30 to about 40 nm or about 120 to about 200 nm, and wherein the buffer comprises about 50 mM NaNO_3 in about 10 mM Tris at about pH 6.8.

[056] In yet another embodiment, the particle has a diameter of about 30 to about 200 nm, and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 1.0 mg/ml in the solution containing the plurality of self-assembling enzymatic components. In another embodiment, the particle has a diameter of about 600 nm, and wherein the plurality of self-assembling enzymatic components are present at a concentration

of about 3 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

[057] In a further embodiment, the particle has a diameter of about 200 nm, and wherein the buffer comprises about 6 mM MgCl_2 at about pH 7.5 and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 0.5 mg/ml in the solution containing the plurality of self-assembling enzymatic components. In another embodiment, the particle has a diameter of about 633 nm, and wherein the buffer comprises about 8 mM MgCl_2 at about pH 7.0 and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 1 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

[058] The present inventors have demonstrated the ability to switch an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state.

[059] Accordingly, in one embodiment, the present invention provides a method of switching an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state, said method comprising contacting a solution comprising a supramolecular assembly as described herein, wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of increased pH to unassemble the supramolecular assembly. In one embodiment the buffer of increased pH is a buffer is at about pH 8.0.

[060] The present invention also provides a method of switching an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state, said method comprising contacting a solution comprising an unassembled supramolecular assembly of a supramolecular assembly as described herein, wherein the self-assembling

enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of decreased pH to assemble the supramolecular assembly. In one embodiment the buffer of decreased pH is a buffer is at about pH 6.0.

[061] In one embodiment, the self-assembly polypeptide is a P₁₁ family polypeptide. In another embodiment, the self-assembly polypeptide is P₁₁-4.

[062] In one embodiment, the method further comprises the step of recovering the supramolecular assembly from solution.

[063] In another embodiment the present invention provides a method of forming an enzymatically active supramolecular assembly having a diameter 'd', the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii) contacting the solution containing a plurality of self-assembling enzymatic components with a buffer to form a solution having a plurality of self-assembling enzymatic components at a concentration 'c' wherein the solution formed has a pH 'a' and comprises MgCl₂ at a concentration 'd' to form an enzymatically active supramolecular assembly, wherein:

$$D \text{ (nm)} = (127.32 - 15.77a + 2.44c - 2.22d + 0.42 ad)^2 + 2.0 \dots\dots\dots \text{(Formula I)}.$$

[064] In another embodiment the present invention provides a method of modulating the size of an enzymatically active supramolecular assembly, said method comprising the steps of contacting a solution comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, with a buffer to form an enzymatically active supramolecular assembly, wherein the buffer comprises a component to modulate the size of the enzymatically active supramolecular assembly formed.

[065] In a further embodiment, the component to modulate the size of the enzymatically active supramolecular assembly formed is Mg^{2+} and/or a pH modulating compound.

[066] In one embodiment, the enzyme is a bovine carbonic anhydrase.

[067] In one embodiment, the self-assembly polypeptide is a P_{11} family polypeptide. In another embodiment, the self-assembly polypeptide is P_{11} -4.

[068] In another aspect, a linker binds the self-assembly polypeptide to the enzyme of the self-assembling enzymatic component. In one embodiment, the linker covalently binds the self-assembly polypeptide to the enzyme. In another embodiment, the linker comprises a polypeptide. In a further embodiment, at least 90% of the amino acids in the linked polypeptide are glycine or serine or a combination thereof. For example, in one embodiment, the linker is a glycine-serine (GS) linker, and in another embodiment the GS linker comprises SEQ ID NO: 2.

BRIEF DESCRIPTION OF THE DRAWINGS

[069] The present invention will now be described with reference to the Figures of the accompanying drawings, which illustrate particular preferred embodiments of the present invention, wherein: The present invention will now be described with reference to the figures of the accompanying drawings, which illustrate particular preferred embodiments of the present invention, wherein:

[070] Figure 1 shows the self-assembling enzymatic component BCA- P_{11} -4, which comprises an enzyme, bovine carbonic anhydrase, fused to a self-assembly polypeptide, BCA- P_{11} -4.

[071] Figure 2 shows self-assembling enzymatic components can form a supramolecular assembly. TEM Image of BCA-P₁₁-4 nanoparticles at two different lower magnifications.

[072] Figure 3 shows self-assembling enzymatic components can form a supramolecular assembly. TEM Image of BCA-P₁₁-4 nanoparticles at high magnification.

[073] Figure 4 shows enzymatic activity of a supramolecular assembly comprising self-assembling enzymatic components. A hydrase activity test shows colour change of BTB dye from black to grey indicating the conversion of CO₂ to bicarbonate ion. Sample name 1- Blank without enzyme; 2- BCA-P₁₁-4 enzyme nanoparticle; 3- Commercial BCA (Sigma). a) Reaction at time t=0 seconds before addition of CO₂ saturated water; b) Reaction at time t=25 seconds; c) Reaction at time t= 500 seconds.

[074] Figure 5 shows the stability and enzymatic activity of a supramolecular assembly comprising self-assembling enzymatic components. Comparison of particle size and relative esterase activity of BCA-P₁₁-4 at different temperatures. The % relative activity at different temperatures was calculated relative to the esterase activity of BCA-P₁₁-4 at 25°C.

[075] Figure 6 shows free enzyme does not form supramolecular assemblies. TEM Images of free enzyme (WT-BCA).

[076] Figure 7 shows a supramolecular assembly comprising self-assembling enzymatic components has the same unfolding curve/three dimensional structure as the free enzyme. Derivative curves of DSF for BCA-P₁₁4 (light grey) and WT-BCA (dark grey) in Tris-chloride and 200mM NaCl pH 8.0 buffer.

[077] Figure 8 shows the size distribution of a supramolecular assembly. Size distribution of nanoparticles measured using Dynamic Light Scattering technique.

[078] Figure 9 shows the nanoparticle size of BCA-P₁₁-4 over time using Dynamic Light Scattering technique. Pdl; polydispersity index.

[079] Figure 10 shows a schematic of A; a self-assembling enzymatic component, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme via a linker, and B; a self-assembling enzymatic component, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme via a tandemly repeated linker.

[080] Figure 11 shows the formation of BCA-P₁₁-4 nanoparticles. (a) Comparison of particle size distribution of BCA-P₁₁-4 with WT-BCA using dynamic light scattering method; (b) TEM image of uranyl acetate stained BCA-P₁₁-4 nanoparticles; (c) Size distribution data of BCA-P₁₁-4 nanoparticles observed in (b) using ImageJ software.

[081] Figure 12 shows the effect of temperature on (a) enzyme activity of BCA-P₁₁-4 nanoparticles and WT-BCA measured by pNPA assay. The relative activity (%) at different temperatures was calculated using the esterase activity of respective enzymes at 25°C as standard (b) Particle size distribution of BCA-P₁₁-4 nanoparticles measured at various temperatures via dynamic light scattering.

[082] Figure 13 shows the evaluation of BCA-P₁₁-4 under CO₂ capture conditions at two temperatures (a) formation of bicarbonate and hydrogen ion measured by pH reduction curves of the reaction at ambient temperature (22 ± 2°C) compared with WT-BCA; (b) TEM image of BCA-P₁₁-4 at end of reaction under ambient condition (22 ± 2°C); (c) pH reduction curves of the reaction at 50°C; (d) TEM image of BCA-P₁₁-4 at end of reaction at 50°C.

[083] Figure 14 shows the influence of pH, ionic strength and added salts on BCA-P₁₁₄ assembly size formation (a) low ionic strength buffer of 10 mM Tris (b) increased ionic strength buffer of 50 mM Tris (c) 50 mM NaCl in 10 mM Tris (d) 50 mM NaNO₃ in 10 mM Tris. Protein concentration 0.5 mg/mL; pH 8.0; pH 7.5; pH 6.8; pH 6.5 and pH 5.6.

[084] Figure 15 shows the forces affecting BCA-P₁₁₄ nanoparticle formation (a) Illustration of the attractive and repulsive forces between two P₁₁₄ peptide monomers arranged in an anti-parallel formation at neutral pH depicting uncharged residues (Q), positively charged residue (R), aromatic residues (F or W) and negatively charged residues (E). Attractive forces are represented by black double-ended arrows and repulsive forces by shaded arrows in the inter-peptide region. (b) Representation of the attractive electrostatic interactions between surface charges of enzyme-peptide monomers at pH <7, where enzyme (shaded pie-shape) bears one positive charge and the peptide (black bars) bears two negative charges.

[085] Figure 16 shows dynamic light scattering results showing effect of protein concentration BCA-P₁₁₄ in 50 mM Tris buffer at pH 6.8.

[086] Figure 17 shows dynamic light scattering results showing effect of temperature on 0.5mg/mL of BCA-P₁₁₄ in 50mM Tris buffer at pH 8.0

[087] Figure 18 shows BCA-P₁₁₄ nanoparticle assembly facilitated by MgCl₂ in 10mM Tris pH 8.0 buffer (a) Effect of 25 mM MgCl₂ on wild-type BCA and BCA-P₁₁₄ (b) Effect of MgCl₂ concentration on BCA-P₁₁₄ nanoparticle size; (c) TEM image of BCA-P₁₁₄ nanoparticles formed in 5 mM MgCl₂; (d) Size distribution by dynamic light scattering illustrating the inhibition of Mg²⁺-facilitated BCA-P₁₁₄ self-assembly by increased ionic strength.

[088] Figure 19 shows the proposed mechanism of BCA-P₁₁4 self-assembly in the presence of Mg²⁺ (a) Mg²⁺ with large Debye sphere (shaded circle) coordinated to four glutamic acid (E) residues (light grey dashed arrow bonds) and two water molecules (dark gray filled arrow bonds). Self-assembly is further stabilized by electrostatic interaction (dashed line) between arginine (R) and glutamic acid (E) residues of adjacent strands. (b) Under high ionic strength, smaller Debye sphere of Mg²⁺ crowded by Cl⁻ ions preventing coordination with glutamic acids (E) that are surround by Na⁺. Crowding of Cl⁻ and Na⁺ ions around arginine (R) and glutamic acid (E) residues prevent electrostatic interaction disrupting self-assembly.

[089] Figure 20 shows two-level full factorial model describing factors that control BCA-P₁₁4 nanoparticle formation. (a) Half-Normal Plot indicating factors with significant effect - A: pH, C: Protein concentration, D: MgCl₂ concentration, AD: pH and MgCl₂ concentration interaction (un-labelled symbols show positive effect, labelled symbols show negative effect and the triangles are error estimates); (b) 3-D Contour plot showing interaction effect between MgCl₂ and pH; (c) Comparison of predicted and actual particle size measured by dynamic light scattering. Condition 1: 0.5 mg/mL BCA-P₁₁4, pH 7.5, 6 mM MgCl₂ (Actual size - solid bold curve, predicted value-vertical dashed line). Condition 2: 1.0 mg/mL BCA-P₁₁4, pH 7.0, 8 mM MgCl₂ (Actual size -dotted curve, predicted value-vertical dashed line).

[090] Figure 21 shows esterase activity of BCA-P₁₁4 nanoparticles of various sizes measured as a percentage of the individual BCA-P₁₁4 fusion protein. Shaded areas on the graph indicate various particle sizes. Average activity of all the sizes (solid bold line) along with standard deviations (black dashed lines) are shown.

[091] Figure 22 shows purification of BCA-(P₁₁4)₃ by Immobilised Metal-ion Affinity Chromatography a) Chromatography profile b) SDS-PAGE showing

various chromatography fractions; lane 1-Protein load, lane 2-flow through, lane 3-wash, lane 4-Eluate E1 fraction, lane 5-Eluate E2 fraction, lane 6-Regeneration, M-Molecular weight marker.

[092] Figure 23 shows SDS-PAGE gel showing recombinant expression of BCA-(P₁₁₄)₃ enzyme-peptide fusion systems a) using different induction method (S-soluble; IN-insoluble) b) comparison of expression levels of WT-BCA, BCA-P₁₁₄ and BCA-(P₁₁₄)₃ induced at 1 mM IPTG 20°C for 16 h. Lanes 1-5: WT-BCA-uninduced soluble, uninduced insoluble, soluble 12 h, soluble 16 h, insoluble 16 h. Lanes 6-9: BCA-P₁₁₄ - uninduced soluble, uninduced insoluble, soluble 16hr, insoluble 16 h. Lanes 10-14: BCA-P₁₁₄(3) - uninduced soluble, uninduced insoluble, soluble 12 h, soluble 16 h, insoluble 16 h (All samples were diluted to OD600 -4.0 prior to gel loading). Arrows indicate BCA-(P₁₁₄)₃ protein band.

[093] Figure 24 shows polishing of BCA-(P₁₁₄)₃ by AIEx a) Chromatography profile b) and c) SDS-PAGE showing various chromatography fractions; b) lane 1-Protein load, lane 2-flow through, lane 3-wash, lane 4-Eluate E1 fraction, lane 5-Eluate E2 fraction, lane 6-Regeneration, M-Molecular weight marker. c) lane 1-E8+E9 fraction, lane2-E10+E11 fraction, lane3-E12 to E16 fraction, E17 fraction and lane5-E18 fraction. Arrows indicate bands analysed for Intact mass.

[094] Figure 25 shows intact mass data of protein bands from AIEx SDS-PAGE (Figure 24c) using MALDI-TOF

[095] Figure 26 shows a) pH induced precipitation of BCA-(P₁₁₄)₃ and b) SDS-PAGE comparing supernatant and pellet sample of BCA-(P₁₁₄)₃ following pH induced precipitation; lane-1 BCA-P₁₁₄, lane-2 BCA-(P₁₁₄)₃ supernatant showing cleaved protein bands, lane-3 BCA-(P₁₁₄)₃ pellet and M-molecular weight marker.

[096] Figure 27 shows Dynamic Light scattering data comparing particle states of BCA-(P₁₁4)₃ initial at pH8.0 (solid line), as a precipitate at pH 6.0 (dashed line) and after re-solubilisation (dotted line).

[097] Figure 28 shows SDS-PAGE showing comparing WT-BCA and BCA-P₁₁4 nanoparticle separation using 100kDa MWCO Centriscart® device. Lane-1, 2, 3: BCA-P₁₁4 load, retentate and permeate respectively. Lanes-4, 5, 6: WT-BCA load, retentate and permeate respectively.

[098] Figure 29 shows BCA-P₁₁4 nanoparticle separation using 100 kDa MWCO Centriscart a) Dynamic light scattering data comparing particle sizes of Load (solid line), permeate (dot-dash line) and retentate (dashed line) samples. b) TEM image showing BCA-P₁₁4 nanoparticle present in retentate sample.

[099] Figure 30 shows SDS-PAGE showing comparing WT-BCA and BCA-(P₁₁4)₃ particle separation using 300 kDa MWCO Centriscart® device. Lane-1, 2, 3: WT-BCA load, permeate and retentate respectively. Lanes-4, 5, 6: BCA-(P₁₁4)₃ load, permeate and retentate respectively. Note fractions were diluted prior to SDS-PAGE analysis.

[0100] Figure 31 shows SDS-PAGE showing BCA-(P₁₁4)₃ particle separation by centrifugation following three biocatalytic cycles. M- Molecular weight marker, lane 1- BCA-(P₁₁4)₃ reaction 1 sample, lane 2-supernatant 1, lane 3- supernatant 2 and lane 4- final pellet after 3 reactions.

[0101] Figure 32 shows protein expression (a) WT-TmCA: Lane 1- Uninduced soluble, lane 2-Uninduced insoluble, lane 3-Induced soluble, lane 4-Induced insoluble (b) TmCA-P₁₁4: Lane 1- Uninduced soluble, lane 2-Uninduced insoluble, lane 3-Auto-induction soluble (1L), lane 4-Auto-induction insoluble (1L) lane 5- Auto-induction soluble (0.5L), lane 6-Auto-induction insoluble (0.5L), lane 7-IPTG

induced soluble (1L), lane 8-IPTG induced insoluble (1L) lane 9-IPTG induced soluble (0.5L), lane 10-IPTG induced insoluble (0.5L).

[0102] Figure 33 shows SDS-PAGE analysis of Ni-IMAC purification fractions (a) WT-TmCA: Lane 1-load, lane 2-Flow thorough, lane 3-Wash 1, lane 4- Wash 2, lane 5-Eluate E1 fraction, lane 6-Eluate E2+E3 fraction (b) TmCA-P₁₁₄ : Lane 1-load, lane 2-Flow thorough, lane 3-Wash 1+2, Eluate E1 fraction, lane 5-Eluate E2 fraction, M-molecular weight marker.

[0103] Figure 34 shows particle sizes measured by Dynamic Light Scattering technique (a) WT-TmCA (b) TmCA-P₁₁₄

[0104] Figure 35 shows TmCA-P₁₁₄ nanoparticles formed at 50°C (a) TEM image (b) Corresponding histogram for (a) TEM image.

[0105] Figure 36 shows temperature stability measured by differential Scanning Fluorimetry (a) Derivative of melting curve for WT-TmCA (b) Melting temperature in various buffers for WT-TmCA (c) Derivative of melting curve for TmCA-P₁₁₄ (d) Melting temperature in various buffers for TmCA-P₁₁

[0106] Figure 37 shows protein expression (a) WT-Tyrosinase (b) Tyrosinase-P₁₁₄. S- Soluble, IN-Insoluble. Arrows indicate protein of interest.

[0107] Figure 38 shows Ni-IMAC purification (a) Chromatography profile for WT-Tyrosinase (b) Chromatography profile for Tyrosinase-P₁₁₄ (c) SDS-PAGE analysis of Ni-IMAC fractions. Lanes 1-7 WT-Tyr: Load, Flow thorough, Wash, Eluate E1 fraction, Eluate E2 fraction, Eluate E3 fraction and Eluate E4 fraction. Lane 8-14 Tyr-P₁₁₄: Load, lane, Flow thorough, Wash, Eluate E1 fraction, Eluate E2 fraction, Eluate E3 fraction and Eluate E4 fraction M-molecular weight marker.

[0108] Figure 39 shows particle sizes measured by Dynamic Light Scattering technique (a) WT-Tyrosinase (b) Tyrosinase-P₁₁₄.

[0109] Figure 40 shows Tyrosinase-P₁₁₄ nanoparticles formed with 10mM MgCl₂ (a) Particle size range 40 – 200nm (b) Large particles of size 100 -500nm.

[0110] Figure 41 shows protein expression (a) WT-Cutinase (b) Cutinase-P₁₁₄. AI- Auto-Induction, IP- IPTG Induction, S- Soluble, IN-Insoluble. Arrows indicate protein of interest in soluble form.

[0111] Figure 42 shows protein expression of Cutinase-P₁₁₄ at various IPTG concentrations. S- Soluble, IN-Insoluble.

[0112] Figure 43 shows Ni-IMAC purification of Cut-P₁₁₄ (a) Chromatography profile (b) SDS-PAGE analysis of Ni-IMAC fractions. Lanes 1-6: Load, Flow thorough, Wash, Eluate E1 fraction, Eluate E2 fraction and regeneration. Box indicates Cutinase-P₁₁₄ band.

[0113] Figure 44 shows nanoparticle formation of Cut-P₁₁₄ (a) Particle size formed with and without 10 mM MgCl₂ added (b) TEM image showing large enzyme particles.

[0114] Figure 45 shows enzyme Activity of Cut-P₁₁₄ measured at different concentration of substrate (pNPB).

[0115] Figure 46 shows protein expression (a) WT-ATA (b) ATA-P₁₁₄. AI- Auto-Induction, IP- IPTG Induction, S- Soluble, IN-Insoluble. Arrows indicate protein of interest in soluble form.

[0116] Figure 47 shows Ni-IMAC purification (a) Chromatography profile for WT-ATA (b) Chromatography profile for ATA-P₁₁₄ (c) SDS-PAGE analysis of Ni-IMAC

fractions. Lanes 1-5 WT-ATA: Load, Flow thorough, Wash 1+2, Eluate E1 fraction, Eluate E2 fraction. Lane 6-11 ATA-P₁₁4: Load, Flow thorough, Wash 1, Wash 2, Eluate E1 fraction, Eluate E2 fraction, M-molecular weight marker.

DETAILED DESCRIPTION OF THE INVENTION

[0117] The present inventors have surprising found that an enzymatically active supramolecular assembly can be formed using an enzyme bound to a self-assembly polypeptide. Accordingly, the present invention provides an enzymatically active supramolecular assembly comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

[0118] In one embodiment, the supramolecular assembly is a nanomaterial, such as a nanoparticle, a nanofibre, a sheet, or a combination thereof.

[0119] In another embodiment, the supramolecular assembly macrostructure, such as a hydrogel or an aggregate.

[0120] Nanomaterials including nanostructures offer certain advantages over conventional supports which include a high surface area to volume ratio, increased enzyme loading, enhanced mobility in flow systems and improved mass-transfer. The small size of nanomaterials provides a large surface area for enzymes to be immobilised. This large surface area increases interfacial area allowing faster reaction rates and conversion efficiency by reducing diffusional limitations which is encountered in conventional immobilisation systems. Nanostructures can have a stabilising effect on enzymes. Also the mobility, nanospatial confinement and solution behaviour of nanoscale systems are very different from conventional enzyme immobilisation systems and can confer unique and desirable properties to enzymes.

[0121] The use of nanomaterials is challenged by problems of reduced enzyme activity, because techniques used to immobilise enzymes on nanomaterials are the same as those used for conventional supports. Furthermore, the enzyme may be denatured as a result of nanoparticle aggregation which occurs when attempting to separate them from bulk solution.

[0122] Without wishing to be bound by theory, the present inventors have demonstrated that a self-assembly polypeptide bound to an enzyme allows the formation of a supramolecular assembly where the catalytic activity of the enzyme in the supramolecular assembly is not reduced relative to the free enzyme.

[0123] In one embodiment the supramolecular assembly is selected from the group consisting of a particle, fibre, sheet or a combination thereof.

[0124] The ability to form the plurality of self-assembling enzymatic components into nanomaterials avoids the problems associated with conventional enzyme immobilisation systems.

[0125] For example, the most robust form of immobilised enzymes is those using the covalent immobilisation technique. However, immobilising enzymes using the covalent method onto solid supports often leads to reduced enzyme activity compared to their free soluble forms. The reasons for this reduced activity is attributed to one or more of the following induced changes in structural conformation, enzyme rigidification or deactivation due to agents used in the immobilisation process. Mateo, C. *et al.* (2007). [Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology*, 40(6), pp.1451–1463] reviews the factors governing the stabilisation of immobilised enzymes. Particular limitations associated with immobilised enzymes include:

- The requirement for highly pure enzymes as starting material to avoid non-specific binding

- Support material must have suitable characteristics as a scaffold material (section Mateo, C. *et al.*, 2007, section 3.2.1)
- Immobilisation must be performed under specific conditions to achieve maximum efficiency of enzyme loading and retention of enzyme activity (Mateo, C. *et al.*, 2007, section 3.2.2)
- Recovered activity of the immobilised enzyme is often less than the free soluble enzyme (Mateo, C. *et al.*, 2007, Table 1)
- Often only a certain proportion of enzyme used for immobilisation process is successfully immobilised on to the support and only a proportion of the immobilised enzymes retain activity (Mateo, C. *et al.*, 2007, table 2)

[0126] Without wishing to be bound by theory, the present invention is based in part on the surprising demonstration that enzymatically active nanoparticles can be formed in the absence of absence of a carrier, a solid support, the need to covalently link the enzyme to a solid support, or to immobilise the enzyme.

[0127] Accordingly, the present invention provides a supramolecular assembly which can be formed without the need for immobilisation of the enzyme on a solid support. For example, the supramolecular assembly can be formed without the need to covalently link the enzyme to a solid support.

[0128] As a result, the supramolecular assemblies of the present invention avoid problems of the immobilized enzymes of the prior art such as those discussed above.

[0129] As used herein, the term "comprising" is intended to mean that the assemblies, components, compositions and methods include the recited elements, but not excluding others.

[0130] The term "enzymatically active" as used herein includes the ability of an enzymatic component, a supramolecular assembly, or an enzyme as described herein to catalyze the conversion of a substrate into a product. A substrate for the enzyme comprises the natural substrate of the enzyme, but can also comprise analogues of the natural substrate, which can also be converted, by the enzyme into a product or into an analogue of a product. The activity of the enzyme can be measured, for example, by determining the amount of product in the reaction after a certain period of time, or by determining the amount of substrate remaining in the reaction mixture after a certain period of time.

[0131] Enzymes suitable for the enzymatically active supramolecular assembly will depend on the desired application for the supramolecular assembly.

[0132] As used herein, the term "enzyme" refers generally to polypeptides that catalyze biochemical reactions. A compound for which a particular enzyme catalyzes a reaction is typically referred to as a "substrate" of the enzyme. In general, six classes or types of enzymes (as classified by the type of reaction that is catalyzed) are recognized. Enzymes catalyzing reduction/oxidation or redox reactions are referred to generally as EC 1 (Enzyme Class 1) Oxidoreductases. Enzymes catalyzing the transfer of specific radicals or groups are referred to generally as EC 2 Transferases. Enzymes catalyzing hydrolysis are referred to generally as EC 3 hydrolases. Enzymes catalyzing removal from or addition to a substrate of specific chemical groups are referred to generally as EC 4 Lyases. Enzymes catalyzing isomerization are referred to generally as EC 5 Isomerases. Enzymes catalyzing combination or binding together of substrate units are referred to generally as EC 6 Ligases.

[0133] In one embodiment the enzyme is selected from the group consisting of enzymes from EC 4.2.1.1 (Lyase), 4.2.1.1 (Lyase), 1.14.18.1 (Oxidoreductase) 3.1.1.74 (Hydrolase), and EC 2.6.1.18 (Transferase).

[0134] Exemplary enzymes used in the present invention include enzymes selected from the group consisting of Bovine carbonic anhydrase (BCA), Microbial carbonic anhydrase (TmCA), Tyrosinase (Tyr), Cutinase (Cut) and Aminotransaminase (ATA).

[0135] Exemplary enzymes used in the present invention include carbon dioxide capture/carbon dioxide converting enzymes selected from the group consisting of formate dehydrogenase, carbonic anhydrase, RuBisCO and combinations thereof.

[0136] In another embodiment, the carbon dioxide capture/carbon dioxide converting enzyme is carbonic anhydrase.

[0137] In another aspect the present invention provides enzymatically active supramolecular assemblies wherein the assembly comprises self-assembling enzymatic components comprising different enzymes. Accordingly, the assembly will have at least two enzymatic activities.

[0138] In one embodiment, the present invention comprises an enzymatically active supramolecular assembly comprising a first self-assembling enzymatic component and a second self-assembling enzymatic component, wherein the first self-assembling enzymatic component comprises a self-assembly polypeptide bound to a first enzyme, and wherein the second self-assembling enzymatic component comprises a self-assembly polypeptide bound to a second enzyme.

[0139] In another embodiment, the enzymatically active supramolecular assembly comprises at least three, at least four or at least five different self-assembling enzymatic components.

[0140] The term “supramolecular assembly” as used herein includes polymers, homo- and/or co- polymers, capable of associating with one another by means of

covalent bonds, non-covalent interactions including ionic interactions and Van der Waals forces, including hydrogen bonding and dispersion forces, or a combination thereof. For example, the term includes polymers, homo- and/or co- polymers of self-assembling enzymatic components as described herein.

[0141] In one embodiment the supramolecular assembly is a nanostructure or a macrostructure. One exemplary nanostructure of the present invention is a nanoparticle. The choice of nanostructure or macrostructure will depend on the desired application of the supramolecular assembly, and can be determined in part by the characteristics of the self-assembly polypeptide to be used in the self-assembling enzymatic component.

[0142] The term “self-assembly” as used herein includes the process of a self-assembling polypeptide or a component comprising the self-assembling polypeptide to form higher order structures or aggregates, including regularly ordered higher order structures or aggregates. The self-assembly may occur in response to conditions in the environment, such as when added to an aqueous medium or in response to a particular environmental condition such as a pH of an aqueous medium.

[0143] The term “self-assembly polypeptide” as used herein includes a peptide comprising a self-assembling motif. Self-assembly polypeptides that are capable of self-assembly or self-assembly of components comprising the self-assembly polypeptide into higher order structures.

[0144] The term “polypeptide”, as used herein, includes amino acid polymers of any length. The protein may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as

conjugation with a labelling component. Also included are, for example, proteins containing one or more analogs of an amino acid (including, for example, unnatural or non-natural amino acids, etc.), as well as other modifications known in the art. Proteins can occur as single chains or associated chains. Associated chains may be joined by non-covalent or covalent interactions.

[0145] Polypeptides of the invention, including self-assembly polypeptides, can be prepared by various means (e.g. isolation and purification from source, recombinant expression, purification from cell culture, chemical synthesis, etc.) and in various forms (e.g. native, fusions, non-glycosylated, lipidated, etc.). They are preferably prepared in substantially pure form (i.e. substantially free from host cell proteins). Typically, the polypeptide is substantially pure when it is at least 60%, by weight, of total protein present. For example, the preparation is at least 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, more preferably at least 90%, by weight, of total protein present.

[0146] Self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme can be prepared by various means (e.g. isolation and purification from source, recombinant expression, purification from cell culture, chemical synthesis, etc.) and in various forms (e.g. native, fusions, non-glycosylated, lipidated, etc.). They are preferably prepared in substantially pure form (i.e. substantially free from host cell proteins). Typically, the self-assembling enzymatic component is substantially pure when it is at least 60%, by weight, of total protein present. For example, the preparation is at least 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, more preferably at least 90%, by weight, of total protein present.

[0147] In one embodiment, the self-assembling enzymatic component is purified from cell culture.

[0148] In one embodiment, the supramolecular assembly comprising self-assembling enzymatic components is substantially free of other components. For example, a supramolecular assembly that contains at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% self-assembling enzymatic component, by weight, of total protein present.

[0149] The present inventors have generated polynucleotides encoding the self-assembling enzymatic components described herein. Accordingly, the present invention provides polynucleotides encoding the self-assembling enzymatic components described herein.

[0150] The term “polynucleotide”, as used herein, includes DNA and RNA, and also their analogues, such as those containing modified backbones (e.g. phosphorothioates, etc.), and also peptide nucleic acids (PNA), etc. The invention includes nucleic acid comprising sequences complementary to those described above (e.g. for antisense or probing purposes). The skilled person understands that strict compliance with the polynucleotide and protein sequences defined herein is not necessary, and functional equivalents are included in the scope of the invention. Various strains and species of organisms may have differences at various amino acid and/or nucleotide residues without substantially affecting enzyme activity or structure of the protein. For example, in respect of proteins it is known that the certain amino acid substitutions can be made without substantially affecting the structure or function of the protein. Such “conservative substitutions” are well known to the skilled person and will not be repeated herein. It is also understood that a protein may be truncated, or have internal deletions without substantially affecting structure or function. Furthermore, certain fragments of a protein may retain important structure and function.

[0151] The degeneracy of the genetic code is such that the same protein may be encoded by a number of different polynucleotide sequences. The present invention includes any alterations that are available by virtue of the degeneracy of the genetic code. Furthermore, the invention provides nucleic acid which can hybridise to these nucleic acid molecules, preferably under "stringent" conditions (e.g. 65°C in a 0.2 x SSC). Nucleic acid according to the invention can be prepared in many ways (e.g. by chemical synthesis, from genomic or cDNA libraries, from the organism itself, etc.) and can take various forms (e.g. single stranded, double stranded, vectors, probes, etc.). They are preferably prepared in substantially pure form (i.e. substantially free from other host cell nucleic acids).

[0152] Similarly, the skilled person understands that strict compliance with any amino acid sequence disclosed herein is not necessarily required, and he or she could decide by a matter of routine whether any further mutation is deleterious or preferred. For example, where the protein has a given biological activity that can be assayed (such as an enzyme activity as described herein) the effect of any mutation on that biological activity may be directly observed. Thus, the polypeptides of the present invention include sequences having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to any protein disclosed herein. The polypeptides also include variants (e.g. allelic variants, homologs, orthologs, paralog, mutants, etc.). The molecules may lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus.

[0153] Functional equivalents of the enzymes are included within the scope of the invention.

[0154] The term "sequence identity", as used herein, includes, in the context of two or more nucleic acids or polypeptide sequences, to two or more sequences or

subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection. Sequence identity, homology and the like may be determined using standard methods known to the skilled person, for example, using any computer program and associated parameters, such as BLAST or FASTA.

[0155] The term “stringent conditions”, as used herein, includes highly stringent conditions, medium stringent conditions, low stringent conditions, including the high and reduced stringency conditions described herein. In alternative embodiments, nucleic acids of the invention as defined by their ability to hybridize under stringent conditions can be between about five residues and the full length of the molecule, e.g., an exemplary nucleic acid of the invention. For example, they can be at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 55, 60, 65, 70, 75, 80, 90, 100, 150, 200, 250, 300, 350, 400 or more residues in length. Nucleic acids shorter than full length are also included. These nucleic acids are useful as, e.g., hybridization probes, labelling probes, PCR oligonucleotide probes, antisense or sequences encoding antibody binding peptides (epitopes), motifs, active sites, binding domains, regulatory domains and the like.

[0156] In one aspect, nucleic acids of the invention are defined by their ability to hybridize under high stringency comprising conditions of about 50% formamide at about 37°C to 42°C. In one aspect, nucleic acids of the invention are defined by their ability to hybridize under reduced stringency comprising conditions in about 35% to 25% formamide at about 30°C to 35°C. Alternatively, nucleic acids of the invention are defined by their ability to hybridize under high stringency comprising conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and a repetitive sequence blocking nucleic acid, such as cot-1 or salmon sperm DNA (e.g., 200 ug/ml sheared and denatured salmon sperm DNA). In one aspect, nucleic acids of

the invention are defined by their ability to hybridize under reduced stringency conditions comprising 35% formamide at a reduced temperature of 35°C.

[0157] Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be "moderate" conditions above 25% formamide and "low" conditions below 25% formamide. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 10% formamide.

[0158] The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Nucleic acids of the invention are also defined by their ability to hybridize under high, medium, and low stringency conditions as set forth in Ausubel and Sambrook. Variations on the above ranges and conditions can be used to practice the invention and are well known in the art.

[0159] The term "native promoter", as used herein, includes a promoter that is endogenous to the organism or virus and is unmodified with respect to its nucleotide sequence and its position in the viral genome as compared to a wild-type organism or virus.

[0160] The term "heterologous promoter", as used herein, includes a promoter that is not normally found in the wild-type organism or that is at a different locus as compared to a wild type organism. A heterologous promoter is often not endogenous to a cell or virus into which it is introduced, but has been obtained from another cell or virus or prepared synthetically. A heterologous promoter can refer to a promoter from another cell in the same organism or another organism, including the same species or another species. A heterologous promoter, however, can be endogenous, but is a promoter that is altered in its sequence or

occurs at a different locus (e.g., at a different location in the genome or on a plasmid). Thus, a heterologous promoter includes a promoter not present in the exact orientation or position as the counterpart promoter is found in a genome. A synthetic promoter is a heterologous promoter that has a nucleotide sequence that is not found in nature. A synthetic promoter can be a nucleic acid molecule that has a synthetic sequence or a sequence derived from a native promoter or portion thereof. A synthetic promoter can also be a hybrid promoter composed of different elements derived from different native promoters.

[0161] A heterologous nucleic acid (also referred to as exogenous nucleic acid or foreign nucleic acid) includes a nucleic acid that is not normally produced in vivo by an organism from which it is expressed or that is produced by an organism but is at a different locus, expressed differently, or that mediates or encodes mediators that alter expression of endogenous nucleic acid, such as DNA, by affecting transcription, translation, or other regulatable biochemical processes.

[0162] Heterologous nucleic acid is often not endogenous to a cell or virus into which it is introduced, but has been obtained from another cell or prepared synthetically. Heterologous nucleic acid can refer to a nucleic acid molecule from another cell in the same organism or another organism, including the same species or another species. Heterologous nucleic acid, however, can be endogenous, but is nucleic acid that is expressed from a different locus or altered in its expression or sequence (e.g., a plasmid). Thus, heterologous nucleic acid includes a nucleic acid molecule not present in the exact orientation or position as the counterpart nucleic acid molecule, such as DNA, is found in a genome. Generally, although not necessarily, such nucleic acid encodes RNA and proteins that are not normally produced by the cell or virus or in the same way in the cell in which it is expressed. Any nucleic acid, such as DNA, that one of skill in the art recognizes or considers as heterologous, exogenous or foreign to the cell in which the nucleic acid is expressed is herein encompassed by heterologous nucleic acid.

[0163] The invention provides nucleic acid (e.g., DNA) sequences of the invention operatively linked to an expression regulatory sequence (including transcriptional regulatory sequence or translational regulatory sequence) e.g., promoters or enhancers, to direct or modulate RNA synthesis/expression. The expression control sequence can be in an expression vector. Exemplary bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*, lambda PR, PL and *trp*. Exemplary eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein I. In one embodiment the promoter is *trc*. In one embodiment, the expression control sequence is inducible.

[0164] As used herein, "portion" is understood to refer to a portion of a polypeptide of the invention which maintains a defined characteristic or activity of the full-length polypeptide. For example, having the ability to self-assemble.

[0165] Thus, a portion or a biologically active fragment of a polypeptide of the invention may be capable of forming higher order structures or aggregates, including regularly ordered higher order structures or aggregates.

[0166] Exemplary self-assembly polypeptides include short peptides of 2-20 amino acid residues which self-assemble through molecular recognition to form structures of various orders. Factors that drive molecular recognition and self-assembly include weak non-covalent interactions like hydrogen bonds, ionic interactions, Van der Waals forces, dispersion forces and hydrophobic interactions. Without wishing to be bound by theory, though these forces are weak, the present inventors propose they can collectively interact among individual units to yield stable and robust peptide structures. At a critical polypeptide concentration, self-assembly can be triggered by external stimuli.

[0167] In one embodiment the external stimulus is selected from the group consisting of salt concentration, pH, temperature, ions, light or an enzyme.

[0168] Accordingly, in one embodiment, the self-assembly polypeptide is an external stimuli responsive self-assembly polypeptide.

[0169] In one embodiment, the self-assembly polypeptide is selected from the group consisting of a pH responsive self-assembly polypeptide, a temperature responsive self-assembly polypeptide; a metal ion responsive self-assembly polypeptide, an enzyme responsive self-assembly polypeptide, and a light responsive self-assembly polypeptide.

[0170] The self-assembly polypeptide can be designed or chosen depending on the desired characteristics of the self-assembly and/or the supramolecular assembly.

[0171] For example, Table 1 below, includes exemplary self-assembly polypeptides used in the present invention.

Stimuli	Mechanism	Effect	Example of self-assembly polypeptide
pH	Protonation/Deprotonation of charged residues	Switch/change secondary structure; changes net charge of peptide	P11-2,4,8,9
Temperature	Folding/unfolding of peptides	Initiate hydrophobic interactions	Elastin-like polypeptide
Metal ions	Co-ordination interaction between metal ion and peptide residue	Induce conformational change; charged interactions	Alkyl-C4G3S(P)RGD-COOH
Enzymes	Phosphorylation/Dephosphorylation; Cleavage of peptide bonds	Induce conformational change Imbalance in ratio of hydrophobicity/hydrophilicity	HAAHHELH
Light	Moieties that absorb light	Induce conformational change	MAX7CNB

[0172] Self-assembly polypeptides can be classified based on the secondary structure of the monomeric units, namely, α -helix peptides, β -sheet peptides and peptide amphiphiles. Peptide amphiphiles (PA) are divided into peptide-only amphiphiles and lipidated amphiphiles. Alternate classification types can be based on the self-assembled structure formed and/or based on the stimuli responsive property of the self-assembly peptide.

[0173] Self-assembly polypeptides can form several types of higher order structures, including nanostructures and macrostructures.

[0174] The term “nanostructures” and “nanomaterials” as used herein includes nanofibres, nanoparticles, nanospheres, vesicles and sheets.

[0175] As used herein, the term “nanoparticle” refers to particles sized between about 0.5 to about 1500 nanometers in at least one dimension, although the nanoparticles need not be spherical in shape. Preferably, the particles are sized between about 30 to about 1000 nanometers in at least one dimension, more preferably about 40 to about 1000 nanometers in at least one dimension, more preferably about 100 to about 1000 nanometers in at least one dimension, more preferably about 200 to about 633 nanometers in at least one dimension.

[0176] In one embodiment the nanoparticles have a diameter of from about 30 nm to about 40 nm, from about 120 nm to about 200 nm. In another embodiment, the average diameter is from about 50 nm to about 100 nm.

[0177] Without wishing to be bound by theory, the type of nanostructure formed by self-assembly polypeptide depends on two factors: a) sequence of the self-assembly peptide monomer which decides the type of functional side chains and b) the kind of interactions between the side chains of these self-assembly peptide monomers.

[0178] For example, some of the principles that govern higher structure formation are similar to those of protein secondary and tertiary structures. For example, proline is a rigid amino acid well-known as a “ β -sheet breaker”, and this feature can be used to control the nanostructures formed.

[0179] For example, a β -strand peptide FI, H-PKFKIIEFEP-OH, forms nanofibers instead of nanosheets; the proline residues on either ends of the peptide prevented the hydrogen bonding required for sheet formation and favoured elongation.

[0180] A nano-web or beads-on-a-thread structure can be formed by using the peptide Ac-PSFCFKFEP-NH₂ which has both a β -turn and sheet in its structure; and this favours formation of filaments and globular structures to yield the nano-web or beads-on-a-thread structure.

[0181] Single amino acid substitutions may also be used to effect nanostructure formation. Fmoc-dipeptides with combinations of phenylalanine (F) and glycine (G) show different forms at the nanoscale. For example, Fmoc-FF can be used to form fibre bundles, whereas Fmoc-GG can be used to form thin fibres with entangled network morphology and the combination Fmoc-FG can be used to form twisted ribbons.

[0182] Peptide amphiphiles of sequence X₆K_n (X = alanine, valine, or leucine; K = lysine; n = 1–5) can be used to form nanostructures at 0.2mM in water; those having a longer hydrophobic segment (A6K, V6K2, and L6K3) can be used to form nanofibres whereas those having more lysine residues (A6K2, V6K3, and L6K4) can be used to form nano-vesicles.

[0183] Accordingly, the self-assembly polypeptides used in the present invention can form different nanostructures depending on the individual peptide sequence despite having the same secondary structure.

[0184] The interaction between self-assembly peptide monomers used in the present invention can also be used to influence nanostructure morphology. For example, in case of peptide amphiphiles, the interaction among hydrophobic tails is the major driving force to self-assemble and the hydrophilic head group involve in hydrogen bonding or electrostatic interactions resulting in nanofibers formation. A series of peptide amphiphiles A6D1, V6D1, V6D2 and L6D2 with tails of increasing hydrophobicity ($A < V < L$) can be used to form both nanofibres as well as nano-vesicles.

[0185] The type of amino acid used as head group in a peptide amphiphiles influences the morphology of the nanostructure. Long, helical fibres are formed with aliphatic amino acids whereas short, straight fibres are formed with aromatic amino acids. Electrostatic forces are also employed to initiate fibril formation which uses two different peptides monomers having complementary charges. Examples of this kind are peptide pairs P₁₁-13 and P₁₁-14 (negative glutamic acid and positive ornithine residues respectively) and the self-complementary EAK16 peptides.

[0186] Model peptides like EAK16/RADA16 and certain peptide amphiphiles can be used for nanotube and nano-vesicle formation respectively.

[0187] In some embodiments, the invention provides macrostructures and macromaterials.

[0188] Beyond a certain critical peptide concentration and/or in response to an external stimulus (pH, temperature etc.), the formed nanostructures can lead to the formation of macrostructures.

[0189] The term “macrostructures” as used herein includes hydrogels and aggregates.

[0190] Several self-assembly polypeptides self-assemble and progress to form macroscopic hydrogels or aggregates.

[0191] As used herein the term “hydrogel” includes a three dimensional network structure that itself is insoluble in water but which is capable of retaining large quantities of water to form a stable, often soft and pliable structure via surface tension. A hydrogel may contain over 90% water, or preferably over 99% water.

[0192] Without wishing to be bound by theory, the general mechanism for hydrogel formation involves the initial stage of fibre/fibril formation which grows into network structures resulting in hydrogel. The formation of hydrogels can be initiated by one or a combination of stimuli, the most common stimuli being temperature, pH and salt ions.

[0193] The self-assembly polypeptides of the present invention include polypeptides that are responsive to pH.

[0194] As used herein, the term “pH responsive self-assembly polypeptide” refers to a self-assembly polypeptide that drives assembly or disassembly of a supramolecular assembly in response to a particular pH or a pH change.

[0195] The present inventors have shown herein that when a pH responsive self-assembly polypeptide, P₁₁-4, is bound an enzyme, the enzyme-self-assembly polypeptide component assembles into an enzymatically active supramolecular structure.

[0196] Accordingly, in one embodiment, the self-assembly polypeptide is the P₁₁-4, which is a member of the P₁₁ family of polypeptides.

[0197] “P₁₁-4” as used herein relates to a self-assembling polypeptide comprising the sequence Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln (SEQ ID NO: 1).

[0198] In another embodiment, the self-assembly polypeptide is a P₁₁ family polypeptide.

[0199] The P₁₁ family of polypeptides are β -sheet forming 11 residue polypeptides with anti-parallel arrangement. The P₁₁ family of polypeptides self-assemble to form nanostructures like tapes and fibrils, and beyond a certain critical peptide concentration (CPC) they form reversible hydrogels. This transition is triggered by pH stimuli and can be fine-tuned by varying salt concentration.

[0200] The peptides in this family can be used to respond to change in pH to switch between a stable fluid and stable gel states.

[0201] Based on the specific amino acids in the sequence, the peptide gelation occurs either at acidic pH (P₁₁-4) or at basic pH (P₁₁-5).

[0202] Chiral tri-peptides using both D-and L-amino acids can be used to create pH responsive hydrogels. The D-amino acid at the N-terminus is essential for molecular packing and fibre elongation. The tri-peptides Val-Phe-Phe, Phe- Phe-Val and Leu-Phe-Phe can be used to form hydrogels at pH 7.4 in sodium phosphate buffer.

[0203] Table 2 sets out exemplary P₁₁ self-assembly polypeptides:

Peptide name/sequence	Critical Peptide concentration (mM)	Low pH	Neutral and near neutral pH	Basic pH
P ₁₁ -4 n-QQEFWEFRQQ-c	6.3	pH< 3cloudy gels	pH 7.5 -8.0; β -sheet to random coil	pH> 8; monomer fluid state

P ₁₁ -8 n-QQEFOWOFRQQ-c	6.4	pH<6; monomer fluid state	pH 6.0-10.3; biphasic-fluid +gel particles	pH> 10.3; nematic gels
P ₁₁ -9 n-SSEFEWEFRSS-c	7.0	pH< 3.2 gels	pH 6.8 -7.2 clear, viscous fluid	pH> 7.2; monomer fluid state
P ₁₁ -12 n-SSEFOWOFRSS-c	7.1	pH<6; monomer fluid state	pH 6.0-8.0; fluid with nanoparticles	pH 8.2-9.5; viscous fluid pH> 9.5; gels

[0204] The optimal pH of the enzyme of the present invention can be paired with a self-assembly polypeptide with desired characteristics at that pH. For example, bovine carbonic anhydrase has its optimal activity is at pH ~8.0 and therefore the P₁₁-4 peptide is an ideal choice as it exists in soluble form at pH > 7.0 and gels at pH < 3.

[0205] The self-assembly polypeptides of the present invention include polypeptides that are responsive to salt.

[0206] For example, the EAK16 peptide can be used to spontaneously form membranes when Na⁺ ions are added. This is attributed to the complementary ionic interactions of alternating amino acids in its sequence. Chiral forms of the same peptide (d-EAK16 and l-EAK16) form gels in the presence of Na⁺/K⁺ ions at concentrations of 1 mg/mL. Using rheological tests, the d-form is more responsive to the ions than its l-form.

[0207] Peptide (TIGYG) based on potassium ion channel epitope can be used to gel in the presence of K⁺ ions but not with Na⁺ ions. At 0.05 wt. %, the peptide gels at various concentrations of K⁺ ions but Na⁺ ions have no effect. Without wishing to be bound by theory, this is attributed to the ability of K⁺ ions to crosslink between nanofibers and act as glue to promote gelation.

[0208] Ca²⁺ ions can be also used to induce gelation. For example, short peptides of sequence n-FGLDD, n-FFCGLD, n-FFCGLDD and longer peptide amphiphile

(C₁₅H₃₁CONH–A4G3ERGD) can be used to form gels with Ca²⁺ and this can be reversed by addition of EDTA solution.

[0209] Peptide amphiphiles can also be used to respond to divalent cations (Ca²⁺ and Mg²⁺) but not to monovalent cations (Na⁺), to form gels. Without wishing to be bound by theory, this is attributed it to the ability of divalent ions to form effective salt bridges.

[0210] Dual-responsive peptides that respond to pH and Ca²⁺ ions can also be used in the present invention. The ability of ions to be involved in electrostatic interactions depends on the charged state of the ions as well as peptide. Since charge depends on pH, their effects are often cumulative and result in peptides that can respond to both pH and ions. Peptide amphiphiles (PA) all having four distinct regions: lipophilic tail (palmityl), β -sheet segment, spacer segment and biological epitope can be used to form gels at pH 7.4 or in 50mM CaCl₂ solution.

[0211] The rate of gelation can be varied by varying the amino acid residues used in the β -sheet and spacer segments. Acidic β -sheet forming peptides of sequence Pro-Y-(Z-Y)₅-Pro (where Y-Leu/Phe and Z-Asp/Glu) were investigated for dual response. Peptide concentration of ~1.6 %w/v formed gels at pH range 6-8 and the exact gelling pH depended on the residues. However, the addition of ~20mM CaCl₂ solution yields gels that are stable and formed instantly even at lower peptide concentration ~1.34 % w/v.

[0212] The self-assembly polypeptides of the present invention include polypeptides which respond to temperature and enzyme action.

[0213] Tri-peptides with hydrophobic residues (Val, Leu and Phe) dissolved under basic conditions (pH 11.5-13.5) with application of heat and on cooling to room temperature can be used to form self-supporting gels. Synthetic peptides modified to have phosphate groups (e.g. Nap-GFFYp-OMe) can be used to form stable

gels by addition of alkaline phosphatase. The dephosphorylation of the peptides triggers the formation of hydrogel at peptide concentration as low as ~0.01 wt. %.

[0214] Another macroscopic form of the supramolecular assembly of the present invention is an aggregate.

[0215] As used herein, the term “aggregate” refers to a bulk material composed of a plurality of hydrogel particles held together by inter-particle and particle-liquid forces, such as, without limitation, hydrogen bonds.

[0216] A salt/ion responsive peptide used in the present invention is the bolaamphiphile bis (N-R-amido- glycyglycine)-1,7-heptane dicarboxylate. At low Ni^{2+} concentration these peptides can be used to form nanofibres and at moderate Ni^{2+} concentration the nanofibres bundle together to form macroscopic aggregates. Addition of EDTA can be used to revert back the bundle form to nanofibres. Similar effects can be achieved using with Cu^{2+} ions but not Na^+ , emphasizing the role of divalent cations to stabilise salt bridge interactions.

[0217] Elastin-like polypeptides (ELP) which have repetitive penta-peptides (Val-Pro-Gly-Xaa-Gly; Xaa-any amino acid except proline) derived from the tropoelastin protein found in muscles can be used for temperature responsive aggregation. Beyond a certain critical solution temperature, ELP converts from soluble to insoluble aggregates. The critical solution temperature of the peptide can be tuned by changing the amino acid sequence.

[0218] In another aspect, the self-assembling enzymatic component comprises at least two self-assembly polypeptides bound to an enzyme. In one embodiment, the self-assembling enzymatic component comprises at least 3 self-assembly polypeptides bound to an enzyme. In another embodiment, the self-assembling enzymatic component comprises at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 self-assembly polypeptides bound to an enzyme.

[0219] In one aspect, the plurality of self-assembling enzymatic components are treated to fix the supramolecular assembly in the assembled state.

[0220] For example, the supramolecular assembly may be treated such that the self-assembling enzymatic components are covalently linked. In one embodiment, the self-assembling enzymatic components are covalently linked via disulphide bonds or amines such that the supramolecular assembly is not responsive to external stimuli. For example, the assembly does not switch between the assembly state and the soluble component state in response to a stimulus. In one embodiment the non-responsive assembly can be reverted to its soluble component state by disrupting the covalent bonds linking the plurality of self-assembling enzymatic components.

[0221] As used herein the term “bound” includes both covalent and non-covalent interactions bridging one polypeptide chain to another. For example, an enzyme polypeptide may be covalently or non-covalently bound to a self-assembly polypeptide. Non-covalent interactions include ionic interactions, Van der Waals forces, hydrogen bonding and dispersion forces, or a combination thereof. In polypeptides, bonds may form between backbone atoms, side chain atoms or both.

[0222] As used herein the term “plurality” refers to more than one. For example, at least two self-assembling enzymatic components. In one aspect the supramolecular assembly comprises at least two self-assembling enzymatic components. In one embodiment the supramolecular assembly comprises at least two self-assembling enzymatic components comprising a first enzyme. In another embodiment the supramolecular assembly comprises at least two self-assembling enzymatic components comprising a first enzyme, and at least two self-assembling enzymatic components comprising a second enzyme.

[0223] In one embodiment, the self-assembly polypeptide and the enzyme of the self-assembling enzymatic components are not covalently linked.

[0224] The short and repetitive nature of self-assembly polypeptides can cause expression problems in the host cell when produced recombinantly. For example, when expressed at high levels, these peptides can associate within the cell and induce a response similar to viral particles. They often lead to cell toxicity or eventually get degraded by proteases.

[0225] The present inventors have surprisingly demonstrated that a self-assembly polypeptide can be expressed as a fusion protein fused to an enzyme of the invention, to form a component that can self-assemble into a supramolecular structure.

[0226] Accordingly, in one embodiment, the self-assembling polypeptide and the enzyme of the self-assembling enzymatic components are covalently linked.

[0227] In another embodiment, the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides.

[0228] To improve self-assembly, in one embodiment the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides. The repeats of the self-assembly polypeptide can be separated by one or more amino acid residues.

[0229] For example, the present inventors have demonstrated that a self-assembly polypeptide can be expressed as a fusion protein fused to an enzyme of the invention, to form a component that can self-assemble into a supramolecular structure, wherein a linker binds the self-assembly polypeptide to the enzyme.

[0230] In one embodiment, the self-assembling enzymatic component comprises at least 2 tandemly repeated self-assembly polypeptides bound to an enzyme. In another embodiment, the self-assembling enzymatic component comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 tandemly repeated self-assembly polypeptides bound to an enzyme.

[0231] A linker as used herein includes one or more amino acids which function to allow a space between the enzyme and the self-assembly polypeptide.

[0232] Accordingly, a variety of linkers with different amino acid sequences are suitable to function to allow a space between the enzyme and the self-assembly polypeptide.

[0233] Glycine, which does not have any functional group attached to the primary carbon atom allowing free rotational movement about the carbon-carbon bond axis, can be used as a linker in the components described herein.

[0234] The Gly-Gly linker can be used to link short coils or domains to allow flexibility and space for interaction.

[0235] Glycines can be interspersed with serine molecules to form a GS-linker. Due to individual characteristics of glycine and serine, the resultant GS-linker is flexible and a random coil with no defined secondary structure.

[0236] In specific cases, a link with stable secondary structure is desired to improve stability of the fusion protein. Short sequences that form α -helical structures can be used to link fusion proteins. If a more rigid linker is needed then proline residues can be used in the linker sequence.

[0237] Poly-proline sequences can be used to form rigid rod structures and as molecular rulers.

[0238] Protease cleavage sites can be incorporated into the linker region. The linker allows for sufficient space for proteases to bind to the specific site to initiate cleavage.

[0239] The length of the linker depends on the specific application of the supramolecular assembly. Linker length can be chosen to ensure there is sufficient space for enzyme-substrate interaction to take place.

[0240] The different types of linkers used in recombinant protein expression and their specific role in the application has been summarized in Table 3 below:

Table 3

Linker*	Secondary structure	Application	Reference
1. GGGGSGGGGS 2. GSGGGG	Random coil	To link two β -strand amyloid peptides	(Baldwin et al., 2006)
1.A(EAAAK)2A - H2 link 2.A(EAAAK)3A - H3 link 3.A(EAAAK)4A - H4 link 4.[A(EAAAK)4A]2 - H4-2	α -helical	Improve stability of fusion protein	(Bai and Shen, 2006)
1. (AGAGAGPEG)2 2. (AGAGAGPEG)4.5	Random coil	Flexibility to protein domains	(Wong Po Foo et al., 2009)
1. (AGAGAGPEG)10 2. (GGGGS)2	Random coil	Flexibility to protein domains	(Ramirez et al., 2013)
(AGAGAGPEG)10	Random coil	Flexibility to protein domains	(Kim et al., 2013)
1. GS 2. GSSGGGS 3. GSSGGGSGS 4. GSSGGSGGSGGSGS 5. GSSGGSGGSGGSGGSGS	Random coil	Improve lipase activity on yeast cell surface	(Washida et al., 2001)
GSSGGSGGSGGSGS	Random coil	Improve lipase activity on yeast cell surface	(Yuzbasheva et al., 2011)
PTPPTTPTPPTTPTPTP	Rigid	Enzyme-peptide fusion	(Wu et al., 2011)
1. EGKSSGSGSESKEF 2. (GGGGS)3	Random	To link antibody domains	(Newton et al., 1996)
SSGIDGR	-	Factor Xa protease cleavage site	(Tenno et al., 2004)
QTNSITSLYKSAGSE ONLY FQ	-	TEVp protease cleavage site	(Hartmann et al., 2009)
GSGSG DDDDK	-	Enteropeptidase cleavage site	(LaVallie et al., 1993)

*Letters in bold highlight the protease cleavage site

[0241] The present inventors have demonstrated that a self-assembly polypeptide can be expressed as a fusion protein fused to an enzyme of the invention, to form

a component that can self-assemble into a supramolecular structure, wherein a GS linker binds the self-assembly polypeptide to the enzyme.

[0242] Accordingly, in one embodiment the linker is a GS linker, wherein at least 90% of the amino acids in the linked polypeptide are glycine or serine or a combination thereof.

[0243] An exemplary GS linker is GGGGSGGGGS (SEQ ID NO: 2).

[0244] In one embodiment where the enzyme is bovine carbonic anhydrase joined via a GS linker to the self-assembly polypeptide P₁₁₋₄, the amino acid sequence of the self-assembling enzymatic component is:

GMSHHWGYGKHNGPEHWHKDFPIANGERQSPVDIDTKAVVQDPALKPLALVY
GEATSRRMVNNGHSFNVEYDDSQDKAVLKDGPLTGTYRLVQFHFHWGSSDDQ
GSEHTVDRKKYAAELHLVHWNTKYGDFGTAAQQPDGLAVVGVFLKVGDNAPAL
QKVLDA LDSIKTKGKSTDFPNFDPGSLLPNVLDYWTYPGSLTTPPLLESVTWIVL
KEPISVSSQQMLKFRTLNFNAEGEPELLMLANWRPAQPLKNRQVRGFPKGGGG
SGGGGSQQRFEWEFEQQ (SEQ ID NO: 3).

[0245] In one embodiment where the enzyme is microbial carbonic anhydrase joined via a GS linker to the self-assembly polypeptide P₁₁₋₄, the amino acid sequence of the self-assembling enzymatic component is:

MANNVAAPLIDLGA EAKKQAQKSAATQSAVPEKESATKVAEKQKEPEEKAKPEP
KKPPHWGYFGEEGPQYWGE LAPEFSTCKTGKNQSPINLKPQTAVGTTSLPGFD
VYYRETALKLINNGHTLQVNIPLGSYIKINGHRYELLQYHFHTPSEHQ RDGFNYP
MEMHLVHKDGDGNLAVIAILFQEGEENETLAKLMSFLPQTLKKQEIHESVKIHPA
KFFPADKKFYKYSGLTTPPCSEGVYWMVFKQPIQASVTQLEKMHEYLG SNAR
PVQRQNARTLLKSWPDRNRANTVYEFYGGGGSGGGGSQQRFEWEFEQQ
(SEQ ID NO: 4).

[0246] In one embodiment where the enzyme is tyrosinase joined via a GS linker to the self-assembly polypeptide P₁₁₋₄, the amino acid sequence of the self-assembling enzymatic component is:

MSNKYRVRKNVLHLDTEKRDFVRTVLILKEKGIYDRYIAWHGAAGKFHTPPGS
DRNAAHMSSAFLPWHREYLLRFERDLQSINPEVTLPYWEWETDAQMQDPSQS
QIWSADFMGGNGNPIKDFIVDTGPFAAGRWTIDEQGNPSGGLKRNFGATKEA
PTLPTRDDVLNALKITQYDTPPWDMTSQNSFRNQLEGFINGPQLHNRVHRWVG
GQMGVGPTAPNDPVFFLHHANVDRIWAVWQIIHRNQNYQPMKNGPFGQNFRD
PMYPWNTTPEDVMNHRKLGYYVDIELRKSQRSSGGGGSGGGGSQQRFWEF
EQQ (SEQ ID NO: 5)

[0247] In one embodiment where the enzyme is cutinase joined via a GS linker to the self-assembly polypeptide P₁₁₋₄, the amino acid sequence of the self-assembling enzymatic component is:

MLPTSNPAQELEARQLGRTRDDLINGNSASCADVIFIYARGSTETGNLGTGSPS
IASNLESAFGKDGWVIQGVGGAYRATLGDNALPRGTSSAAIREMLGLFQQANTK
CPDATLIAGGYSQGAALAAASIEDLDSAIRDKIAGTVLFGYTKNLQNRGRIPNYP
A DRTKVFCNTGDLVCTGSLIVAAPHLAYGPDARGPAPEFLIEKVRVAVRGSAGGGG
SGGGGSQQRFWEFEFEQQ (SEQ ID NO: 6)

[0248] In one embodiment where the enzyme is aminotransaminase joined via a GS linker to the self-assembly polypeptide P₁₁₋₄, the amino acid sequence of the self-assembling enzymatic component is:

MASMDKVFAGYAARQAILESTETTNPFAGIAWVEGELVPLAEARIPLLDQGM
HSDLTYDVPSVWDGRFFRLDDHITRLEASCTKLRLRLPLPRDQVKQILVEMVAKS
GIRDAFVELIVTRGLKGVGRTRPEDIVNNLYMFVQPYVWVMEPDMQRVGGS
AV VARTVRRVPPGAIDPTVKNLQWGDIVRGMFEAADRGATYPFLTDGDAHLTEGS

GFNIVLVKDGVLVTPDRGVLQGVTRKSVINAAEAFGIEVRVEFVPVELAYRCDEIF
MCTTAGGIMPITTLTGMPVNGGQIGPITKKIWDGYWAMHYDAAYSFEIDYNERN
GGGGSGGGGSQQRFWEFEQQ (SEQ ID NO: 7)

[0249] In one embodiment, the present invention provides a self-assembling enzymatic component as described herein, wherein the self-assembling enzymatic component is capable of switching between a soluble and insoluble (e.g. assembled into a supramolecular assembly) state.

[0250] As discussed herein, a self-assembling enzymatic component comprising a self-assembly polypeptide bound to an enzyme can self-assemble into a supramolecular assembly in response to an external stimulus.

[0251] In another embodiment, a supramolecular assembly as described herein can reverse back to the soluble component state of the self-assembling enzymatic components, in response to an external stimulus.

[0252] The P₁₁β-sheet peptide family can be used to switch between soluble-gel states in response to pH at peptide concentration of 6-7mM. Without wishing to be bound by theory, the mechanism controlling this behaviour is due to three main interactions between peptide monomers which are charged interactions between arginine and glutamic acid residues, hydrophobic interactions of glutamate and $\pi-\pi$ interaction between aromatic residues of tryptophan and phenylalanine residues.

[0253] The choice of the charged residue in the peptide decides the net charge of the peptide and this helps to tune the switching behaviour at different pH. A peptide amphiphile (C₁₂-GAGAGAGY) based on the silk fibroin protein can be used to form reversible hydrogels. At 0.2 wt. % and at pH 11, the peptide is soluble; at pH 8 it shows aggregation before forming a gel at pH 4. When the

tyrosine residue was replaced by serine, the gelling property is lost but reversible aggregates are formed at similar pH values.

[0254] An α -helical peptide AFD19 can be used for pH responsive reversible gelation at 0.1 wt. %. Importantly, this peptide it has two gelation and solution states unlike the other systems which have only one. At the extremes of pH 3.0 and 11.5 the peptide remains soluble but at pH 6 and 10.5 it forms a stable gel and at pH 7.5 it forms a precipitate.

[0255] Peptide amphiphiles SA2 and SA7 can be used to form switchable aggregates; the peptides self-assemble into nano-vesicles of diameter ~ 60 nm at pH 7.0 and at pH 5.0 the vesicles clump into visible aggregates. However, once vesicles are formed they cannot revert back to the monomeric state, but the aggregation of vesicles is reversible.

[0256] The ELPs can be used as a reversible stimuli-responsive system. These peptides can be modified in different ways to alter their switchable behaviour. A grafted 20 repeat sequence of ELP using methacryloyl polymer forms cylindrical polymeric brushes. These brushes show dual response to high level of salt and temperature to form aggregates which were reversible at lower levels. Incorporation of cysteine residues in the ELP promotes cross-linking hydrogels. At 2.5 wt. % the peptides exhibit dual response to both temperature and oxidative conditions resulting in reversible gels.

[0257] An advantage of such supramolecular assemblies is that the enzymatic components can be selectively separated from the reaction mixture on demand by switching between their soluble and insoluble states. For example; enzymes which respond to external stimuli could be temporarily immobilised in an enzymatically active supramolecular assembly and then switched back to their soluble component state to be reused. Such a property will be beneficial for

enzymes used in the pharmaceutical and fine chemical industries where free or soluble enzymes are preferred over immobilised forms.

[0258] The present inventors have also demonstrated that a plurality of self-assembling enzymatic components as described herein are able to form an enzymatically active supramolecular assembly without the need for additional self-assembling peptides to drive the formation the assembly.

[0259] Accordingly, in one embodiment, the enzymatically active supramolecular assembly consists of a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

[0260] In another embodiment, the enzymatically active supramolecular assembly consists essentially of a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

[0261] As used herein, "consisting essentially of" when used to define compositions and methods, refers to excluding other elements of any essential significance to the composition and methods. For example, an assembly consisting essentially of the a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, would not exclude other elements that do not materially affect the of the invention.

[0262] As discussed above, immobilising enzymes using the covalent method on to solid supports often leads to reduced enzyme activity compared to their free soluble forms.

[0263] The present inventors have surprisingly demonstrated that the catalytic activity of the enzyme in the supramolecular assembly is at least 70% of the catalytic activity of the free enzyme, such as at least 70%, 74%, 80%, 85%, 90%, 95%, or 98% of the catalytic activity of the free enzyme.

[0264] Without wishing to be bound by theory, the present inventors have shown that the self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme interact primarily between the self-assembly polypeptides of the plurality of components, and this is expected to minimise any distortion to the enzyme active site allowing greater retention of activity in the nanoparticle form compared to conventional immobilisation strategies.

[0265] The present inventors have also demonstrated the supramolecular assembly is stable between 25 to 50°C, such as between 25 to 40°C.

[0266] The present inventors have surprisingly demonstrated that the catalytic activity of the enzyme in the supramolecular assembly is at least 70% of the catalytic activity of the free enzyme, such as at least 70%, 74%, 80%, 85%, 90%, 95%, or 98% of the catalytic activity of the free enzyme at a range of temperatures.

[0267] The present inventors have also demonstrated the supramolecular assembly is at least as catalytically active as the free enzyme between 25 to 50°C.

[0268] The present inventors have also demonstrated the supramolecular assembly is at least as catalytically active as the free enzyme at 4°C.

[0269] The present inventors have also demonstrated the supramolecular assembly is stable over a storage period of at least 2 months.

[0270] The present inventors have produced a self-assembling enzymatic component comprising a first enzyme, and a self-assembling enzymatic component comprising a second enzyme.

[0271] Accordingly, in another aspect, the present invention provides a process for forming an enzymatically active supramolecular assembly as described herein, wherein the process comprises the step of contacting a first self-assembling enzymatic component with at least a second self-assembling enzymatic component, wherein each self-assembling enzymatic components each comprise a self-assembly polypeptide bound to an enzyme.

[0272] Without wishing to be bound by theory, the formation of a supramolecular assembly comprising a first self-assembling enzymatic component comprising a first enzyme and a second self-assembling enzymatic component comprising a second enzyme brings the first and second enzymes into close proximity, thereby facilitating the catalytic activity of the two enzymes and of the supramolecular assembly, and/or increases the catalytic activity such that reversible reactions can be driven to completion more frequently compared to free enzymes in solution and/or compared to enzymes immobilised using conventional immobilisation systems, and/or decreases the reaction time compared to free enzymes in solution and/or compared to enzymes immobilised using conventional immobilisation systems, and/or allows the enzymes to act co-operatively.

[0273] As used herein, the term "co-operatively" refers to two or more enzymes functioning in a concerted manner. One example is that the two or more enzymes form an enzyme complex, or alternatively react with the same substrate simultaneously, to carry out their catalytic activities.

[0274] In one embodiment, the product of the first enzyme is a substrate for the second enzyme. For example, wherein the first enzyme is carbonic anhydrase and the second enzyme formate dehydrogenase, the bicarbonate produced by

the self-assembling enzymatic component comprising carbonic anhydrase is used as a substrate for the self-assembling enzymatic component comprising formate dehydrogenase.

[0275] In one embodiment the first and second enzymes form part of a cascade reaction.

[0276] As used herein the term "cascade reaction" includes a series of enzymatic reactions where the product of one reaction is a substrate for a subsequent reaction. For example, a first enzyme reacts with the substrate first, producing a product, which in turn, is a substrate that reacts with a second enzyme.

[0277] The enzymatic reactions of the cascade reaction can be part of the same metabolic pathway.

[0278] As used herein, the term "metabolic pathway" refers to a series of chemical reactions occurring within a cell. In each pathway, a principal chemical is modified by a series of chemical reactions. Lists of metabolic pathways are available publicly from, e.g., the KEGG Pathway Database (available at www.genome.jp/kegg/pathway.html).

[0279] In another aspect the present invention provides a self-assembling enzymatic component capable of forming an enzymatically supramolecular assembly, wherein the self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

[0280] As discussed above, the choice of enzyme will depend on the desired application of the enzymatically active supramolecular assembly.

[0281] The present inventors have demonstrated enzymatic activity of the supramolecular assemblies.

[0282] Accordingly, the assemblies provided herein can be used for a number of industrial applications.

[0283] For example, the assemblies described herein can be used in the conversion of carbon dioxide to bicarbonate as part of carbon capture and sequestration processes. To date, the capture of CO₂ is the bottle neck of the three-step process of carbon capture and sequestration (CCS) process including its transport and storage, and contributes to up to 82% of capital cost. Hence capture methods are the area of focus for improvement in the CCS process to improve overall efficiency and reduce cost. Chemical based capture technologies like electrochemical pumps, chemical looping and selective membranes focus mainly on concentrating CO₂ content in flue gas rather than conversion. Chemical methods also use toxic, corrosive substances and demand additional energy costs for catalyst and adsorbent regeneration.

[0284] Biological methods using carbonic anhydrase (CA) enzyme accelerates CO₂ absorption rate from flue gas streams and converts them to bicarbonate forms CA enzymes have been integrated with CO₂ capture methods in both their free form such as the Carbozyme permeator or immobilised to support materials for industrial scrubbers. The form of CA used strongly influences the overall efficiency of the capture process. Increased hydration of CO₂ at gas-liquid interface is achieved by free CA that cannot be reused. To recover free CA, ultrafiltration and nanofiltration membranes are used but are limited by low flow rates, high transmembrane pressure drop and membrane fouling.

[0285] The present inventors have demonstrated that a carbon dioxide capture/ carbon dioxide converting enzyme can be formed into an enzymatically active supramolecular assembly.

[0286] Accordingly, in one embodiment, the carbon dioxide converting/carbon dioxide converting enzyme is selected from the group consisting of formate dehydrogenase, carbonic anhydrase, RuBisCO and combinations thereof.

[0287] As discussed herein, in one aspect, the present invention comprises an enzymatically active supramolecular assembly comprising a first self-assembling enzymatic component and a second self-assembling enzymatic component, wherein the first self-assembling enzymatic component comprises a self-assembly polypeptide bound to a first enzyme, and wherein the second self-assembling enzymatic component comprises a self-assembly polypeptide bound to a second enzyme. In one embodiment the first enzyme is carbonic anhydrase and the second enzyme is formate dehydrogenase.

[0288] The present inventors have demonstrated that enzymatically active bovine carbonic anhydrase can be formed into an enzymatically active nanoparticle.

[0289] Accordingly, in one embodiment the invention provides an enzymatically active supramolecular assembly comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to carbonic anhydrase.

[0290] As shown in the examples, the enzyme bovine carbonic anhydrase (BCA) was fused with the self-assembly polypeptide P₁₁-4 connected by a GS-linker (Figure 1) to form a self-assembling enzymatic component comprising a self-assembly polypeptide bound to an enzyme via a linker.

[0291] The self-assembling peptide P₁₁-4 has a β -strand structure and spontaneously self-assembles to form nanofibers under suitable conditions. The P₁₁-4 peptide is relatively small in size compared to the enzyme BCA itself and sufficient space is provided for peptide interaction to promote self-assembly of two

or more fusion proteins. BCA and P₁₁-4 were connected by the GS-linker to provide flexibility between protein domains.

[0292] Following bacterial growth and expression, BCA-P₁₁-4 was purified and the formation of nanoparticles was demonstrated with TEM images (Figure 2). BCA-P₁₁-4 samples show the presence of uniform distribution of particles with spherical morphology. This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce a supramolecular assembly, wherein the assembly is a nanoparticle.

[0293] Accordingly, in one aspect the present invention provides an enzymatically active supramolecular assembly comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to carbonic anhydrase, wherein the assembly is a nanoparticle.

[0294] Figure 3 demonstrates the particles observed had an average size in the range of 50 – 100 nm with some as large as 180 nm. In contrast, there was no formation of spherical particles observed in the TEM images of free enzyme WT-BCA (Figure 6), confirming that the self-assembly polypeptide plays an important role in driving the self-assembly of individual monomers to form nanoparticles of defined size and shape.

[0295] Unlike spherical aggregates that are commonly the form with misfolded proteins, the structure and folding properties of individual BCA-P₁₁.4 monomers that form the nanoparticle are preserved in the nanoparticles. This is illustrated by the similar melting temperatures of BCA- P₁₁-4 nanoparticles and WT-BCA, as measured by differential scanning fluorimetry (Figure 7).

[0296] Figure 3 demonstrates a supramolecular assembly comprising BCA- P₁₁-4 is enzymatically active. Furthermore, the enzymatically active nanoparticles retain full catalytic activity of the enzyme, and the enzymatically active nanoparticles retain full catalytic activity of the enzyme at 4°C. As demonstrated in Figure 3, the hydase activity of BCA- P₁₁-4 nanoparticles is comparable to that of free enzyme (WT-BCA) using the Wilbur-Anderson method. After 25 seconds, both enzyme solutions changed color to green indicating enzymatic conversion of CO₂. The Wilbur-Anderson units [WAU] for BCA- P₁₁-4 and WT-BCA were calculated to be 18.70WAU and 18.99WAU respectively, confirming that BCA- P₁₁-4 nanoparticles retained 98% of hydase activity in comparison to WT-BCA.

[0297] Accordingly, the present invention provides an assembly wherein the catalytic activity of the enzyme is at least 98% of the catalytic activity of the free enzyme.

[0298] In addition to high activity, BCA-P₁₁-4 nanoparticles also have attractive high stability at increased temperatures, an important feature for CO₂ capture at high temperature. The influence of high temperature on the nanoparticle stability and enzyme activity was tested. Results from dynamic light scattering technique showed that an average particle size range of 100 – 120nm was stable between 25°C to 50°C (Figure 4).

[0299] Accordingly, in one embodiment the present invention provides an assembly wherein the assembly is stable between 25 to 50°C.

[0300] Catalytic performance measured using p-nitrophenyl acetate (p-NPA) showed that BCA-P₁₁-4 retains almost all of its initial catalytic activity at higher temperatures, with maximum activity observed at 40°C similar to the free enzyme WT-BCA (data not shown). These results illustrate that the BCA- P₁₁-4 nanoparticles retain both their spherical self-assembled structure as well as their catalytic activity at higher temperatures making them beneficial for CO₂ capture

applications. Additionally, the nano-structural feature of these self-assembled nanoparticles was retained over a storage period of 2 months (Figure 8). No significant change in size of the particles was observed which highlight the role of the specific interactions involved in self-assembly process leading to the formation of well-defined nanoparticles.

[0301] Accordingly, in one embodiment the present invention provides an assembly wherein the assembly is catalytically active between 25 to 50°C.

[0302] The structural stability of the BCA-P₁₁-4 nanoparticles is essential for its integration with existing methods for CO₂ capture process. The relatively large size of BCA-P₁₁-4 nanoparticles allows its integration with large pore membrane as opposed to the small pore membranes used in existing methods to retain free carbonic anhydrase. The preservation of nanoparticle structure of BCA-P₁₁-4 over time would ensure long-term containment within membrane minimizing the replenishing of fresh enzyme. The use of BCA-P₁₁-4 within large pore membrane will allow operations at high flow rates and easy membrane maintenance, thereby improving the overall process efficiency.

[0303] Accordingly, in one aspect the present invention provides a process for sequestering carbon dioxide from a carbon dioxide containing fluid or gas, wherein the method comprises the steps of:

- providing an enzymatically active supramolecular assembly described herein, wherein the enzyme is carbonic anhydrase;
- contacting the assembly with the carbon dioxide containing fluid or gas; and
- converting carbon dioxide in the carbon dioxide containing fluid or gas to bicarbonate.

[0304] In one embodiment, the present invention provides a self-assembling enzymatic component wherein the self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and wherein the

self-assembling enzymatic component is capable of forming an enzymatically active supramolecular assembly in the presence of at least one further self-assembling enzymatic component comprising a self-assembly polypeptide bound to an enzyme.

[0305] In one embodiment of a process described herein, the supramolecular assembly is immobilized. For example, in one embodiment the present invention provides a process for sequestering carbon dioxide from a carbon dioxide containing fluid or gas, wherein the method comprises the steps of:

- providing an enzymatically active supramolecular assembly described herein, wherein the enzyme is carbonic anhydrase;
- contacting the assembly with the carbon dioxide containing fluid or gas; and
- converting carbon dioxide in the carbon dioxide containing fluid or gas to bicarbonate;

wherein the assembly is immobilized on a film or a membrane.

[0306] In another aspect, the present invention provides a process for sequestering carbon dioxide from a carbon dioxide containing fluid or gas, wherein the method comprises the steps of:

- providing an enzymatically active supramolecular assembly described herein, wherein the enzyme is carbonic anhydrase;
- contacting the assembly with the carbon dioxide containing fluid or gas; and
- converting carbon dioxide in the carbon dioxide containing fluid or gas to bicarbonate,

wherein the carbon dioxide containing fluid or gas is a fluid or gas stream.

[0307] In another embodiment, an assembly as described herein is formulated as an agent designed to ameliorate or treat a condition such as a pathological condition. The agent may be administered via a conventional route normally used to administer a medicament including, but not limited to, oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous, intraarticular, and

intramedullary), intraperitoneal, transmucosal (including nasal), transdermal, rectal and topical (including dermal, buccal, sublingual and intraocular) routes. Intravenous delivery may take place via a bolus injection or via infusion; infusion may be done over a period ranging from less than a minute to several hours to continuously. In certain embodiments, a course of treatment will involve administration by a combination of routes.

[0308] The present inventors have demonstrated that the formation of the enzymatically active supramolecular assemblies described herein can be controlled, allowing the formation of supramolecular assemblies of different sizes.

[0309] Importantly, the present inventors have demonstrated that the formation of the enzymatically active supramolecular assemblies described herein can be controlled, allowing the formation of supramolecular assemblies of different sizes.

[0310] As described in the Examples (e.g. Example 11), the present inventors have demonstrated the controlled formation of BCA-P₁₁4 nanoparticles using pH and metal-ion Mg²⁺ as two independent key parameters, and using difference concentrations of self-assembling enzymatic components. It is also demonstrated that salt type and ionic strength of the solution influence nanoparticle size and degree of self-assembly. A two-level full factorial model was used to determine that pH and MgCl₂ concentration can be varied alone or combined to control nanoparticle size.

[0311] Accordingly, in one embodiment, the present invention provides a method of forming an enzymatically active supramolecular assembly, the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii) contacting the solution containing a plurality of self-assembling enzymatic

components with a buffer to form an enzymatically active supramolecular assembly.

[0312] As used herein, the term "buffer" includes those agents which maintain a solution pH in an acceptable range. A buffer is an aqueous solution consisting of a mixture of a weak acid and its conjugate base or a weak base and its conjugate acid. Its pH changes very little when a small amount of strong acid or base is added to it and thus it is used to prevent any change in the pH of a solution. Buffer solutions are used in protein formulations as a means of keeping proteins within a narrow pH range to optimize shelf life. As used herein, the term "buffer" refers to a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer.

[0313] The present inventors have demonstrated that pH reduction in a buffer of sufficient ionic strength allows adjacent self-assembling enzymatic components monomers to interact and self-assemble.

[0314] An example of a suitable buffer is a buffer comprising 50mM Tris-HCl. In one embodiment the buffer comprises 50 mM NaNO₃ in 10 mM Tris. In another embodiment the buffer comprises 50 mM NaNO₃ in 10 mM Tris.

[0315] Accordingly, in one embodiment, the present invention provides a method of forming an enzymatically active supramolecular assembly, the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii) contacting the solution containing a plurality of self-assembling enzymatic components with a buffer to form an enzymatically active supramolecular assembly, wherein the buffer comprises 50mM Tris-HCl.

[0316] Importantly, as demonstrated in Example 11, the size of the supramolecular assembly formed increased with reducing pH.

[0317] In one embodiment the solution formed has a pH in the range from about 5.6 to about 6.8.

[0318] Accordingly, in one embodiment the present invention provides a method as described herein, wherein the particle has a diameter of about 100 nm, and wherein the buffer comprises 50mM Tris-HCl at pH 6.8.

[0319] In another embodiment, the present invention provides a method as described herein, wherein the particle has a diameter of about 400 nm, and wherein the buffer comprises 50mM Tris-HCl at pH 6.5.

[0320] In another embodiment, the present invention provides a method as described herein, wherein the particle has a diameter of about 1500 nm, and wherein the buffer comprises 50mM Tris-HCl at pH 5.6.

[0321] The present inventors have also demonstrated in Example 11 that supramolecular assembly size is influenced by the concentration of the self-assembling enzymatic component. Importantly, formation of enzyme nanoparticles requires a relatively low concentration of self-assembling enzymatic components (e.g. 0.025 mg/mL P₁₁₄ in 0.5 mg/mL BCA-P₁₁₄) compared to the concentration of self-assembling polypeptide alone (e.g. peptide P₁₁₄ alone requires ≥ 10 mg/mL for self-assembly).

[0322] In one embodiment the solution formed has a concentration of self-assembling enzymatic components in the range from about 0.5 mg/mL to about 3mg /mL.

[0323] Accordingly, in one embodiment the present invention provides a method as described herein, wherein the particle has a diameter of about 30 to about 200 nm, and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 1.0 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

[0324] In another embodiment the present invention provides a method as described herein, wherein the particle has a diameter of about 600 nm, and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 3 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

[0325] In another embodiment the present invention provides a method as described herein, wherein the particle has a diameter of about 200 nm, and wherein the buffer comprises 6 mM MgCl_2 at pH 7.5 and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 0.5 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

[0326] In another embodiment the present invention provides a method as described herein, wherein the particle has a diameter of about 633 nm, and wherein the buffer comprises 8 mM MgCl_2 at pH 7.0 and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 1 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

[0327] The present inventors have shown that in addition to ionic strength, the type of salts affect supramolecular assembly. For example, in Example 11 the type of salt affects BCA- P_{114} nanoparticle formation.

[0328] In one embodiment the solution formed comprises an ionic strength of less than 200 mM. In another embodiment, the solution formed comprises NaNO_3 at 50mM, NH_4Cl at 50 mM or NH_4Cl at 100 mM. In a preferred embodiment the solution formed does not comprise NaCl .

[0329] Accordingly, in one embodiment the present invention provides a method as described herein, wherein the particle has a diameter of about 30 to about 40 nm or about 120 to about 200 nm, and wherein the buffer comprises 50 mM NaNO_3 in 10 mM Tris at pH 6.8.

[0330] The present inventors have also shown that, divalent cations affect supramolecular assembly. For example, in Example 11, 25 mM Mg^{2+} promoted BCA-P_{114} self-assembly.

[0331] Accordingly, in one embodiment the divalent cation is Mg^{2+} .

[0332] In a preferred embodiment, the Mg^{2+} is provided by MgCl_2 present at a concentration of about 5 mM MgCl_2 , about 10 mM MgCl_2 , or about 25 mM MgCl_2 .

[0333] In another embodiment, the present invention provides a method as described herein wherein the particle has a diameter of about 1500 nm, and wherein the buffer comprises 10 mM MgCl_2 at pH 6.1.

[0334] In another embodiment, the present invention provides a method as described herein wherein the buffer comprises 25 mM MgCl_2 in 10mM Tris at pH 8.0.

[0335] In another embodiment, the present invention provides a method as described herein, wherein the particle has a diameter of about 200 nm, and wherein the buffer comprises 5 mM MgCl_2 in 10mM Tris at pH 8.0.

[0336] In another embodiment, the present invention provides a method as described herein, wherein the particle has a diameter of about 400 nm, and wherein the buffer comprises 50 mM MgCl₂ in 10mM Tris at pH 8.0.

[0337] Importantly, the present inventors have described the factors and interactions that can be used to predict supramolecular assembly size under various conditions. For example, Example 11 demonstrates that protein concentration, MgCl₂ and pH allow prediction of BCA-P114 particle size under various conditions.

[0338] Accordingly, in one embodiment, the present invention provides a method of forming an enzymatically active supramolecular assembly having a diameter 'd', the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii) contacting the solution containing a plurality of self-assembling enzymatic components with a buffer to form a solution having a plurality of self-assembling enzymatic components at a concentration 'c' wherein the solution formed has a pH 'a' and comprises MgCl₂ at a concentration 'd' to form an enzymatically active supramolecular assembly, wherein:

$$D \text{ (nm)} = (127.32 - 15.77a + 2.44c - 2.22d + 0.42 ad)^2 + 2.0 \dots\dots\dots(\text{Formula I}).$$

[0339] In another embodiment the present invention provides a method of modulating the size of an enzymatically active supramolecular assembly, said method comprising the steps of contacting a solution comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, with a buffer to form an enzymatically active supramolecular assembly, wherein the

buffer comprises a component to modulate the size of the enzymatically active supramolecular assembly formed.

[0340] In a further embodiment, the component to modulate the size of the enzymatically active supramolecular assembly formed is Mg^{2+} and/or a pH modulating compound.

[0341] In one embodiment, the enzyme is a bovine carbonic anhydrase.

[0342] In one embodiment, the self-assembly polypeptide is a P_{11} family polypeptide. In another embodiment, the self-assembly polypeptide is P_{11-4} .

[0343] In another aspect, a linker binds the self-assembly polypeptide to the enzyme of the self-assembling enzymatic component. In one embodiment, the linker covalently binds the self-assembly polypeptide to the enzyme. In another embodiment, the linker comprises a polypeptide. In a further embodiment, at least 90% of the amino acids in the linked polypeptide are glycine or serine or a combination thereof. For example, in one embodiment, the linker is a glycine-serine (GS) linker, and in another embodiment the GS linker comprises SEQ ID NO: 2.

[0344] The present inventors have demonstrated that it is possible to generate supramolecular assemblies that are recoverable.

[0345] The recoverability of the supramolecular assemblies as described herein has a number of advantages. For example, membrane filtration can be used as an alternate processing route because it offers benefits of continuous operation mode, improved product recovery and reduced energy consumption. Cross-flow filtration composed of a series of membranes is being used as an efficient purification method for the recovery of proteins and enzymes from complex solutions. Particularly, membrane based processes are useful for enzymatic

reactions since they provide high-surface area for enzyme loading, easy separation of products and reusability of the enzymes when immobilised on the membrane surface. However, due to smaller size of free enzymes, the selection of membrane is often limited.

[0346] Supramolecular assemblies and particles formed by self-assembly do not require additional chemical agents. Also they can be efficiently retained within available membrane systems and also eliminate the need to immobilise them on the membrane surface, consequently offering additional benefits of reduced process time and cost associated with membrane preparation and maintenance. In addition to the membrane-based recovery method, the supramolecular assemblies such as the precipitates formed from the enzyme fused with 3 peptides offer another mode to recover enzymes without the need for membranes. Hence reusability of enzyme particles can be achieved in two complementary modes: with and without membranes.

[0347] The self-assembled, enzymatically active supramolecular assemblies of the present invention present a carrier-free method to immobilise enzymes with retained activities and reusability for advanced biocatalysis.

[0348] The present inventors have demonstrated that a supramolecular assembly with self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides forms an about 1000-1100 nm precipitate at pH 6.0, but this precipitate can be solubilized at pH 8.0.

[0349] Accordingly, the present invention provides a method of switching an enzymatically active supramolecular assembly between an insoluble state and a soluble state, said method comprising contacting a solution comprising an insoluble supramolecular assembly as described herein wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly

polypeptides, with a buffer of increased pH to solubilize the supramolecular assembly. In one embodiment, the buffer of increased pH is at pH 8.0.

[0350] Accordingly, the present invention provides a method of switching an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state, said method comprising contacting a solution comprising a supramolecular assembly as described herein wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of increased pH to unassemble the supramolecular assembly. In one embodiment, the buffer of increased pH is at pH 8.0.

[0351] Accordingly, the present invention provides a method of switching an enzymatically active supramolecular assembly between an insoluble state and a soluble state, said method comprising contacting a solution comprising a plurality of self-assembling enzymatic components as described herein wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of increased pH to solubilize the supramolecular assembly. In one embodiment, the buffer of decreased pH is at pH 6.0.

[0352] The present invention also provides a method of switching an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state, said method comprising contacting a solution comprising a plurality of self-assembling enzymatic components as described herein, wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of decreased pH to assemble the supramolecular assembly. In one embodiment, the buffer of decreased pH is at pH 6.0.

[0353] The present inventors have demonstrated it is possible to recover supramolecular assemblies, for example using both ultrafiltration and precipitation

methods. Both methods are routinely employed at large industrial scales for product recovery and hence can be easily incorporated into existing processes. Despite the difference in particles sizes formed by BCA-P₁₁4 and BCA-(P₁₁4)₃, both forms can be recovered from solution by membrane filtration with appropriate membrane pore size.

[0354] Accordingly, in one embodiment the present invention provides a method as described herein, wherein the method further comprises the step of recovering the supramolecular assembly.

[0355] Methods of recovering nanoparticles are known in the art. In one embodiment, filtering can be performed to recover supramolecular assemblies.

[0356] The filtering of the supramolecular assemblies can be performed using a back-pulse or back- flush filter array, a cross flow membrane, a flow-through membrane, a sintered metal filter, a single media filter, a multimedia filter, or a combination thereof.

[0357] The filtering of the supramolecular assemblies can further comprise a back flush of a filter with liquid medium to produce the recovered supramolecular assemblies.

[0358] Generally, various types of filter configurations for solid/liquid filtration can be used. These filter configurations include (1) outside-in filtration where a traditional solid/liquid barrier separation occurs on the outer perimeter of a tubular filter element, (2) inside-out filtration where a solid/liquid barrier separation occurs on the inside of a tubular filter element, (3) inside-out (multimode) filtration where a solid/liquid (barrier or crossflow) separation that occurs on the inside of open-ended tubular filter element and filtration is with multi-option top or bottom feed inlet.

[0359] Further, the supramolecular assemblies can be separated from liquid medium using a gravity-assisted separation technique.

[0360] In one embodiment, the supramolecular assemblies can be separated from liquid medium using centrifugation.

[0361] In one embodiment, the supramolecular assemblies can be separated from liquid medium using precipitation.

[0362] The present inventors have demonstrated that by increasing the number of self-assembly polypeptides in the self-assembling enzymatic component, the self-assembly behavior of these components can be controlled. For example, the present inventors have demonstrated that increasing the number of self-assembly polypeptides in the self-assembling enzymatic components form large aggregates, rather than nanoparticles, that were easily precipitated. Therefore altering the peptide length and number of repeats in the fusion protein design provides an option to specifically design enzyme-peptide fusion systems with a controllable assembly feature.

[0363] The present inventors have generated self-assembling enzymatic components wherein the self-assembling enzymatic component comprises a self-assembly polypeptide bound to a number of enzymes. For example, in Example 14, the inventors have fused a self-assembly polypeptide to thermostable carbonic anhydrase from *Thiomicrospira crunogena* (TmCA), Tyrosinase (Tyr), Cutinase (Cut) and ω -Aminotransferase (ATA). All four enzymes fused a self-assembly polypeptide were successfully expressed and purified, and their chromatogram profiles were similar to those of their wild-type counterparts. All enzyme-fusion systems investigated in the Examples showed similar activity and stability in comparison to their wild-type counterpart, and in all cases, the fusion systems demonstrated reaction rates comparable to wild-type enzyme.

[0364] The enzyme particles formed using TmCA-P₁₁₄, Tyr-P₁₁₄ and Cut-P₁₁₄ are similar in size to those reported for BCA-P₁₁₄ and BCA-(P₁₁₄)₃ described above, and therefore are expected to be recovered and reused using membrane separation processes as described herein.

[0365] Such reproducible expression and purification performance of the fusion systems in comparison to wild-type enzymes confirm that the fused peptide does not significantly influence the basic characteristics of the enzymes. This is beneficial from a commercial production point of view as these enzyme-peptide fusion systems can therefore be produced at bulk scale with similar ease to regular enzymes.

[0366] The present inventors have demonstrated that TmCA-P₁₁₄ self-assembly can be controlled using temperature. That temperature can be used as a trigger for certain types of enzymes without comprising their structure and function is useful for forming nanoparticles of thermostable enzymes which have a wide range of industrial applications.

[0367] Control of Tyr-P₁₁₄ self-assembly using Mg²⁺ ions demonstrates that self-assembly controlled by metal-ions can be broadly applied to different types of enzymes if fused with the P₁₁₄ peptide.

[0368] Importantly, the enzymes characterized in the Examples are of different classes, source, structure and function, demonstrating supramolecular assemblies can be formed using the methods of the invention for a wide array of enzymes. All enzyme-fusion systems investigated showed similar activity and stability in comparison to their wild-type counterparts.

[0369] The four enzymes are important for industrial application in sectors such as energy, pharmaceutical and fine-chemical manufacturing. For example, due to its attractive stability at high pH, TmCA is one promising candidate of the a number

of carbonic anhydrases being explored for CO₂ removal in flue gas scrubbing systems, and carbonic anhydrases have already be tested in immobilised form on solid supports to allow reuse and continuous biocatalytic processes.

[0370] As used herein, the term “Carbonic Anhydrase” (EC 4.2.1.1) is synonymous with CA, carbonate dehydratase; anhydrase; carbonate anhydrase; carbonic acid anhydrase; carboxyanhydrase; carbonic anhydrase A; and carbonate hydro-lyase and refers to an enzyme that catalyzes the reversible hydration of CO₂ to bicarbonate - $\text{H}_2\text{CO}_3 = \text{CO}_2 + \text{H}_2\text{O}$. ‘BCA’ refers to bovine carbonic anhydrase.

[0371] There is no particular restriction to a method for measuring carbonic anhydrase activity, and it can be measured using the methods described herein.

[0372] As used herein, the term “carbonic anhydrase gene” includes a cDNA molecule, genomic gene or nucleotide sequence which is capable of encoding a CA enzyme or a polypeptide fragment thereof or alternatively, a nucleotide sequence which is complementary to said cDNA molecule, genomic gene or nucleotide sequence. Carbonic anhydrase gene encoding a carbonic anhydrase are well known in the art.

[0373] Tyrosinase is used in the manufacture of 3,4-dihydroxyphenylalanine (L-DOPA), a potent drug used in treatment of several neural diseases (Zaidi et al., 2014). Attempts to reuse this enzyme for L-DOPA production by immobilising on DEAE-Granocel (cellulose) support showed that this method was not feasible due to enzyme inactivation and use of native enzyme would be more productive.

[0374] As used herein, the term “tyrosinase” is an enzyme that is also referred to as monophenol monooxygenase; phenolase; monophenol oxidase; cresolase; monophenolase; tyrosine-dopa oxidase; monophenol monooxidase; monophenol dihydroxyphenylalanine:oxygen oxidoreductase; N-acetyl-6-hydroxytryptophan

oxidase; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase; o-diphenol:O₂ oxidoreductase; phenol oxidase.

[0375] Tyrosinase catalyzes an oxidation reaction of phenols. For example, tyrosinase catalyzes the reactions: (1) L-tyrosine+L-dopa+O₂→L-dopa-dopaquinone+H₂O; (1a) L-tyrosine + ½ O₂ = L-dopa; (1b) L-dopa + ½ O₂ = dopaquinone + H₂O; and (2) 2 L-dopa + O₂ = 2 dopaquinone + 2 H₂O.

[0376] Tyrosinase (EC 1.14.18.1) is a type III copper protein found in a broad variety of bacteria, fungi, plants, insects, crustaceans, and mammals, which is involved in the synthesis of betalains and melanin. The enzyme, which is activated upon binding molecular oxygen, can catalyse both a monophenolase reaction cycle (reaction 1, above) or a diphenolase reaction cycle (reaction 2, above). During the monophenolase cycle, one of the bound oxygen atoms is transferred to a monophenol (such as L-tyrosine), generating an o-diphenol intermediate, which is subsequently oxidized to an o-quinone and released, along with a water molecule. The enzyme remains in an inactive deoxy state, and is restored to the active oxy state by the binding of a new oxygen molecule. During the diphenolase cycle the enzyme binds an external diphenol molecule (such as L-dopa) and oxidizes it to an o-quinone that is released along with a water molecule, leaving the enzyme in the intermediate met state. The enzyme then binds a second diphenol molecule and repeats the process, ending in a deoxy state. The second reaction is identical to that catalysed by the related enzyme catechol oxidase (EC 1.10.3.1). However, the latter cannot catalyse the hydroxylation or monooxygenation of monophenols.

[0377] There is no particular restriction to a method for measuring tyrosinase activity, and it can be measured using the methods described herein. As used herein, the term “tyrosinase gene” includes a cDNA molecule, genomic gene or nucleotide sequence which is capable of encoding a tyrosinase enzyme or a polypeptide fragment thereof or alternatively, a nucleotide sequence which is

complementary to said cDNA molecule, genomic gene or nucleotide sequence. Tyrosinase genes encoding a tyrosinase are well known in the art.

[0378] Tyrosinase substrates products, and commercial applications are shown below:

Substrate	Product	Application	Industry
Tyrosine, DOPA	Melanin	Melanin biosynthesis	Medical and Pharmaceutical
Tyrosine	DOPA	Production of Psychoactive drugs	Pharmaceutical
Tyrosol	Hydroxytyrosol	Anti-oxidant	Food
Phenols and Catechols	Polyphenolic materials	Organic semiconductors/ photovoltaics	Electrical/electronic
Sericin	Conjugated sericin	Protein cross-linking	Silk textile industry
Phenolic species	Quinine species	Biosensors for pollutant detection; Herbicides determination	Waste water treatment
Monophenols, diphenols (from proteins) along with p-coumaric acid and caffeic acid and (from carbohydrates)	o-diquinones	Cross-linked Biopolymers	Food industry
α -lactalbumin	Cross-linked α -lactalbumin	Dairy Processing	Food industry
wheat dough	2-S-cysteinyl-DOPA, 2,5-di-S-cysteinyl-DOPA, 6-S-cysteinyl-DOPA,	Cereal Processing.	Food industry

	5-S-cysteinyl-3, 4-DOPA, and di-DOPA cross-links in gluten proteins		
Pork and chicken meat	Improves gel formation ability of meat	Meat Processing	Food industry
Tyrosine present in silk and wool fibres	L-DOPA mediated crosslinking in silk and wool	Hydrogels for skin substitutes, matrices for drug delivery and tissue engineering	Medical

[0379] Cutinase has wide industrial applications ranging from oil industry, production of flavour and phenolic compounds to polymer synthesis and enantioselective esterification reactions.

[0380] As used herein, the term “cutinase” is an enzyme that is also referred to as cutin hydrolase. Cutinase catalyzes the reaction: cutin + H₂O = cutin monomers.

[0381] Cutinase (EC 3.1.1.74) acts on cutin a polymeric structural component of plant cuticles, which is a polymer of hydroxy fatty acids that are usually C16 or C18 and contain up to three hydroxy groups. The enzyme from several fungal sources also hydrolyses the p-nitrophenyl esters of hexadecanoic acid. It is however inactive towards several esters that are substrates for non-specific esterases.

[0382] There is no particular restriction to a method for measuring cutinase activity, and it can be measured using the methods described herein.

[0383] As used herein, the term “cutinase gene” includes a cDNA molecule, genomic gene or nucleotide sequence which is capable of encoding a cutinase

enzyme or a polypeptide fragment thereof or alternatively, a nucleotide sequence which is complementary to said cDNA molecule, genomic gene or nucleotide sequence. Cutinase genes encoding a cutinase are well known in the art.

[0384] Cutinase substrates, products, and commercial applications are shown below:

Substrate	Product	Application	Industry
Apple, tomato orange peel	ricinoleic acid	Industrial chemical	Waste valorisation
p-nitrophenyl ester, triolein and tricaprilin	emulsified triacylglycerols	synthesis of structured triglycerides, polymers, surfactants	production of personal care products, in agrochemicals and pharmaceutical chemistry
chemical modification of fatty acids (hydrogenation), reorganization and breakdown of fatty acids in the triglyceride main chain	Oils and fats	Oil and fat industry	Food industry
geranyl diphosphate	Acetates, propionates, and butyrates of acyclic alcohols. Eg: geraniol and citronellol	Terpenic esters synthesis	Flavour industry
transesterification of butyl acetate and hexanol	hexyl acetate	Fruit flavour synthesis	Flavour industry
organophosphate malathion	Degradation	Waste treatment	Insecticide and pesticide degradation
hydrolysis of ester bonds only at the fiber surface	improvement in polyester fiber quality	Textiles	Textile industry
Polycaprolone, Dihexylphthalate	Degradation	Biodegradation of plastics	Polymer industry
resolution of diols 1–4.	Respective enantiomers	Enantioselective esterification reactions	Fine chemical industry

[0385] As used herein, the term “amino transferase” is an enzyme that catalyzes a reaction between an amino acid and an α -keto acid; an amino acid contains an amine (NH₂) group, and a keto acid contains a keto (=O) group. In transamination, the NH₂ group on one molecule is exchanged with the =O group on the other molecule. The amino acid becomes a keto acid, and the keto acid becomes an amino acid.

[0386] As used herein, the term “ ω -amino transferase (ATA)” is an enzyme that catalyzes the transfer of an amino group from a primary amino donor to a carbonyl acceptor with pyridoxal 5'-phosphate (PLP) as cofactor. The reaction can be divided into two half reactions, where the amino group is first transferred to PLP to form PMP (pyridoxamine phosphate) and then from PMP onto the carbonyl group. ω -amino transferase (ATA) which is a highly valuable enzyme for the R-selective transamination reactions used to produce pharmaceutically-important drug intermediates (Höhne and Bornscheuer, 2012). Due to the high cost of making ATA, reusability of ATA is preferred and recent attempts have reported immobilisation of ATA via fused his-tag on magnetic beads for ATA recycling and reuse (Dold et al., 2016). These reports suggest that industrially important enzymes are continuously been investigated with variety of immobilisation methods for reusability and for developing continuous biocatalytic processes.

[0387] (S)-selective ω -transaminases are well known, and (R)-amines have mainly been prepared by kinetic resolution of racemic amines by (S)-transaminases. However, with this method (R)-amines are obtained only with a maximum yield of 50%. Recently, several (R)-selective enzymes have been described.

[0388] There is no particular restriction to a method for measuring aminotransferase activity, and it can be measured using the methods described herein.

[0389] As used herein, the term “aminotransferase gene” includes a cDNA molecule, genomic gene or nucleotide sequence which is capable of encoding a cutinase enzyme or a polypeptide fragment thereof or alternatively, a nucleotide sequence which is complementary to said cDNA molecule, genomic gene or nucleotide sequence. Aminotransferase genes encoding aminotransferases are well known in the art.

[0390] Aminotransferase substrates, products, and commercial applications are shown below:

Substrate	Product	Application	Industry
Amine enantiomers along with pyruvate and alanine as co-substrates	Ketone enantiomers	Chiral amine and drug precursor production	Pharmaceutical industry
Ketones along with pyruvate and alanine as co-substrates	Chiral amines	drug precursor production	Pharmaceutical industry
δ -keto acid ester	δ -amino acid ester	Asymmetric synthesis	Pharmaceutical industry
acetophenone	1-phenylethylamine	Asymmetric synthesis	Pharmaceutical industry
1-phenylethylamine	Small aliphatic amines, Sterically demanding aliphatic amines, Aryl-alkyl amines, Cyclic amines, amino alcohols	Asymmetric synthesis	Pharmaceutical industry
ketone and isopropylamine	sitagliptin	Asymmetric synthesis of sitagliptin	Pharmaceutical industry

[0391] The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only, and the invention is not limited to these examples, but rather encompasses all variations that are evident as a result of the teachings provided herein.

EXAMPLES

EXAMPLE 1: Fusion-protein expression and purification

[0392] DNA encoding bovine carbonic anhydrase (BCA) with linker and P₁₁₋₄ peptide were codon optimized, synthesized and ligated into pET28a expression plasmid between *NcoI* and *XhoI* restriction sites by GenScript (Piscataway, NJ, USA). The recombinant plasmid was transformed using NEB High efficiency transformation protocol into BL21 (DE3) competent *E.coli* cells (New England Biolabs Inc.). Cells were grown using terrific broth (TB) medium and kanamycin (50 µg/mL) for 4 hours at 37°C. Protein production was induced by adding 1mM isopropyl-β-D-thiogalactopyranoside to the medium after 4 hours and incubated at 20°C for 16 hours. Harvested cells were suspended in lysis buffer (50mM Tris-HCl pH8.0, 50mM NaCl, 1mM EDTA, 0.5% Triton-X) and incubated for 20 minutes at room temperature. Cells were lysed using a homogeniser (Avestin Emulsiflex C5, Ottawa, Canada) for 2 passes at 15,000 psi. Cell supernatant solution was collected by centrifugation at 14,000 rpm 4°C for 20 minutes. BCA-P₁₁₋₄ was purified by immobilized metal-ion affinity chromatography on a Profinity IMAC (BioRad laboratories) column (1.5 × 8 cm) charged with nickel ion with BioRad BioLogic DuoflowTM Chromatography system. Fusion protein was eluted step-wise from 24 to 200 mM imidazole in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5M NaCl. Eluate containing the fusion protein was desalted using size exclusion chromatography on a G25 Sephadex (GE Healthcare) column (1.5 × 17 cm) with 20mM Tris-HCl buffer (pH 8.0). Purified BCA-P₁₁₋₄ was stored at 4°C in desalting buffer for subsequent analysis. Wild-type BCA (Sigma Aldrich) dissolved in

desalting buffer at 0.5-1.0 mg/mL concentration was used for comparative studies.

EXAMPLE 2: Transmission electron microscopy

[0393] Protein samples of 10 μ L volumes having concentration 0.5 -1.0 mg/mL were applied on individual freshly glow-discharged carbon coated 400 mesh copper grids and left on grids for 5 minutes. Grids were washed with 5 μ L of water and blotted with filter paper and stained using 2%v/v uranyl acetate for 30 seconds. ^[23] Samples were viewed using transmission electron microscope at 80 kV (Hitachi, HT7710 120kV FEG) and at 200 kV (FEI, Tecnai G2 T20 TWIN LaB6) and electron micrographs were recorded using CCD camera and Gatan "Digital Micrograph" software.

EXAMPLE 3: Differential Scanning Fluorimetry (DSF)

[0394] The unfolding characteristics of protein samples were determined using differential scanning fluorimetry and method described by Niesen and co-workers. ^[24] Protein samples with fluorescent dye SYPRO orange and buffer were incubated in 96 well plates. Samples were subjected to steady increased temperature cycles using real-time PCR instrument (Bio-Rad iCycler5). The melting temperature was calculated from the thermal transition curves and its derivative curves using the BioRad CFX Manager Software.

EXAMPLE 4: Hydrase activity test

[0395] The hydrase activity of enzyme was determined using the Wilbur Anderson method. ^[22] For the assay, 2mL of chilled assay buffer (50 mM Tris-HCl, 0.1 M sodium sulphate, pH 8.0) was added in a 4mL disposable cuvette. To this 20 μ L of 0.05% Bromothymol blue (BTB) dye was added and stirred well. Chilled enzyme of volume 20 μ L corresponding to 10 μ g of protein (determined by

Bradford's method – Sigma Bradford reagent) was added. The reaction was initiated by the addition of chilled CO₂ saturated water. The reaction was monitored by recording the pH change and color change of solution. The pH drop in the solution due to conversion of CO₂ to HCO₃³⁻ is visualized using the indicator bromothymol blue (BTB) which changes color from blue (pH >7.6) to green (pH 6.0 – 7.6) and yellow (pH < 6.0) accordingly. Blank solution was prepared as mentioned above and volume of enzyme was replaced with water. The time taken for the pH change from 8.0 to 7.0 was recorded in seconds and the hydase activity was calculated in terms of Wilbur Anderson Units (WAU) using the formula $(T_c - T_e) / T_e$; where T_c is the time in seconds for change in pH to 7.0 for the blank solution and T_e is the time in seconds for change in pH to 7.0 for the enzyme solution.

EXAMPLE 5: Dynamic Light Scattering (DLS)

[0396] Nanoparticle sizes were determined using Zetasizer Nano (Malvern Instruments). Protein particle sample of volume 1mL was taken in cuvette and measured for particle size using the zetasizer software. For temperature dependent DLS measurement, the sample chamber was set at specific temperature using in-built temperature control and protein was incubated at respective temperature for 5 minutes before taking measurements. Average particle sizes were determined from 3 runs each comprising 16 cycles

EXAMPLE 6: Esterase activity using para-Nitrophenyl acetate (pNPA) assay

[0397] The esterase activity was performed in a 96 well assay plate using reaction buffer (50mM sodium sulphate + 50mM HEPES pH 8.0) with a sample volume containing 3µg of enzyme. To this *p*-nitrophenyl acetate was added as a substrate at a final concentration of 1mM. Absorbance was measured at 405 nm every 15 seconds for 10 minutes using microplate reader (Infinite 200PRO, Tecan). Similar blank solution was prepared using water instead of enzyme solution. Following

blank correction of absorbance, the slope in terms of absorbance per second was calculated to determine reaction rate and esterase activity. For temperature dependent assay, enzyme was incubated at specific temperature in a heating block for 5 minutes before adding to the reaction buffer.

EXAMPLE 7: Formation of nanoparticles comprising bovine carbonic anhydrase bound to a self-assembly polypeptide.

[0398] The enzyme bovine carbonic anhydrase (BCA) was fused with the self-assembly polypeptide P₁₁₋₄ connected by a GS-linker (Figure 1).

[0399] The self-assembling peptide P₁₁₋₄ was selected because of its β -strand structure and its ability to spontaneously self-assemble to form nanofibers under suitable conditions. The P₁₁₋₄ peptide is relatively small in size compared to the enzyme BCA itself and sufficient space is required for peptide interaction to promote self-assembly of two or more fusion proteins. BCA and P₁₁₋₄ were connected by the GS-linker known to provide flexibility between protein domains.

[0400] Following bacterial growth and expression, BCA- P₁₁₋₄ was purified and the formation of nanoparticles was demonstrated with TEM images (Figure 2). BCA- P₁₁₋₄ samples show the presence of uniform distribution of particles with spherical morphology. This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce a supramolecular assembly. This data also demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce a supramolecular assembly wherein the assembly is a nanoparticle.

[0401] Figure 3 demonstrates the particles observed had an average size in the range of 50 – 100nm with some as large as 180nm. In contrast, there was no formation of spherical particles observed in the TEM images of free enzyme WT-

BCA (Figure 6), confirming that the self-assembly polypeptide plays an important role in driving the self-assembly of individual monomers to form nanoparticles of defined size and shape.

[0402] Unlike spherical aggregates that are commonly the form with misfolded proteins, the structure and folding properties of individual BCA-P₁₁-4 monomers that form the nanoparticle are preserved in the nanoparticles. This is illustrated by the similar melting temperatures of BCA- P₁₁-4 nanoparticles and WT-BCA, as measured by differential scanning fluorimetry (Figure 7). This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce a supramolecular assembly wherein the three dimensional structure of the enzyme is preserved in the supramolecular assembly.

EXAMPLE 8: Enzymatically active supramolecular assemblies.

[0403] Figure 4 demonstrates a supramolecular assembly comprising BCA- P₁₁-4 is enzymatically active. Furthermore, the enzymatically active nanoparticles retain full catalytic activity of the enzyme. As demonstrated in Figure 4, the hydrase activity of BCA- P₁₁-4 nanoparticles is comparable to that of free enzyme (WT-BCA) using the Wilbur-Anderson method. After 25 seconds, both enzyme solutions changed color to green indicating enzymatic conversion of CO₂. The Wilbur-Anderson units [WAU] for BCA- P₁₁-4 and WT-BCA were calculated to be 18.70WAU and 18.99WAU respectively, confirming that BCA-P₁₁-4 nanoparticles retained 98% of hydrase activity in comparison to WT-BCA. This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce an enzymatically active supramolecular assembly.

EXAMPLE 9: Enzymatically active supramolecular assemblies are highly stable at increased temperatures.

[0404] In addition to high activity, BCA- P₁₁-4 nanoparticles also have attractive high stability at increased temperatures, an important feature for CO₂ capture at a high temperature. The influence of high temperature on the nanoparticle stability and enzyme activity was tested. Results from dynamic light scattering technique showed that an average particle size range of 100 – 120nm was stable between 25°C to 50°C (Figure 5). This data demonstrates that the enzymatically active supramolecular assembly is stable between 25°C to 50°C.

[0405] Catalytic performance measured using p-nitrophenyl acetate (p-NPA) showed that BCA-P₁₁-4 retains almost all of its initial catalytic activity at higher temperatures, with maximum activity observed at 40°C similar to the free enzyme WT-BCA (data not shown). These results illustrate that the BCA- P₁₁-4 nanoparticles retain both their spherical self-assembled structure as well as their catalytic activity at higher temperatures making them beneficial for CO₂ capture applications. This data demonstrates that the enzymatically active supramolecular assembly is stable and as catalytically active as the free enzyme between 25°C to 50°C (Figure 7).

[0406] Additionally, the nano-structural feature of these self-assembled nanoparticles was retained over a storage period of 2 months (Figure 8). No significant change in size of the particles was observed which highlights the role of the specific interactions involved in self-assembly process leading to the formation of well-defined nanoparticles. This data demonstrates that the enzymatically active supramolecular assembly is stable upon storage.

[0407] The structural stability of the BCA-P₁₁-4 nanoparticles is essential for its integration with existing methods for CO₂ capture process. The relatively large size of BCA-P₁₁-4 nanoparticles allows its integration with large pore membrane

as opposed to the small pore membranes used in existing methods to retain free CA. The preservation of nanoparticle structure of BCA-P₁₁-4 over time would ensure long-term containment within membrane minimizing the replenishing of fresh enzyme. The use of BCA-P₁₁-4 within large pore membrane will allow operations at high flow rates and easy membrane maintenance, thereby improving the overall process efficiency.

EXAMPLE 10: Enzymes bound to self-assembly polypeptides.

[0408] Table 4 sets out self-assembling enzymatic components produced, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.

[0409] Table 4:

Enzyme	E.C number and details	Enzyme molecular weight (kDa)	Enzyme Fusion name	Enzyme fusion molecular weight (kDa)
Bovine carbonic anhydrase (BCA)	4.2.1.1 Lyase	29.17	BCA-P ₁₁ 4	31.34
Microbial carbonic anhydrase (TmCA)	4.2.1.1 Lyase	33.65	TmCA-P ₁₁ 4	35.82
Tyrosinase (Tyr)	1.14.18.1 Oxidoreductase	34.37	Tyr-P ₁₁ 4	36.53
Cutinase (Cut)	3.1.1.74 Hydrolase	22.39	Cut-P ₁₁ 4	24.56
Aminotransaminase (ATA)	2.6.1.18 Transferase	36.17	ATA-P ₁₁ 4	38.34

[0410] The fusion-proteins of table 4 are expressed and purified using the methods of Example 1.

[0411] Nanoparticle formation of the fusion-proteins of table 4 is examined using transmission electron microscopy as exemplified in Example 2.

[0412] Tyrosinase monophenolase and diphenolase activity is determined by measuring the formation of L-dopachrome from L-tyrosine or L-Dopa using a colorimetric assay. The assay is performed using 200 μ l of 50 mM Tris HCl buffer pH 7.5, 0.01 mM CuSO_4 , 28°C, 3 μ g/mL of purified enzyme. Substrate concentrations typically range between 0.1 to 6.0 mM for L-Dopa, and 0.02–6.0 mM for L-tyrosine. The formation of L-dopachrome ($\epsilon=3600 \text{ M}^{-1} \text{ cm}^{-1}$) is monitored by measuring the absorbance at 475 nm. (Goldfeder, Kanteev, Adir, & Fishman, 2013).

[0413] Cutinase hydrolyses the colourless molecule p-nitrophenyl butyrate to the yellow molecule p-nitrophenol, which allows for colorimetric analysis of cutinase activity. For the assay 0.25-1.5 μ M cutinase is analysed by adding 1 μ L of 0.1 M p-nitrophenyl butyrate in dimethyl sulfoxide and measured p-nitrophenol absorbance at 405 nm for 3 min using a spectrophotometric plate reader. The initial velocity, v_o , of p-nitrophenyl butyrate hydrolysis to p-nitrophenol is calculated from the linear portion of a plot of $A_{405 \text{ nm}}$ versus time.

[0414] The catalytic activity of aminotransaminase from cell lysate can be determined by using a standard photometric assay at 25°C containing 0.25 mg/mL of total lysate protein, 0.1 mM PLP, 5mM (R)- α -methylbenzylamine and 5 mM pyruvate or 5 mM butanal in 50 mM potassium phosphate buffer pH 7.5. The increase of acetophenone was measured at 300 nm (extinction coefficient= $0.28 \text{ mM}^{-1} \text{ cm}^{-1}$).

[0415] Microbial carbonic anhydrase activity is determined using the methods of Example 4 and Example 6.

[0416] Nanoparticle sizes are determined using the method of Example 5.

EXAMPLE 11: Modulation of enzymatically active supramolecular assembly formation**[0417] Expression and Purification of BCA-P₁₁₄ fusion protein**

[0418] The fusion protein BCA-P₁₁₄ was expressed in *E.coli* cells and purified as described above. Briefly gene expressing enzyme bovine carbonic anhydrase linked to P₁₁₄ peptide via a GS-linker was cloned as a single construct into pET28a between NcoI and XhoI (GenScript, Piscataway, NJ, USA). The plasmid pET28a-BCA-P₁₁₄ was transformed into *E.coli* BL21 (DE3) and cells were grown in terrific broth. Expression of BCA-P₁₁₄ was initiated by 1 mM IPTG and culturing continued for 16 h at 20°C. Cells were lysed and the supernatant collected after centrifugation was purified using immobilized metal-ion affinity chromatography (Profinity IMAC, Biorad laboratories). Fusion protein was eluted using step gradients from 24 to 200 mM imidazole in 50 mM Tris-HCl + 0.5 M NaCl pH 8.0 buffer. Pure protein fractions were buffer exchanged (G25 Sephadex, GE Healthcare) against either 10 mM Tris-HCl buffer (pH 8.0) for metal-ion screening and full-factorial experiments and 50 mM Tris-HCl buffer (pH 8.0) as the desalting buffer for all other experiments.

[0419] Size measurement using Dynamic Light scattering

[0420] Enzymatically active nanoparticle formation and size was determined using a Zetasizer Nano (Malvern Instruments). BCA-P₁₁₄ in solution (1 mL) was measured under a range of conditions for particle size using the zetasizer software. Data were collected from 3 independent experiments with each comprising 12 cycles.

[0421] Characterization of BCA-P₁₁₄ nanoparticle formation

[0422] The effect of four factors namely pH, protein concentration, temperature and metal-ions on enzymatically active nanoparticle formation of BCA-P₁₁4 was evaluated. The effect of pH was studied at a fixed protein concentration of 0.5 mg/mL and pH adjusted using 5 M or 1 M acetic acid for fine adjustment. The effect of varying protein concentration was evaluated at pH 6.8±0.1. To test temperature dependence, the sample chamber of Zetasizer Nano was adjusted using the in-built temperature control and BCA-P₁₁4 solution, diluted to 0.5 mg/mL in 50 mM Tris-HCl pH 8.0 to final volume of 1 mL, was incubated at the respective temperature in a heating block for 5 min before taking measurements. Stock solutions of 0.5 M NH₄Cl, MgCl₂, NaCl, NaNO₃ and Na₂SO₄ were prepared in 10 mM Tris-HCl pH 8.0 and the required volume added to BCA-P₁₁4 sample diluted to 0.5 mg/mL in 10 mM Tris-HCl pH 8.0, to achieve a final concentration of 50, 100 and 200 mM. For evaluation at pH 6.5, BCA-P₁₁4 with added salts was prepared as above and adjusted to pH 6.5 ± 0.1 using 1 M acetic acid.

[0423] Transmission Electron Microscopy

[0424] Negative staining of enzymatically active supramolecular assemblies was performed on carbon coated 200 mesh copper grids (GSCu200CC, Proscitech, QLD Australia) by an established protocol (Booth et al., 2011) using uranyl acetate stain. Samples were visualized at 200 kV using FEI, Tecnai G2 T20 TWIN LaB6 and electron micrographs were recorded using CCD camera and Gatan “Digital Micrograph” software.

[0425] Two-level Full factorial Design of enzymatically active supramolecular assemblies

[0426] A Factorial Design experiment using a two-level design was used to model the BCA-P₁₁4 enzymatically active supramolecular assembly formation. This method examined 4 factors (pH, temperature, MgCl₂ concentration and protein concentration) at two levels (Table 5) to determine the major factors and

interaction effects on BCA-P₁₁4 supramolecular assembly formation. A 2⁴ full factorial design with 4 center points was performed in 20 experiments where supramolecular assembly sizes measured by dynamic light scattering were used as the response variable. The center point values were determined as the average of low and high level values. Response data were analyzed using statistical software Design- Expert 10 (Stat-Ease, Inc. Minneapolis, USA).

[0427] Table 5 shows Two-level Factorial Design:

Factor	Low Level (-1)	Centre point (0)	High Level (+1)
pH	6.8	7.4	8.0
Temperature (°C)	25	32.5	40
Protein concentration (mg/mL)	0.5	1.25	2.0
MgCl ₂ concentration (mM)	2	11	20

[0428] Esterase Activity of BCA-P₁₁4 using para-nitrophenyl acetate (pNPA) assay

[0429] The enzyme activities of BCA-P₁₁4 in monomeric and nanoparticle forms were determined using the p-nitrophenylacetate (pNPA) assay (Pocker Y, 1968) with minor modifications. Enzyme samples (3 µg protein) were incubated in (50mM HEPES pH 8.0 containing 50 mM Na₂SO₄) and 1 mM of pNPA at 25°C in a final volume of 0.2 mL in a 96-well microplate. The product formation was monitored at 405 nm over 10 min by spectrophotometer microplate reader (Infinite 200Pro, Tecan).

[0430] Effect of pH and Ionic strength of buffer on assembly

[0431] The effect of varying pH on BCA-P₁₁4 at 0.5 mg/mL in low ionic strength buffer of 10 mM Tris-HCl was examined. As shown in Figure 14, when pH was reduced to 6.8, no nanoparticle formation of BCA-P₁₁4 was observed. As shown

in Figure 14, nanoparticles with an average diameter of 100 nm were formed at pH 6.8 and gradually increased in size with further reduction in pH. This result demonstrates that self-assembly requires sufficient ionic strength in the buffer to allow adjacent BCA-P₁₁4 monomers to interact and self-assemble.

[0432] This data demonstrates that assembly of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme can be controlled by pH, and that the size of the enzymatically active supramolecular assembly can be controlled by pH.

[0433] Without wishing to be bound by theory, the present inventors propose two interactive forces that influence BCA-P₁₁4 self-assembly: firstly, there is the interaction between peptide-peptide monomers (F_{p-p}) in anti-parallel orientation that is a combination of the attractive and repulsive forces as shown in Figure 15, where attractive forces exist between oppositely positioned arginine and glutamic acid residues and the π - π interaction of aromatic residues while glutamic acid residues at the 5th and 6th peptide position impart repulsive forces. Secondly, there is a pH-dependent interaction between enzyme and peptide monomers (F_{e-p}) as illustrated in Figure 15.

[0434] Decreasing pH from 8 to 6.8 results in an insignificant change in charge of P₁₁4 from -2.5 to -2 but the charge of BCA changes from -3.4 to a positive +0.7 (Table 6). Without wishing to be bound by theory, it is therefore expected that lowering pH has no significant effect on peptide-peptide interaction F_{p-p} but prompts a positive intermolecular enzyme-peptide interaction F_{e-p} that favors assembly (Figure 15). However, the positive F_{e-p} alone is not sufficient to form nanoparticles at pH 6.8. It requires an enhancement of peptide-peptide interaction F_{p-p} by increase in buffer concentration from 10 to 50 mM. This ionic strength-dependent result is consistent with the observation of self-assembly of pure P₁₁4 peptide regulated by ionic strength to form hydrogels near neutral pH. A

combination of the two forces F_{p-p} and F_{e-p} that are regulated by pH and ionic strength controls the self-assembly of BCA-P₁₁₄ to form nanoparticles.

[0435] Table 6 shows estimated charge of peptide P₁₁₄, enzyme BCA and fusion protein BCA-P₁₁₄ from pH 4.0 to pH 8.0 calculated using PROTEIN CALCULATOR v3.4:

pH	Estimated charges		
	P ₁₁₄	BCA	BCA-P ₁₁₄
4	0.3	29.5	29.7
4.2	-0.1	26.4	26.3
4.4	-0.5	23	22.5
4.6	-0.8	19.5	18.7
4.8	-1.1	16.3	15.2
5	-1.4	13.7	12.3
5.2	-1.6	11.6	10
5.4	-1.7	9.9	8.2
5.6	-1.8	8.5	6.7
5.8	-1.9	7.3	5.4
6	-1.9	6.1	4.2
6.2	-2	4.8	2.8
6.4	-2	3.4	1.4
6.6	-2	2	0
6.8	-2	0.7	-1.3
7	-2.1	-0.4	-2.4
7.2	-2.1	-1.3	-3.3
7.4	-2.2	-2	-4
7.6	-2.3	-2.6	-4.6
7.8	-2.4	-3	-5
8	-2.5	-3.4	-5.4

[0436] Effect of temperature and protein concentration on assembly

[0437] The influence of two additional parameters: protein concentration and temperature was examined. Increasing BCA-P₁₁₄ concentration from 0.5 to 1 mg/mL resulted in the formation of nanoparticles in the size range of 30-200 nm when maintaining the same buffer and pH conditions. Larger nanoparticles (~600nm) were formed at 3 mg/mL BCA-P₁₁₄ (Figure 16). Formation of enzyme nanoparticles requires a relatively low concentration of peptide (e.g. 0.025 mg/mL P₁₁₄ in 0.5 mg/mL BCA-P₁₁₄) compared to peptide P₁₁₄ alone which requires ≥ 10 mg/mL for self-assembly (Riley et al., 2009). Conversely, varying temperature from 25 to 50°C (Figure 17) showed no significant impact on BCA-P₁₁₄ self-assembly at pH 8.0.

[0438] This data demonstrates that assembly of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme into an enzymatically active supramolecular assembly can be controlled by the concentration of self-assembling enzymatic components, and that the size of the enzymatically active supramolecular assembly can be controlled by the concentration of self-assembling enzymatic components.

[0439] Effect of salts on assembly

[0440] Following the pH experiments with BCA-P₁₁₄, whether the peptide-peptide force F_{p-p} may be influenced by addition of salts to increase ionic strength and thus promote BCA-P₁₁₄ nanoparticle formation was examined. 50 mM NaCl or NaNO₃ to 10 mM Tris-HCl at pH 8.0 was added followed by pH adjustment to 6.8. Surprisingly, these two salts had opposite effects. With the addition of NaCl no nanoparticle formation was observed at pH 8.0 or 6.8 (Figure 14c) while the addition of NaNO₃ resulted in nanoparticles with two size peaks (30 and 200 nm) at pH 6.8 (Figure 14d). This result suggests that not only ionic strength but also the type of salt affects BCA-P₁₁₄ nanoparticle formation.

[0441] The effect of the salts Na_2SO_4 , NaCl , NaNO_3 and NH_4Cl which are known to influence protein stability or solubility was examined. Formation of nanoparticles was studied at 3 concentrations for each salt and two pH values, pH 6.5 and 8.0, which favor or disfavor formation of nanoparticles in the absence of salt, respectively (Table 7). Irrespective of the salt used, no self-assembly was observed with addition at 200 mM. This we attribute to the effect of high ionic strength which weakens attractive charge interactions between enzyme and peptide Fe-p, and also decreases attractive peptide-peptide force Fp-p as reported in literature (Aggeli et al., 2003).

[0442] Nanoparticles were produced in 50 mM NaNO_3 at pH 6.5 (Figs 14c-d). The effect of NaNO_3 on nanoparticle formation may be attributed to its specific interaction with wild-type BCA through the first hydration shell confirmed by molecular dynamic simulation studies (Warden et al., 2015). The most effective salt for facilitating BCA- P_{114} self-assembly in low ionic strength buffer was NH_4Cl . Ammonium chloride promoted self-assembly at pH 6.5 and salt concentrations up to 100 mM. Without wishing to be bound by theory, this behavior might be attributed to the unique ability of the ammonium cation to form cation- π interactions (Dougherty, 1996) with aromatic amino acids present in the peptide.

[0443] Table 7: Influence of added salts and pH on self-assembly of BCA-P₁₁4.

Salt	Concentration (mM)	pH 8.0	pH 6.5
Na ₂ SO ₄	50	-	-
	100	-	-
	200	-	-
NaCl	50	-	-
	100	-	-
	200	-	-
NaNO ₃	50	-	+
	100	-	-
	200	-	-
NH ₄ Cl	50	-	+
	100	-	++
	200	-	-

[0444] Particles formed in 10 mM Tris buffer with added salts and size measured by dynamic light scattering indicated by “+” for particle size < 500nm, “++” for particle size > 500nm and “-” no nanoparticle.

[0445] This data demonstrates that assembly of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme into an enzymatically active supramolecular assembly can be controlled by the concentration of salt, and that the size of the enzymatically active supramolecular assembly can be controlled by the concentration of salt.

[0446] Role of divalent cation – Mg²⁺ ions on assembly

[0447] The effect of divalent cations on BCA-P₁₁4 nanoparticle assembly independent of pH change was examined. For example, zinc ions promote the peptide AM1 to form strong films at neutral pH (Dexter et al., 2006) and similarly calcium cross-links beta roll peptides which form hydrogels (Dooley et al., 2012). The effects of the chloride salts of Ca²⁺ and Mg²⁺ on BCA-P₁₁4 self-assembly

were evaluated in parallel with wild-type BCA in order to understand their binding to protein surfaces. Ca^{2+} promoted formation of BCA- P_{114} nanoparticles at pH 8.0 but also with wild-type BCA which lacks the self-assembling peptide (data not shown) suggesting a non-specific effect of this cation between residues at the protein surface. 25 mM Mg^{2+} promoted BCA- P_{114} self-assembly at pH 8.0 but showed no influence on enzyme size with wild-type BCA alone under similar conditions (Figure 18a), suggesting a specific Mg^{2+} interaction with the peptide region of BCA- P_{114} . In terms of controlled assembly of nanoparticles, Mg^{2+} is the preferred cation because its interaction is primarily with the peptide region and with reduced enzyme surface interactions as demonstrated with the wild-type BCA.

[0448] The nanoparticle-forming effect observed with MgCl_2 on BCA- P_{114} also presents a new approach to generating nanoparticles using certain metal ions without adjusting pH. We examined the effect of different MgCl_2 concentrations on BCA- P_{114} self-assembly by dynamic light scattering. Nanoparticles were formed in 10mM Tris pH 8.0 buffer at MgCl_2 concentration as low as 5 mM while larger nanoparticles were favored at 25 mM MgCl_2 (Figure 18b). Transmission electron microscopy imaging confirmed the presence of BCA- P_{114} nanoparticles with diameter in the range of 100-200 nm formed at 5 mM MgCl_2 (Figure 18c). At concentrations above 25 mM MgCl_2 , a reduction in particle size was observed with no self-assembly beyond 75 mM. Self-assembly of BCA- P_{114} in the presence of MgCl_2 can be explained by the Debye length of Mg^{2+} ion calculated using the formula $K^{-1} \text{ (nm)} = 0.176/[\text{MgCl}_2]^{1/2}$. At concentrations < 5mM, the chance of Mg^{2+} ions interacting with BCA- P_{114} is greatly reduced due to dilution. With increasing concentrations (5-25 mM), Mg^{2+} ions have Debye length ranging from 1.44–0.64 nm favoring ionic interactions. Beyond 25 mM, the Debye length is significantly reduced, with a value of 0.37 nm at 75 mM, as a result of complete saturation of the cation effect by Cl^- ions.

[0449] This data demonstrates that assembly of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme into an enzymatically active supramolecular assembly can be controlled by the concentration of Mg^{2+} , and that the size of the enzymatically active supramolecular assembly can be controlled by the concentration of Mg^{2+} .

[0450] Without wishing to be limited by theory, the present inventors propose a mechanism for the formation and inhibition of BCA- P_{114} nanoparticles with Mg^{2+} . In solution, Mg^{2+} ions exist in a stable coordination complex with 6 oxygen atoms from water molecules (Bock et al., 1994). However, in solution with proteins, Mg^{2+} has preference to interact with oxygen atoms from carboxyl and hydroxyl groups in the side chain residues of amino acids (Zheng et al., 2008). Figure 19a illustrates the interaction of Mg^{2+} with the carboxyl oxygen of glutamic acid residues in P_{114} monomers. Since the preferred coordination number for Mg^{2+} is 6, it is possible that this state may be satisfied by 2 or more oxygen atoms of glutamic acid residues from 2 or more BCA- P_{114} monomers and the rest with nearby water molecules. The preference of oxygen atoms of glutamic acid over those of water molecules depends directly on the proximity of Mg^{2+} to these molecules, or indirectly, to the size of the Debye sphere whose radius equals the Debye length. In high ionic strength solutions such as 0.5 M NaCl, it is expected that the abundance of ions in the solution results in Na^+ surrounding the negatively charged glutamic acid residues and Cl^- ions saturating the Debye sphere of Mg^{2+} (Figure 19b). This results in loss of interaction between Mg^{2+} and glutamic acid residues in BCA- P_{114} , preventing self-assembly. The effect of 0.5 M NaCl was confirmed by particle size measured by dynamic light scattering (Figure 18d) which showed a complete prevention of nanoparticle self-assembly that were formed in the absence of NaCl. The inventors propose that Mg^{2+} promotes self-assembly through ionic interactions with P_{114} peptide in BCA- P_{114} and the size of particle can be controlled by Mg^{2+} concentration and ionic strength of solution.

[0451] Following statistical analysis of BCA-P₁₁₄ nanoparticle formation, a regression model was generated with the level of significance set at $\alpha = 0.01$ corresponding to 1% chance of error in the observed response. The points lying away from the straight line in the half-normal plot (Figure 20a) showed that individual factors pH (A) and MgCl₂ concentration (D) have the most significant effect on self-assembly followed by protein concentration of less significance. Interestingly the model also indicated a significant interaction effect (AD) between pH and MgCl₂ concentration. This interaction and its effect on nanoparticle size is shown in Figure 20b as a 3-D contour plot with regions of monomeric, native size (blue) at pH 8.0 and 2 mM MgCl₂, and progresses to small particle sizes (green) with changing pH and MgCl₂ to largest particle sizes (orange) at pH 6.8 and 20 mM MgCl₂. The model generated using the two-level full factorial design, not only encompasses the aforementioned findings regarding the effects of individual factors in self-assembly but it also identified an interaction between pH and MgCl₂ concentration. The model generates Formula I which describes the factors and interactions that can be used to predict BCA-P₁₁₄ particle size under various conditions:

$$\text{Particle size diameter 'D'(nm)} = (127.32 - 15.77a + 2.44c - 2.22d + 0.42 ad)^2 + 2.0 \dots\dots\dots(\text{Formula I})$$

[0452] Where; a is pH, c is protein concentration, and d is MgCl₂ concentration.

[0453] Applying this equation, BCA-P₁₁₄ particle were predicted to be formed with sizes of 253 and 633 nm under the following conditions – (1) 0.5 mg/mL BCA-P₁₁₄, pH 7.5, 6 mM MgCl₂, and (2) 1.0 mg/mL BCA-P₁₁₄, pH 7.0, 8 mM MgCl₂, respectively. Experimental results confirmed the predicted sizes whereby peak centers for conditions 1 and 2 measured by dynamic light scattering were 201 and 669 nm (Figure 20c), respectively. Thus, the empirical formula predicts particle size of BCA-P₁₁₄ under specified conditions and encompasses the parameters that allow robust control over particle size.

[0454] This data demonstrates that assembly of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme into an enzymatically active supramolecular assembly can be controlled by the concentration of Mg^{2+} , concentration of self-assembling enzymatic components, and/or pH, and that the size of the enzymatically active supramolecular assembly can be controlled by the concentration of Mg^{2+} , concentration of self-assembling enzymatic components, and/or pH.

[0455] This data also demonstrates that using the factorial design described herein, the conditions required to form a desired size of enzymatically active supramolecular assembly can be determined, and allows formation of enzymatically active supramolecular assemblies of a desired size.

[0456] Esterase activity of BCA-P₁₁4 enzyme nanoparticles

[0457] Esterase activities were determined for the various enzyme nanoparticles formed under the two-factorial design conditions and expressed relative to the activity of the monomeric protein-peptide unit. Average activities of the protein particles of various sizes were 108% of the relative activity of individual enzyme-peptide units as shown in Figure 21. This demonstrates that the self-assembly process allows proper orientation and access to active sites of individual enzyme molecules thereby retaining complete functionality. This is a much desired feature when designing enzyme nanoparticles and other protein assemblies with functionality for specific applications.

[0458] This data confirms that assembly of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme forms an enzymatically active supramolecular assembly.

EXAMPLE 12: Formation of a reusable enzymatically active supramolecular assembly

[0459] Plasmid and Bacterial expression

[0460] The gene encoding BCA and a single peptide (BCA-P₁₁₄) was inserted into pET28a vector and expressed in BL21 (DE3) *E.coli* cells as described above. The second fusion construct was designed to attach three P₁₁₄ peptides in series to the C-terminus of BCA via GGGGSGGGGS linker sequence and is designated as BCA-(P₁₁₄)₃. The gene sequence for BCA-P₁₁₄(3) was codon optimised, synthesized and ligated into pET28a expression plasmid between *NcoI* and *XhoI* restriction sites by GenScript (Piscataway, NJ, USA). The plasmid pET28a-BCA-(P₁₁₄)₃ was transformed into BL21 (DE3) *E.coli* cells using NEB High Efficiency Transformation protocol. Cells were grown in terrific broth (TB) medium and kanamycin (50 µg/mL) for 4 hours at 37 °C followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside at 20 °C for 16 hours. For Auto-Induction method, 2% v/v lactose was added in the initial TB medium.

[0461] *Amino acid sequence of BCA- (P₁₁₄)₃*

[0462] GMSHHWGYGKHNGPEHWHKDFPIANGERQSPVDIDTKAVVQDPALKPL
ALVYGEATSRRMVNNGHSFNVEYDDSQDKAVLKDGPLTGTYRLVQFHFHWGS
SDDQGSEHTVDRKKYAAELHLVHWNTKYGDFGTAAQQPDGLAVVGVFLKVG
DANPALQKVLDALDSIKTKGKSTDFPNFDPGSLLPNVLDYWTYPGSLTTPPLLESV
TWIVLKEPISVSSQQMLKFRTLNFNAEGEPELLMLANWRPAQPLKNRQVRGFPK
GGGGSGGGGSQQRFWEFEQQQQRFWEFEQQQQRFWEFEQQ (SEQ ID
NO: 8)

[0463] Scheme showing sequence regions corresponding to: Enzyme (normal text), Linker (bold) and self-assembly peptide tandem repeat (underline).

[0464] Cell harvest and protein purification

[0465] At the end of fermentation, cells were harvested by centrifugation (Sigma, John Morris Scientific) at 10,000 rpm for 10 mins. Cell pellet of 2.5g was re-suspended in 60 mL lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% Triton-X 100, and 0.5 mM ZnCl₂) and incubated for 20 minutes at room temperature on low shaking. Cells were placed on ice bath and lysed using sonicator (QSonica) equipped with 2 mm probe at 90% amplitude with 10 sec pulse ON and 20 sec pulse OFF for total time of 5 min. Cell supernatant was collected by centrifugation at 11,000 rpm 10 °C for 20 min. Sodium chloride was added to the supernatant to give a final concentration of 0.5 M, followed by filtration through 1.2 and 0.45 µm filters prior to purification. BCA-P₁₁-4(3) was purified using 5 mL HisTrapFF (GE Healthcare, Sydney) immobilised metal-ion affinity chromatography charged with nickel ion using AKTA start Chromatography system. The column was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl before loading 60 mL supernatant. After washing with 20 mL washing buffer (10 mM Tris-HCl buffer pH 8.5), fusion protein was eluted step-wise from 10, 24, 50 and 200 mM imidazole in 10 mM Tris-HCl buffer (pH 8.5). Eluate containing the fusion protein was diluted with water to decrease the conductivity 4-6mS/cm before loading on 1mL HiTrap QFF anion exchange chromatography column (GE Healthcare). Protein fractions was eluted with 0-100% gradient over 15 column volume (CVs) using 10 mM Tris-HCl buffer (pH 8.5) containing 1 M NaCl. Fractions were pooled based on purity determined by SDS-PAGE 4-12% Bis-Tris Bolt gels and eluate sample was desalted into 10 mM Tris-HCl buffer (pH 8.5) using size exclusion chromatography on a G25 Sephadex (GE Healthcare) column (1.5 × 17 cm). Purified BCA-(P₁₁4)₃ was stored at 4 °C in desalting buffer for subsequent analysis.

[0466] Preparation of enzyme particles

[0467] a. BCA-P₁₁4 nanoparticles

[0468] Purified BCA-P₁₁4 was diluted to a protein concentration of 0.8 mg/mL with 10 mM Tris-HCl buffer (pH 8.0). Using 5 M acetic acid solution, the pH of the enzyme solution was adjusted to 6.1±0.1 to which MgCl₂ stock solution prepared in 10 mM Tris-HCl buffer (pH 8.0) was added to a final concentration of 10 mM. Nanoparticles were formed instantaneously and their size measured by Dynamic Light scattering technique using Zetasizer Nano (Malvern Instruments).

[0469] b. BCA-(P₁₁4)₃ reversible particles

[0470] Purified BCA-(P₁₁4)₃ was diluted to 0.5 mg/mL protein concentration with 10 mM Tris-HCl buffer (pH 8.0). Using 5 M acetic acid, the pH of solution was adjusted to 5.7±0.1, the pI of BCA-(P₁₁4)₃, to induce instant formation of aggregates. BCA-(P₁₁4)₃ solution was then incubated under this condition for 15 min at 50 rpm. The solution was centrifuged at 8000 rpm for 5 min (Sigma, John Morris Scientific). The supernatant was collected in a separate tube and BCA-(P₁₁4)₃ particles were collected as a precipitated pellet. To the pellet, 10 mM Tris-HCl buffer (pH 8.0) was added to its initial volume and gently shaken for 5 min to completely solubilise the pellet for subsequent activity analysis.

[0471] Enzyme kinetic measurements by pNPA assay

[0472] The kinetic parameters of the enzyme were determined by measuring the esterase activity of BCA using para-nitrophenol acetate (pNPA) as substrate. The assay was performed in a 96 well assay plate using reaction buffer (50 mM sodium sulphate + 50 mM HEPES pH 8.0) containing 3 µg of enzyme in a final sample volume of 0.2mL. Substrate was added at the following concentrations 0.1, 0.25, 0.5, 0.75 and 1 mM and absorbance measured at 405 nm every 30 sec for 15 min using a microplate reader (Infinite 200PRO, Tecan). Similarly, a blank solution was prepared with the same components except that water was added instead of enzyme solution. Following correction of absorbance for blank solution

value, the slope in terms of change in absorbance per sec was calculated. To determine reaction rate, a standard curve of product p-nitrophenol was used to calculate concentration in terms of micromole of product released per minute and thus the reaction rate. The kinetic constants K_m and V_{max} were determined using the Lineweaver-Burke plot.

[0473] Enzyme particle recovery by ultrafiltration

[0474] Two Centriscart (Sartorius) centrifugal ultrafiltration device each with different molecular weight cut offs (MWCO) were used to demonstrate the recovery of enzyme particles. For BCA- P_{114} nanoparticle separation, 100 kDa MWCO membranes were used and for BCA- $(P_{114})_3$ particles 300kDa MWCO membranes were used. About 1 mL of enzyme solution was added into the outer tube of the device and the inner tube containing the filter membrane was placed into it. The loaded vessels were centrifuged at 1000 rpm for 4 min and then the inner tube containing permeate removed. The retentate fraction was retrieved from the outer tube. Similar separation was carried out using WT-BCA at same protein concentration and buffer solution as a control.

[0475] Enzymatically active supramolecular assembly reuse for CO₂ conversion and recovery through precipitation

[0476] A volume of 1 mL BCA- $(P_{114})_3$ at 0.8 mg/mL in 10 mM Tris-HCl buffer (pH 8.0) buffer solution was taken into a microcentrifuge tube. The enzyme activity was determined for a small subsample of this solution by removing 3.5 μ L (targeting 3 μ g) and testing CO₂ hydration using the Wilbur Anderson method modified to 0.2 mL total volume in a microtiter plate set-up. The initial enzyme activity was designated Reaction 1. Following this, the pH of BCA- $(P_{114})_3$ was adjusted to pH 5.7 ± 0.1 with 5 M acetic acid and incubated for 15 min at 50 rpm. The solution was centrifuged at 8000 rpm for 5 min. The supernatant was collected in a separate tube and the enzyme precipitate was solubilized with 10

mM Tris-HCl buffer (pH 8.0). This was repeated for two more cycles and enzyme performance was measured after every precipitation and solubilisation step. The supernatant and final resuspended pellet were analyzed for protein concentration using the Bradford method and SDS-PAGE analysis in 4-12% Bis-Tris Bolt gels.

[0477] Influence of peptide length on enzyme production and activity

[0478] The BCA-(P₁₁₄)₃ fusion system was designed to have 3 repeats of peptide in contrast to the single peptide in BCA-P₁₁₄ fusion system as illustrated in Figure 10. All other design parameters such as linker length and C-terminal fusion position were maintained.

[0479] Expression of BCA-(P₁₁₄)₃ in *E.coli* BL21 (DE3) cells was carried out using both auto-induction and IPTG induction methods. Highest soluble expression was achieved under conditions of 1mM IPTG at 20°C (Figure 22a) while mostly insoluble expression was observed at 37°C. This trend is similar to expression of WT-BCA and BCA-P₁₁₄ for which maximum soluble expression was observed at 20°C. To study the impact of peptide length on the yield of fusion protein, WT-BCA, BCA-P₁₁₄ and BCA-(P₁₁₄)₃ were expressed under the optimal condition of 1mM IPTG at 20°C using exactly the same condition (shaking speed, volume of shaking flasks). SDS-PAGE analysis revealed that the expression yield of BCA-(P₁₁₄)₃ was significantly reduced in comparison to WT-BCA and BCA-P₁₁₄ as shown in Figure 22b. The reduction in protein yield for BCA-(P₁₁₄)₃ illustrates the direct consequence of the additional peptide units in this design, possibly due to its repetitive nature. The *E.coli* expression system is known to perform poorly when expressing peptides or proteins rich in repetitive sequences. For example, a 60% reduction in peptide yield was reported when 6-repeats of P₁₁₄ peptide fused to KSI was expressed using *E.coli* system. Also low yield of BCA-(P₁₁₄)₃ may indicate the need for increased protein folding time due its longer peptide length during the translational phase. Without wishing to be bound by theory, fusion to C-

terminus may further slow-down protein folding due to the presence of a C-terminal knot structure in BCA which is inherently difficult to refold.

[0480] The soluble fraction from BCA-(P₁₁₄)₃ cell lysate was purified by nickel-ion affinity chromatography (Figure 23a). Due to the lower levels of protein expression, elution fractions showed lower purity BCA-(P₁₁₄)₃ (34kDa) as a result of competitive binding by impurity protein (~90kDa) as shown in SDS-PAGE (Figure 23b). Consequently this necessitated a second purification step using anion exchange chromatography (AEx) to improve protein purity and increase protein concentration. Chromatography profile for AEx (Figure 24a) shows the presence of two peaks. However SDS-PAGE analysis (Figure 24b) reveals fractions of similar or closely related molecular weight proteins of BCA-(P₁₁₄)₃ rather than impurity protein. A more detailed analysis of the pre-peak and peak tail fractions (Figure 24c) illustrates two distinct bands of slightly different molecular weights. Mass spectrometry analysis using trypsin digested samples showed that both bands corresponded to the protein sequence of BCA-(P₁₁₄)₃ (data not shown). However this data alone did not explain the variation in the molecular weights observed in SDS-PAGE. To further investigate this difference, both protein bands were subjected to MALDI-TOF for intact mass analysis which revealed interesting results. Figure 25a shows that the pre-peak fraction corresponds to a mass of 31.75 kDa while Figure 25b shows that the peak-tail fraction to have a mass of 34.41 kDa which corresponds to the size of BCA-(P₁₁₄)₃. The probable sequence of 31.75 kDa protein mass was determined using Protparam computational tool and identified as a partially cleaved sequence of BCA-(P₁₁₄)₃, with cleavage of the last 19 amino acids of the C-terminal sequence. Due to the close sequence similarity and similar peptide fragment length, this difference was unidentifiable through mass spectrometry of trypsin digested samples, but is revealed by exact mass measurement. Importantly, the data provides evidence that repeat sequences coupled with complex knot formation at the C-terminal of the enzyme were truncated by the *E.coli* expression system.

[0481] Without wishing to be bound by theory, the present inventors propose the reduced yield of $\text{BCA}-(\text{P}_{114})_3$ may relate to the C-terminal knot structure of BCA which is specific only to certain enzyme families.

[0482] The kinetic parameters and enzyme performance of $\text{BCA}-(\text{P}_{114})_3$ in comparison with WT-BCA and $\text{BCA}-\text{P}_{114}$ are compared in table 8. Initial rate of reaction shows a 50% decrease for $\text{BCA}-(\text{P}_{114})_3$ in comparison to the WT-BCA, whereas the performance of $\text{BCA}-\text{P}_{114}$ was similar to WT-BCA. This is quantified by the change of $V_{\max}$ of the three enzymes using the Double-reciprocal plot method. A 30-fold increase in K_m value of $\text{BCA}-(\text{P}_{114})_3$ indicates a reduced affinity of the enzyme towards the substrate. The reduction in $V_{\max}$ and increased K_m values of $\text{BCA}-(\text{P}_{114})_3$ is proposed to be an effect of the length of the 3 peptide repeats causing steric effect on accessibility of the substrate to the enzyme as well as aforementioned difficulty in folding at the C-terminal knot region. The presence of knot structures in proteins is known to provide a stabilization effect protecting it from unfolding under denaturing conditions. The tightening of the C-terminal knot in BCA has been associated with formation of the active site and thereby has a direct influence on the enzyme activity.

[0483] Table 8: Kinetic parameters of WT-BCA, $\text{BCA}-\text{P}_{114}$ and $\text{BCA}-(\text{P}_{114})_3$

Enzyme	Initial velocity U_o ($\mu\text{M}/\text{min}$)*	Specific Activity* (U/mg)	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM)	k_{cat} (s^{-1})
WT-BCA	1.898	632.74	3.476	0.113	563.11
$\text{BCA}-\text{P}_{114}$	1.320	440.17	3.028	0.193	532.75
$\text{BCA}-(\text{P}_{114})_3$	0.990	330.12	0.547	3.883	104.59

*- at 1 mM pNPA concentration

[0484] This data demonstrates the assembly of self-assembling enzymatic components comprising a tandemly repeated self-assembly polypeptide bound to an enzyme into a supramolecular assembly.

[0485] This data also demonstrates the assembly of self-assembling enzymatic components comprising a tandem repeat of a self-assembly polypeptide bound to an enzyme form an enzymatically active supramolecular assembly.

[0486] Reversible precipitation of BCA-(P₁₁₄)₃ system

[0487] Following the study of expression and activity of the BCA-(P₁₁₄)₃ the effect of peptide length on the self-assembly behaviour was investigated. A gradual decrease of pH from 8.0 to 5.0 using 5 M acetic acid showed that BCA-(P₁₁₄)₃ did not result in the self-assembly into nanoparticles as it does for the single peptide fusion. While no change in particle size was observed at pH 6.8±0.1 as was the case for BCA-P₁₁₄, at pH 6.0, BCA-(P₁₁₄)₃ instantly formed visible precipitates that were easily separated by centrifugation. This effect of pH was not noted for WT-BCA and BCA-P₁₁₄ (Figure 26a) indicating that the increased number of peptides steered the complex to precipitation rather than soluble nanoparticle formation. When sample containing mixture of BCA-(P₁₁₄)₃ and the cleaved protein forms was precipitated only BCA-(P₁₁₄)₃ was obtained in the precipitate. SDS-PAGE analysis (Figure 26b) demonstrates the pellet contains purely the intact form of BCA-(P₁₁₄)₃ ~34.41 kDa, whereas the supernatant comprised of mainly the cleaved form ~31.75 kDa. This confirms that only the full length form of BCA-(P₁₁₄)₃ with 3 peptide repeat has the ability to form precipitates. An explanation for the pH induced precipitation of BCA-(P₁₁₄)₃ as opposed to that of the single peptide is attributed to the increased number of glutamine residues in the 3 peptide repeat. The BCA-(P₁₁₄)₃ has in total 12 glutamines whereas the BCA-P₁₁₄ has only 4. The recurrence of “-QQQQ-” between peptide repeats in the BCA-(P₁₁₄)₃ system mimics the poly-glutamine rich sequence known to form amyloid-like protein aggregates in neurodegenerative diseases such Huntington.

[0488] This data demonstrates the assembly of self-assembling enzymatic components comprising a tandemly repeated self-assembly polypeptide bound to an enzyme into a supramolecular assembly can be unassembled into the self-assembling enzymatic components using pH. This allows recovery of the self-assembling enzymatic components for re-use.

[0489] Surprisingly, unlike the amyloid peptides, the BCA-(P₁₁₄)₃ formed precipitates that were completely soluble when resuspended in 10mM Tris-HCl pH 8.0 buffer. Dynamic light scattering results (Figure 27) shows that the resolubilised pellet had a particle size close to the initial monomeric form of BCA-(P₁₁₄)₃. Further, the reversible precipitation is also induced by switching pH from 8.0 to 6.0 and vice-versa using 5 M acetic acid and 1 M NaOH. To verify that precipitates formed as a result of this drastic pH switching is an attribute of the peptide length rather than the enzyme charge itself, all three enzymes WT-BCA, BCA-P₁₁₄ and BCA-(P₁₁₄)₃ were subjected to pH switching. Following the pH switch, any effect on activity was determined. The activity measured by Wilbur-Anderson method (table 9) shows that minimal impact (~10% reduction) was observed with BCA-(P₁₁₄)₃ under pH switch compared to WT-BCA (~25%) and BCA-P₁₁₄ (~80%) which were more strongly affected. This demonstrates that though BCA-(P₁₁₄)₃ had a reduced V_{max}, its molecular design provides robustness against drastic conditions such pH switch.

[0490] Table 9: Impact of pH switching on the enzyme activity of WT-BCA, BCA-P₁₁₄ and BCA-(P₁₁₄)₃ by Wilbur-Anderson method.

Enzyme	Enzyme Activity		% Reduction in Activity*
	Wilbur Anderson units (U)		
	Initial	After pH switch	
WT-BCA	68.3	50.8	25.6

BCA-P ₁₁₄	51.0	9.4	81.5
BCA-(P ₁₁₄) ₃	40.6	36.8	9.3

*-[(Initial activity-Activity after switch)/Initial activity) x 1

EXAMPLE 13: Recovery of enzyme particles by ultrafiltration

[0491] One objective of engineering enzymes with self-assembly feature is to allow recovery and reuse of enzymes.

[0492] BCA-P₁₁₄ nanoparticle recovery

[0493] Separation of BCA-P₁₁₄ nanoparticles from solution using 100 kDa membrane was performed alongside WT-BCA as a control. The membrane was able to retain most of the BCA-P₁₁₄ nanoparticles when compared to WT-BCA as shown by SDS-PAGE analysis (Figure 28) and the efficiency of separation determined from permeate and retentate protein concentrations illustrates that up to 60% of BCA-P₁₁₄ nanoparticles were retained as opposed to only 4% of WT-BCA (Table 10). Retention of WT-BCA is also a consequence of minimum dead volume associated with the ultrafiltration device.

[0494] Table 10: Separation efficiency of BCA-P₁₁₄ nanoparticle using 100kDa MWCO Centrisart device

Enzyme	Protein concentration (mg/mL)#			%Retention or Recovery rate*
	Load	Permeate	Retentate	
WT-BCA	0.525	0.267	0.277	3.6%
BCA-P ₁₁₄	0.511	0.158	0.386	59.1%

*-[1-(Permeate concentration/Retentate concentration)] x100%; # -measured by the Bradford method

[0495] The retention of nanoparticles following membrane separation is illustrated by the particle sizes of BCA-P₁₁₄ measured by Dynamic light scattering (Figure 29a). The BCA-P₁₁₄ nanoparticles formed at pH 6.1 and 10 mM MgCl₂ showed mean particle size of ~1500 nm which is in close agreement with the predicted value of 1333 nm using the formula described above. The retentate fraction shows presence of two populations of particles sizes ~100 nm and ~850nm. This size distribution is further confirmed by TEM image which also shows presence of two distinct particle sizes (Figure 29b). On the contrary, the permeate fraction shows particles size ~5 nm which corresponds to the monomeric form of BCA-P₁₁₄. These results suggest that there may be a few loosely bound BCA-P₁₁₄ monomers that have not been fully incorporated into the self-assembled particle causing them to dissociate when subjected to centrifugation and filtration. Nevertheless, the ultrafiltration method with 100 kDa membrane can be used to recover and reuse BCA-P₁₁₄ nanoparticles.

[0496] This data demonstrates that supramolecular assemblies of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme into can be recovered from solution.

[0497] BCA-(P₁₁₄)₃ particle recovery

[0498] The BCA-(P₁₁₄)₃ fusion system formed visible particles on reduction of pH and hence the largest MWCO (300kDa) ultrafiltration membrane was used to recover the protein while allowing a higher flux and thus shorten operation time. Results from SDS-PAGE (Figure 29) show that the 300 kDa membrane was able to retain most of the BCA-(P₁₁₄)₃ particles in the retentate fraction. In contrast, most of the WT-BCA passed through the large pores of the membrane to be accumulated in the permeate fraction. The separation efficiency based on %retention was calculated to be 86% and 24% for BCA-(P₁₁₄)₃ and WT-BCA, respectively by determining enzyme concentration (Table 11).

[0499] This data demonstrates that supramolecular assemblies of self-assembling enzymatic components comprising a tandemly repeated self-assembly polypeptide bound to an enzyme into can be recovered from solution.

[0500] Table 11: Separation efficiency of BCA-(P₁₁₄)₃ particle using 300 kDa MWCO Centriscart device

Enzyme	Protein concentration (mg/mL) #			%Retention or Recovery rate*
	Load	Permeate	Retentate	
WT-BCA	0.646	0.217	0.285	23.9
BCA-(P ₁₁₄) ₃	0.571	0.108	0.783	86.2

*-[1-(Permeate concentration/Retentate concentration)] x100%; # -measured by the Bradford method

[0501] Reusability of BCA-(P₁₁₄)₃ particles by pH induced precipitation

[0502] BCA-(P₁₁₄)₃ was shown to be robust to the pH switch and particles formed were large enough to be precipitated out of solution. Therefore the reusability of BCA-(P₁₁₄)₃ system was investigated in a coupled process that included biocatalysis in an industrial scenario. For this purpose, pH induced precipitation of the enzyme-peptide for recovery was performed for three consecutive cycles with activity tested after each cycle.

[0503] Supernatant samples collected after every precipitation step showed minimal loss of BCA-(P₁₁₄)₃, with most of the enzyme recovered in the final pellet after three process cycles (Figure 31). Evaluation of enzyme performance for CO₂ capture showed that up to 70% of the initial activity was observed after 3 biocatalytic and pH induced precipitation cycles (Table 12). The small reduction in activity may be attributed to the corresponding loss of enzyme in the supernatant.

Nevertheless, the specific precipitation behaviour of BCA-(P₁₁₄)₃ combined with simple recovery method such as centrifugation offers a feasible route for the recovery and reuse of enzymes without need for a solid-support.

[0504] This data demonstrates that supramolecular assemblies of self-assembling enzymatic components comprising a tandemly repeated self-assembly polypeptide bound to an enzyme into can be recovered from solution and re-used.

[0505] Table 12: Separation efficiency and biocatalytic performance of BCA-(P₁₁₄)₃ particle following three biocatalytic cycles.

Reaction	Enzyme activity (WA-U)*	% Relative activity	% Protein lost in supernatant #
1	12	100%	-
2	9.4	78%	19%
3	8.0	67%	20%

* WA – Wilbur Anderson method; # - Measured by the Bradford method

EXAMPLE 14: Production of enzymatically active supramolecular assemblies using different enzymes.

[0506] Four enzyme candidates were examined in fusion with a single self-assembly polypeptide with a GS-linker. The enzyme candidates are described in Table 13.

[0507] Table 13: Four representative enzyme candidates and their properties

Parameter	TmCA	Tyrosinase	Cutinase	ω -Amino transferase
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Enzyme classification number	4.2.1.1	1.14.18.1	3.1.1.74	2.6.1.18
Enzyme class	Lyases	Oxidoreductase	Serine Esterase	Transferase
Protein data bank ID	4XZ5	4HD6	1AGY	4CE5
Source organism	Bacteria/ <i>Thiomicrospira crunogena</i>	Bacteria/ <i>Bacillus megaterium</i>	Fungal / <i>Fusarium solani</i>	Fungal / <i>Aspergillus terreus</i>
<u>Enzyme properties</u>				
Amino acids	317	297	230	325
Molecular weight (kDa)	35.81	34.41	23.98	36.17
pI	8.76	8.96	8.36	5.18
Metal ion	Zn	Cu	None	None; but needs co-factor Pyridoxal-5'-phosphate (PLP)
Number of Cysteines & disulphide bonds	2 Cys 1 disulphide bond	None	4 Cys 2 disulphide bonds	3 Cys 0 disulphide bonds
Expression host	<i>E.coli</i> BL21 (DE3)	<i>E.coli</i> BL21 (DE3)	<i>E.coli</i> Origami B (DE3)	<i>E.coli</i> BL21 Gold (DE3)
Enzyme assay	Spectrophotometry method using para-nitrophenyl acetate as substrate	Spectrometry method using L-tyrosine and L-DOPA as substrate	Spectrophotometry method using para-nitrophenyl butyrate as substrate	Spectrometry method using α -methylbenzyl amine as substrate
Industrial application	CO2 capture and conversion	Production of catechols Production of L-DOPA	Industrial esterification, transesterification reactions	Production of chiral amines

[0508] Plasmid and Bacterial expression

[0509] Two plasmids were constructed for each of the four selected enzyme candidates, one containing the gene for the wild type (WT)-enzyme and the other with gene for the corresponding enzyme-peptide fusion, giving 8 constructs in total. The fusion construct for each enzyme was designed with one P₁₁₄ peptide attached to the C-terminus via GGGGSGGGGS linker sequence similar to the model system BCA-P₁₁₄ presented described herein. Each of the gene sequences were codon optimised, synthesized and ligated into pET28a expression plasmid between *NdeI* and *XhoI* restriction sites by GenScript (Piscataway, NJ, USA) thereby incorporating an N-terminal 6X-Histag with thrombin cleavage site sequence pre-existing in the pET28a plasmid. The plasmids were transformed into BL21 (DE3) *E.coli* competent cells using NEB High Efficiency Transformation protocol only with the exception of plasmids for WT-Cutinase and Cutinase-P₁₁₄ that were transformed into SHuffle T7 express cells to facilitate correctly formation of disulphide bond for cutinase (4 cysteines and 2 disulphide bonds).

[0510] For TmCA, tyrosinase and cutinase-expressing cells, growth in terrific broth (TB) medium and kanamycin (50 µg/mL) was initiated with 2% v/v inoculum for 4 h at 37°C for the first growth phase. Protein expression was examined by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM in the medium at 37°C for 3 h and at 20 °C for 16 h. For Auto-Induction method, 2% v/v lactose was added in the initial TB medium as an inducer and incubated at 37°C and 20°C for 16 h. In the case of ATA, the above initial growth conditions were followed and protein expression was examined by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside to the medium at 25°C and 15°C for 16 h while for auto-Induction method, 2% v/v lactose was added in the initial TB medium as an inducer and incubated at 25°C and 15°C for 16 h. The amino acid sequences for all four enzyme-peptide fusion constructs with N-terminal Histag and C-terminal

P₁₁₄ peptide are shown below. (Scheme showing sequence regions corresponding to: Histag (*italics*), thrombin cleavage site (underline), enzyme (plain text), linker (**bold underline**) and peptide (**bold italics**).

[0511] Amino acid sequence of TmCA- P₁₁₄ (37.98 kDa)

*MGSSHHHHHHSSGLVPRGSH*MANNVAAPLIDLGA~~EAKKQAQKSAATQSAVPEK~~
 ESATKVAEKQKEPEEKAKPEPKPPHWGYFGEEGPQYWGE~~LAPEFSTCKTGK~~
 NQSPINLKPQTAVGTTSLPGFDVYYRETALKLINNGHTLQVNIPLGSYIKINGHRY
 ELLQYHFHTPSEHQRDGFNYPMEMHLVHKDGDGNLAVIAILFQEGEENETLAKL
 MSFLPQTLKKQEIHESVKIHPAKFFPADKKFYKYSGLTTPPCSEGVYWMVFKQ
 PIQASVTQLEKMHEYLGSNARPVQRQNARTLLKSWPDRNRANTVYEFY**GGGGS**
GGGGSQQRFEWEFEQQ (SEQ ID NO: 9)

[0512] Amino acid sequence of Tyrosinase- P₁₁₄ (38.57 kDa)

*MGSSHHHHHHSSGLVPRGSH*MSNKYRVRKNVLHLTDTEKRDFVRTVLILKEKGI
 YDRYIAWHGAAGKFHTPPGSDRNAAHMSSAFLPWHREYLLRFERDLQSINPEV
 TLPYWEWETDAQMQDPSQSQIWSADFMGGNGNPIKDFIVDTGPFAAGRWTID
 EQGNPSGGLKRNFGATKEAPTLPTRDDVLNALKITQYDTPPWDMTSQNSFRNQ
 LEGFINGPQLHNRVHRWVGGQMGVGPTAPNDPVFFLHHANVDRIWAVWQIIHR
 NQNYQPMKNGPFGQNFRDPMYPWNTTPEDVMNHRKLGYYDIELRKSKRSS**G**
GGGSGGGGSQQRFEWEFEQQ (SEQ ID NO: 10)

[0513] Amino acid sequence of Cutinase- P₁₁₄ (26.59 kDa)

*MGSSHHHHHHSSGLVPRGSH*MLPTSNPAQELEARQLGR~~TTRDDLINGNSASCA~~
 DVIFIYARGSTETGNLGTLGPSIASNLESAFGKDG~~VWIIQGVGGAYRATLGDNALP~~
 RGTSSAAIREMLGLFQQANTKCPDATLIAGGYSQGAALAAASIEDLDSAIRDKIAG
 TVLFGYTKNLQNRGRIPNYPADRTKVFCNTGDLVCTGSLIVAAPHLAYGPDARG
 PAPEFLIEKVRVARGS**GGGSGGGGSQQRFEWEFEQQ** (SEQ ID NO: 11)

[0514] Amino acid sequence of ATA- P₁₁₄ (40.50 kDa)

*MGSSHHHHHHSSGLVPRGSHMASMDKVFAGYAARQAILESTETTNPFAKGIW
VEGELVPLAEARIPLLDQGFMSDLTYDVPSVWDGRFFRLDDHITRLEASCTKLR
LRLPLPRDQVKQILVEMVAKSGIRDAFVELIVTRGLKGVRGTRPEDIVNNLYMFV
QPYVWVMEPDMQRVGGSAAVVARTVRRVPPGAIDPTVKNLQWGDIVRGMFEAA
DRGATYPFLTGDGDAHLTEGSGFNIVLVKDGVLVTPDRGVLQGVTRKSVINAAEA
FGIEVRVEFVPVELAYRCDEIFMCTTAGGIMPITTLTGMPVNGGQIGPITKKIWDG
YWAMHYDAAYSFEIDYNERN**GGGGSGGGGSQQRFEWEFEQQ*** (SEQ ID NO:
12)

[0515] Cell Harvest and Protein purification

[0516] Cell harvest and purification for all enzymes was undertaken using the general procedure as follows with minor modifications with respect to buffers. Briefly, cells were harvested by centrifugation at 10,000 rpm (Sigma 6-16K, John Morris Scientific) for 10 min. Cell pellet of 2.5 to 5 g was re-suspended in 50-60 mL of lysis buffer and incubated for 20 min at room temperature under low speed shaking. Cells were placed in an ice bath and lysed using sonicator (QSonica, Q125) equipped with 2 mm probe at 90% amplitude with 10 sec pulse ON and 20 sec pulse OFF for total time of 5 min. (Note: For cutinase, cell pellet was re-suspended in BugBuster Protein Extraction Reagent lysis buffer (Merck Millipore) and incubated for 20 min at room temperature on low shaking and sonication was avoided). Cell supernatant solution was collected by centrifugation at 11,000 rpm (Sigma 6-16K, John Morris Scientific) 10 °C for 20 min. Supernatant was filtered through 1.2 and 0.45 µm filters prior to purification. Purification was performed on a 5 mL HisTrapFF (GE Healthcare) immobilized metal-ion affinity chromatography charged with nickel ion using AKTA start Chromatography system. The column was equilibrated and washed using binding buffer. In some cases a second washing step was used to remove contaminant proteins. Fusion protein was

eluted step-wise with appropriate ratio of buffers A and B to target specific imidazole concentration for at least 3 column volumes. Fractions were pooled based on purity determined by SDS-PAGE 4-12% Bis-Tris Bolt gels and bulk eluate sample was desalted using size exclusion chromatography on a G25 Sephadex (GE Healthcare) column (1.5 × 17 cm) with specific desalting buffer. Purified samples were stored at 4°C in desalting buffer for subsequent analysis. The specific buffers used for each enzyme are tabulated below (Table 14). Identical purification protocols were performed for WT-enzyme and their corresponding fusion system.

[0517] Table 14:

Enzyme	TmCA	Tyrosinase	Cutinase	ATA
Lysis buffer	50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% Triton-X 100	50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 0.5% Triton-X 100	BugBuster Protein Extraction Reagent lysis buffer (Merck Millipore)	50mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% Triton-X 100 and 0.1mM co-factor Pyridoxal L phosphate-PLP
IMAC buffer A	50 mM Tris-HCl buffer pH 8.0+0.5 M NaCl	50 mM Tris-HCl buffer pH 7.5+0.5 M NaCl	20 mM Tris-HCl buffer pH 8.0+0.5 M NaCl	50 mM Tris-HCl buffer pH 7.5 containing 0.3 M NaCl
IMAC buffer B	50 mM Tris-HCl buffer pH 8.0+0.5 M NaCl +0.2M imidazole	50 mM Tris-HCl buffer pH 7.5+0.5 M NaCl +0.2M imidazole	20 mM Tris-HCl buffer pH 8.0+0.5 M NaCl +1M imidazole	50 mM Tris-HCl buffer+0.3M NaCl pH 8.0+0.5M imidazole
Elution condition	Step elution with 100,150, 200mM imidazole	Step elution with 50,100, 200mM imidazole	Step elution with 50,500, 1000mM imidazole	Step elution with 100, 200, 300, 500mM imidazole
Desalting buffer	50 mM Tris-HCl buffer pH 8.0+0.2 M NaCl	10 mM Tris-HCl buffer pH 7.5	20 mM Tris-HCl buffer pH 8.0	20 mM Tris-HCl buffer pH 7.5+0.01mM co-factor Pyridoxal L phosphate-PLP

[0518] Preparation of enzyme particles

[0519] Physical parameters such as alteration of pH, addition of metal ions, adjusting ionic strength and change of temperature were explored to initiate the formation of enzyme nanoparticles for the four enzymes fused with self-assembly peptide. The optimal method for each enzyme is summarized below.

[0520] TmCA-P₁₁4 nanoparticles

[0521] Purified TmCA-P₁₁4 at 0.4 mg/mL protein concentration in 50 mM Tris-HCl buffer pH 8.0±0.2 M NaCl was incubated for 1 min at different temperatures (25, 30, 40 and 50 °C) within the chamber of the Zetasizer Nano equipment (Malvern Instruments). Nanoparticle formation was monitored by Dynamic Light scattering technique in the Zetasizer Nano and particle sizes determined as triplicate measurements each consisting of 10 cycles of measurement. The same conditions were applied for WT-TmCA as control sample.

[0522] Tyr- P₁₁4 nanoparticles

[0523] Purified Tyr-P₁₁4 at 0.5 mg/mL protein concentration in 10 mM Tris-HCl buffer pH 7.5 was incubated with MgCl₂ (final concentration 10mM) for 2 min at 25°C and nanoparticle formation was monitored by Dynamic Light scattering using a Zetasizer Nano (Malvern Instruments). Particle sizes were determined as triplicate measurements each consisting of 10 cycles and compared with particle size formation without addition of MgCl₂. The same conditions were applied for WT-Tyr as a control sample.

[0524] Cut- P₁₁4 nanoparticles

[0525] Purified Cut-P₁₁₄ at 0.5 mg/mL protein concentration in 20 mM Tris-HCl buffer pH 8.0 was incubated with addition of MgCl₂ (final concentration 10mM) for 2 min at 25°C and nanoparticle formation was monitored by Dynamic Light scattering technique using Zetasizer Nano (Malvern Instruments). Particle sizes were determined as triplicate measurements each consisting of 10 cycles and compared with particle size determined without addition of MgCl₂.

[0526] Enzyme Activity assays

[0527] Esterase assay for TmCA

[0528] The esterase activity using para-nitrophenyl acetate (pNPA) as substrate was used to determine the kinetic parameters of both WT-TmCA and TmCA-P₁₁₄. The assay was performed in a 96 well assay plate using reaction buffer (1 M sodium sulphate + 50 mM HEPES pH 8.0) in final assay volume of 0.2 mL containing 3 µg of enzyme and pNPA at the following concentrations 0.1, 0.25, 0.5, 0.75 and 1 mM. Absorbance was measured at 405 nm every 30 sec for 15 min using a microplate reader (Infinite 200PRO, Tecan). A blank sample was prepared using assay buffer and substrate with water replacing the enzyme solution. Following blank correction of absorbance, the slope in terms of absorbance per sec was calculated. To determine reaction rate, a standard curve for product p-nitrophenol was used to convert slope of the change in absorbance to micromole of product released per minute and thus the reaction rate. The kinetic constants K_m and V_{max} were estimated using a Lineweaver-Burke plot. To study the effect of temperature on esterase activity, enzyme solutions were incubated in a heating block at different temperatures (25, 30, 40, 50 and 60°C) for 5 min and measured for esterase activity as described above with 1 mM pNPA concentration.

[0529] Monophenolase and Diphenolase activity for Tyrosinase

[0530] Enzyme assays for WT-Tyr and Tyr-P₁₁4 were performed in a 96 well plate using reaction buffer (50mM Tris-HCl + 0.01 mM CuSO₄ pH 7.5) in final assay volume of 0.2 mL containing 3 µg of enzyme. For monophenolase activity L-tyrosine was added as substrate to a final concentration of 1 mM and for diphenolase activity 1 mM L-DOPA was added as substrate. Absorbance was measured at 475 nm every 15 sec for 8 min using a microplate reader (Infinite 200PRO, Tecan). A blank solution was prepared using water instead of the enzyme solution. Following blank correction of absorbance, the slope in terms of absorbance per sec was determined.

[0531] pNPB assay for Cutinase

[0532] The esterase activity of Cut-P₁₁4 was determined using para-nitrophenyl butyrate (pNPB) as substrate at concentrations 0.1 and 0.5 mM. The assay was performed in a 96 well assay plate using reaction buffer (50 mM Tris-HCl pH 8.0 +10 mM NaCl) in final assay volume of 0.2 mL containing 0.35 µg enzyme. Absorbance was measured at 405 nm every 15 sec for 2 min using a microplate reader (Infinite 200PRO, Tecan). A blank solution was prepared using water instead of enzyme solution. Following blank correction of absorbance, the slope in terms of absorbance per second was converted to reaction rate determined by calculating the concentration of p-nitrophenol from a standard curve.

[0533] Differential Scanning Fluorimetry

[0534] The folding properties of WT-TmCA and TmCA-P₁₁-4 were determined using the differential scanning fluorimetry method (Niesen et al., 2007) following the protocol developed at the Collaborative Crystallisation Centre (C3, CSIRO Manufacturing, Parkville) (Seabrook et al., 2015). Briefly, protein samples were mixed with fluorescent dye SYPRO orange and buffer and incubated in 96 well plates. Samples were heated from 20 to 100°C at 2°C per min using a real-time PCR instrument (Bio-Rad iCycler5). The melting temperature was calculated from

the thermal transition curves and its derivative curves using the BioRad CFX Manager Software.

[0535] Negative Staining and Transmission Electron Microscopy

[0536] The negative staining procedure was performed as described above. Samples were viewed using transmission electron microscope at 200 kV (FEI, Tecnai G2 T20 TWIN LaB6) and electron micrographs were recorded using CCD camera and Gatan "Digital Micrograph" software. Particle sizes of the protein were determined using ImageJ software to construct the histogram.

[0537] Expression and Purification of WT-TmCA and TmCA-P₁₁₄

[0538] Soluble expression of WT-TmCA and TmCA-P₁₁₄ was achieved with 1 mM IPTG induction at 20 °C. The SDS-PAGE analysis (Figure 6.1) shows that the level of soluble protein expression was comparable for both wild-type and the enzyme-fusion system. There was a significant amount of protein of interest in the insoluble fractions but as these occurred in both wild-type and fusion systems to a similar extent it may be attributed to the characteristics of the TmCA enzyme rather than an effect of the peptide. The expression performance for TmCA-P₁₁₄ was unchanged when the scale was doubled from 0.5 to 1 L flasks (Figure 32b). These results suggest that the fusion of P₁₁₄ peptide with TmCA does not greatly impact its expression and that soluble expression can be achieved at a yield comparable to WT-TmCA

[0539] Protein purification using nickel-IMAC yielded pure fractions of wild-type and fusion proteins. An additional gradient wash step removed significant amount of contaminating proteins and resulted in pure WT-TmCA and TmCA-P₁₁₄ in the later eluting fractions (Figure 33). The similar elution profiles and purity attained for the two proteins following the same protocol suggests that fusion of the

peptide to TmCA has an insignificant impact on its binding to the IMAC column and purification performance.

[0540] Nanoparticle formation of TmCA-P₁₁₄

[0541] The native TmCA enzyme is originally obtained from the deep-sea chemolithoautotroph microorganism *Thiomicrospira crunogena* (Dobrinski et al., 2010), and the enzyme's structure is more stable in buffers containing high salt rather than low salt. For this reason, the TmCA desalting buffer contained 200 mM NaCl which prevents the enzyme from precipitating out of the solution. Conditions were explored to promote self-assembly. As demonstrated above, BCA-P₁₁₄ nanoparticles at pH 6.8 showed increase in particle size at higher temperatures. Based on this observation, temperature was used as a controlling factor to induce particle self-assembly in TmCA-P₁₁₄.

[0542] A short incubation at temperatures between 25-40°C induced no change in particle size of TmCA-P₁₁₄ but at 50 °C, dynamic light scattering analysis showed TmCA-P₁₁₄ nanoparticles of size ranging from 200-500 nm (Figure 34b). On the contrary, WT-TmCA showed no self-assembly at 50 °C (Figure 34a). This confirms that the self-assembly was driven by the fused P₁₁₄ peptide. The formation of TmCA-P₁₁₄ nanoparticles was confirmed by transmission electron microscopy which showed particles of average 200 nm in size which correlated with value shown by dynamic light scattering (Figure 34b).

[0543] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce an supramolecular assembly.

[0544] Temperature and pH stability of TmCA-P₁₁₄

[0545] Since the self-assembly of TmCA-P₁₁4 is triggered by a higher temperature of 50 °C and this condition may unintentionally change enzyme properties, it was essential to confirm that the self-assembly did not alter the enzyme's structural stability. For this purpose, the unfolding characteristics of TmCA-P₁₁4 were investigated by differential scanning fluorimetry. The melting curves shown in Figure 36a and c illustrate that both WT-TmCA and TmCA-P₁₁4 unfold at ~60 °C. This is similar to the unfolding temperature of another dimeric CA isolated from the same microorganism *Thiomicrospira crunogena* (Díaz-Torres et al., 2015). These results demonstrate that the enzyme's folded state is preserved at 50 °C and further confirms that the self-assembly induced at this temperature is mediated through the peptide rather than an unfavourable aggregation phenomenon as a consequence of enzyme unfolding. Furthermore, the unfolding characteristics were examined in different buffers with a pH range from 5.0 to 9.0 at two different NaCl concentrations. Figure 36b and d, shows that both WT-TmCA and TmCA-P₁₁4 were stable in buffers of pH range 7.0-9.0 and the lower melting temperature was observed in acidic buffers. The only exception was the observation with sodium MES pH 6.0 buffer which increased the melting temperature of TmCA-P₁₁4 to 65°C which was absent in the case of wild-type enzyme. Overall, with all buffer types, higher melting temperature was observed with the higher NaCl concentration of 200 mM rather than 50 mM, in line with the salt preferring nature of the TmCA enzyme.

[0546] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce an supramolecular assembly wherein the structure of the enzyme is preserved in the supramolecular assembly.

[0547] Enzyme Activity of TmCA-P₁₁4

[0548] The kinetic parameters of wild-type and TmCA fusion system were determined by the esterase assay method. The various parameters summarised

in Table 15 shows that the fusion system had a similar initial reaction rate compared to the wild-type enzyme. The fusion system showed a slight reduction in $V_{\max}$ with increased K_m compared with WT-TmCA. This difference could be a consequence of the self-assembled nature of TmCA-P₁₁4 which may restrict access of substrate to the active site of the enzyme. When reaction rates were measured at various temperatures, TmCA-P₁₁4 followed a similar trend to WT-TmCA with maximum temperature stability observed at 50°C which reduces at 60°C due to protein unfolding (Table 16). These temperature stability results are in line with the thermostability characteristics of the *Thiomicrospira crunogena* which originates from hydrothermal vents.

[0549] Table 15: Kinetic parameters of WT-TmCA and TmCA-P₁₁4.

Enzyme	Initial velocity U_o ($\mu\text{M}/\text{min}$)*	Specific Activity* (U/mg)	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM)	k_{cat} (s^{-1})
WT-TmCA	0.413	165.06	0.400	9.7	95.9
TmCA-P ₁₁ 4	0.413	137.67	0.325	15.4	68.6

*- at 1 mM pNPA concentration

[0550] Table 16: Reaction rates of WT-TmCA and TmCA-P₁₁4 at different temperatures:

Temperature (°C)	Initial velocity U_o ($\mu\text{M}/\text{min}$)*	
	WT-TmCA	TmCA-P ₁₁ 4
25	0.413	0.413
30	0.249	0.330
40	0.302	0.413
50	0.407	0.413
60	0.224	0.330

*- at 1mM pNPA concentration

[0551] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce an enzymatically active supramolecular assembly.

[0552] Expression and Purification of WT-Tyr and Tyr-P₁₁4

[0553] Preliminary expressions tests for tyrosinase using different induction methods and at two different temperatures showed that the maximum soluble protein expression for both WT-Tyr and Tyr-P₁₁4 was achieved at 20 °C using 1 mM IPTG for induction (Figure 37). The amino acid sequence of both enzymes were confirmed by mass spectrometry following trypsin digestion (data not shown).

[0554] This is the first report of soluble expression of this enzyme at lower temperatures which has been previously conducted at 37 °C. The purification profiles for both WT-Tyr and Tyr-P₁₁4 by nickel-IMAC chromatography were comparable (Figure 38a and b) and yielded fractions of protein with similar and high levels of purity (Figure 38c). From these results the expression and purification characteristics of Tyr-P₁₁4 appear to be similar to WT-Tyr and fusion with peptide did not significantly impact the production and purification of this enzyme.

[0555] Nanoparticle formation of Tyr-P₁₁4

[0556] Two approaches to Tyr-P₁₁4 nanoparticle formation were undertaken: (i) via addition of magnesium alone and (ii) combination of addition of magnesium and change of pH. When 10 mM MgCl₂ was added to purified Tyr-P₁₁4 solution in 10 mM Tris-HCl pH 7.5 buffer, formation of particles of size 100-500 nm was detected by dynamic light scattering (Figure 6.8b) but not observed with WT-Tyr

(Figure 39a). This confirms that self-assembly of Tyr-P₁₁4 induced by Mg ions is driven by the interacting peptides as described above for BCA-P₁₁4. Based on these observations, we envisage that the molecular mechanism for self-assembly of Tyr-P₁₁4 induced by Mg ions is similar to that of BCA-P₁₁4 as discussed above. The presence of particles was also confirmed using electron microscopy which showed both small nanoparticles (50-100 nm) and larger particles (200-500 nm) (Figure 40). The smaller particles observed could be a result of the particle disassembly during sample preparation and drying of microscopy grids as the dynamic light scattering shows a single peak. In contrast to assembly of BCA-P₁₁4 at lower pH, Tyr-P₁₁4 showed no self-assembly behaviour when subjected to a reduced pH even as low as 4.0 (data not shown). This contrasting response to pH of the two fusion proteins may be due to the differences of the two enzymes in their surface charges and the surface charge in tyrosinase may not favour protein-peptide interaction as observed with BCA-P₁₁4 described above.

[0557] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce a supramolecular assembly.

[0558] Enzyme Activity of Tyr-P₁₁4

[0559] The enzyme activities of Tyr-P₁₁4 in terms of monophenolase and diphenolase activity were determined and compared to WT-Tyr. Reaction rates measured at 475 nm showed that the wild-type had a higher diphenolase activity than monophenolase activity (Table 17). This is a consequence of the mutation at V218G in the WT-Tyr sequence done to improve enzyme activity and this result was similar to the previously reported work. On the other hand Tyr-P₁₁4 showed an increase in both mono-and diphenolase activity by 87% and 48% respectively when compared to WT-Tyr. This improvement is particularly useful because conversion of L-tyrosine to DOPA rather than DOPA to dopachrome is preferred for commercial applications.

[0560] Table 17: Reaction rates of WT-Tyrosinase and Tyrosinase-P₁₁₄

Enzyme	Reaction rate (Absorbance 475nm/min)	
	Monophenolase Activity	Diphenolase Activity
WT-Tyrosinase	0.012	0.051
Tyrosinase-P ₁₁₄	0.096	0.099

[0561] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce an enzymatically active supramolecular assembly.

[0562] This data also demonstrates the enzymatically active supramolecular assembly is able to produce DOPA.

[0563] Expression and Purification of WT-Cut and Cut-P₁₁₄

[0564] Expression under various conditions showed that 1 mM IPTG yielded soluble expression of both WT-Cut and Cut-P₁₁₄ whereas no soluble expression was observed by the auto-induction method (Figure 41). Mass spectrometry analysis of the trypsin-digested protein bands confirmed the amino acid sequence of WT-Cut and Cut-P₁₁₄ (data not shown). Overall the soluble expression of the cutinases were very low with a high proportion of the enzyme expressed in the insoluble form. Similar low expression of cutinase in *E.coli* cytoplasm have been previously reported. To attempt to improve soluble expression, induction at lower IPTG conditions were investigated. SDS-PAGE analysis showed soluble expression improved at low IPTG concentration (Figure 42) as had been previously reported. Though the soluble expressions between 0.1 to 0.5 mM IPTG

were similar, 0.2 mM showed least insoluble expression and lower IPTG concentrations also resulted in higher growth rates with an increase in total cell weight (data not shown). Based on these results, 0.2 mM IPTG was selected as the optimum concentration for soluble expression of Cut-P₁₁₄. Purified fractions of Cut-P₁₁₄ were obtained when eluted with 0.5 M imidazole by Ni-IMAC (Figure 43) which was subsequently used to analyse nanoparticle formation and enzyme activity.

[0565] Nanoparticle formation and enzyme activity of Cut-P₁₁₄

[0566] The influence of MgCl₂ on nanoparticle formation for Cut-P₁₁₄ was examined by dynamic light scattering. Cut-P₁₁₄ alone formed large particles of size ~ 800 nm without added MgCl₂ (Figure 44a) and the addition of MgCl₂ showed no significant change in particle size. Cutinase being closely related to the lipase family has several hydrophobic loops in its structure that are accessible to the solvent (Carvalho et al., 1998) and capable of movement upon interfacial binding (Martinez et al., 1992). It is possible that the cutinase is prone to self-aggregation. Under transmission electron microscopy, large aggregate like particles were observed whose size was ~800-900nm (Figure 44b) which correlated with the dynamic light scattering results.

[0567] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce a supramolecular assembly.

[0568] Despite being in an aggregated state, Cut-P₁₁₄ displayed significant enzyme activity. Substrate p-nitrophenyl butyrate at low concentration (0.1 mM) resulted in a slower reaction rate when compared to 0.5 mM which reached enzyme saturation within 45 sec of reaction initiation (Figure 45). This may be attributed to the low solubility of pNPB in aqueous buffers and its subsequent availability to the enzyme for conversion. The increased specific activity of

enzyme with high pNPB concentration (Table 18) indicates that Cut-P₁₁4 displays high activity and conversion if sufficient substrate can be provided to it.

[0569] This data demonstrates that a plurality of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme are able to produce an enzymatically active supramolecular assembly.

[0570] Table 18: Reaction rate and Specific Activity of Cutinase-P₁₁4.

Substrate concentration (mM)	Reaction Rate (Abs/min)	Reaction Rate (μM pNP/min)	Specific Activity (U/μg)
0.1	0.918	12.56	35.68
0.5	3.168	43.34	123.12

[0571] Expression and Purification of WT-ATA and ATA-P₁₁4

[0572] ω-aminotransferase was examined as an example of the transferase class of enzymes. Preliminary expression attempts showed that maximum soluble expression for both WT-ATA and ATA-P₁₁4 was obtained with IPTG induction at 25 °C (Figure 46). This correlates with the expression results reported previously for the WT-ATA. Bands excised from the soluble fractions (indicated by arrows in SDS-PAGE gel; Figure 46) were subjected to mass-spectrometry analysis and the amino acid sequence was confirmed to be that of WT-ATA and ATA-P₁₁4. Soluble fractions of both WT-ATA and ATA-P₁₁4 were purified by Ni-IMAC and showed similar chromatography profiles (Figure 47a and b). High purity fractions were obtained when eluted with 100 and 200 mM imidazole for both wild-type and fusion enzyme (Figure 47c). The above results show that high soluble expression and pure fractions may be obtained with is ATA-P₁₁4 with yield comparable to WT-ATA. Hence the expression and purification characteristics of ATA enzyme remain unaltered when fused with P₁₁4 peptide.

[0573] This data demonstrates the formation of self-assembling enzymatic components comprising a self-assembly polypeptide bound to an enzyme.

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The Claims Defining the Invention are as Follows:

1. An enzymatically active supramolecular assembly comprising:
a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme.
2. An assembly according to claim 1, wherein the plurality of self-assembling enzymatic components are covalently linked.
3. An assembly according to claim 1 or 2, wherein the self-assembly polypeptide and the enzyme are covalently linked.
4. An assembly according to any one of claims 1 to 3, wherein the self-assembly polypeptide is a pH responsive self-assembly polypeptide.
5. An assembly according to any one of claims 1 to 4, wherein the assembly is selected from the group consisting of a particle, fibre, sheet or a combination thereof.
6. An assembly according to claim 5, wherein the assembly is a particle.
7. An assembly according to claim 6, wherein the particle is spherical.
8. An assembly according to any one of claims 5 to 7, wherein the particle has a diameter of from about 20 nm to about 200 nm.
9. An assembly according to claim 8, wherein the particle has a diameter of from about 30 to about 1500 nm.

10. An assembly according to any one of claims 1 to 5, wherein the assembly is a hydrogel or an aggregate.
11. An assembly according to any one of claims 1 to 10, wherein the enzyme converts carbon dioxide to bicarbonate.
12. An assembly according to any one of claims 1 to 10, wherein the enzyme is selected from the group consisting of a formate dehydrogenase, a carbonic anhydrase, RuBisCO and combinations thereof.
13. An assembly according to any one of claims 1 to 10, wherein the enzyme is selected from the group consisting of a cutinase, a tyrosinase and an aminotransferase.
14. An assembly according to any one of claims 1 to 13, wherein the assembly consists of the plurality of self-assembling enzymatic components.
15. An assembly according to any one of claims 1 to 14, wherein the assembly is stable between 25 to 50°C.
16. An assembly according to any one of claims 1 to 15, wherein the catalytic activity of the enzyme is at least 80% of the catalytic activity of the free enzyme.
17. An assembly according to claim 16, wherein the catalytic activity of the enzyme is at least 98% of the catalytic activity of the free enzyme.
18. An assembly according to any one of claims 1 to 17, wherein the assembly is stable for at least 2 months.

19. An assembly according to any one of claims 1 to 18, wherein the assembly is switchable between the supramolecular assembly state and an unassembled state.
20. An assembly according to any one of claims 4 to 18, wherein the assembly is pH responsive.
21. An assembly according to any one of claims 1 to 20, wherein the self-assembly polypeptide is a P₁₁ family polypeptide.
22. An assembly according to any one of claims 1 to 21, wherein the self-assembly polypeptide is P₁₁-4.
23. An assembly according to any one of claims 1 to 22, wherein a linker binds the self-assembly polypeptide to the enzyme.
24. An assembly according to claim 23, wherein the linker covalently binds the self-assembly polypeptide to the enzyme.
25. An assembly according to claim 23 or 24, wherein the linker comprises a polypeptide.
26. An assembly according to claim 25, wherein at least 90% of the amino acids in the linked polypeptide are glycine or serine or a combination thereof.
27. An assembly according to any one of claims 23 to 26, wherein the linker is a glycine-serine (GS) linker.
28. An assembly according to claim 26, wherein the GS linker comprises (SEQ ID NO: 2).

29. An assembly according to any one of claims 1 to 28 wherein the supramolecular assembly self assembles in the absence of a carrier.

30. A process for sequestering carbon dioxide from a carbon dioxide containing fluid or gas, wherein the method comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is carbonic anhydrase;

- contacting the assembly with the carbon dioxide containing fluid or gas;

and

- converting carbon dioxide in the carbon dioxide containing fluid or gas to bicarbonate.

31. A process according to claim 30, wherein the enzymatically active supramolecular assembly is immobilised.

32. A process according to claim 30 or 31, wherein the enzymatically active supramolecular assembly is immobilised on a film or membrane.

33. A process according to any one of claims 30 to 32, wherein the fluid or gas is a fluid or gas stream.

34. An enzymatically active supramolecular assembly comprising a first self-assembling enzymatic component and a second self-assembling enzymatic component, wherein the first self-assembling enzymatic component comprises a self-assembly polypeptide bound to a first enzyme, and wherein the second self-assembling enzymatic component comprises a self-assembly polypeptide bound to a second enzyme.

35. A self-assembling enzymatic component wherein the self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and wherein the self-assembling enzymatic component is capable of

forming an enzymatically active supramolecular assembly in the presence of at least one further self-assembling enzymatic component comprising a self-assembly polypeptide bound to an enzyme.

36. A process for producing a tyrosinase reaction product from a solution comprising a substrate of tryosinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is a tyrosinase;
- contacting the assembly with solution comprising a substrate of the tyrosinase; and
- converting a substrate in the solution comprising the substrate of the tyrosinase to a reaction product of the tryosinase.

37. A process for producing a tyrosinase reaction product from a solution comprising a substrate of tryosinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is a tyrosinase;
- contacting the assembly with a solution comprising a substrate selected from the group consisting of tyrosine, DOPA, Tyrosol, Phenols and Catechols, Sericin, Phenolic species, Monophenols, diphenols, and α -lactalbumin
- converting a substrate in the solution comprising the substrate of the tyrosinase to a reaction product of the tryosinase.

38. A process for producing an aminotransferase reaction product from a solution comprising a substrate of aminotrasferase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is a aminotransferase;
- contacting the assembly with a solution comprising a substrate of the aminotransferase; and

- converting a substrate in the solution comprising the substrate of the aminotransferase to a reaction product of the aminotransferase.

39. A process for producing an aminotransferase reaction product from a solution comprising a substrate of aminotrasferase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is an aminotransferase;
- contacting the assembly with solution comprising at least one substrate selected from the group consisting of amine enantiomers, pyruvate, alanine, ketones, δ -keto acid ester, acetophenone, 1-phenylethylamine, ketone and isopropylamine; and
- converting a substrate in the solution comprising the substrate of the aminotransferase to a reaction product of the aminotransferase.

40. A process for producing chiral amines from a solution comprising amino acids, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is an aminotransferase;
- contacting the assembly with a solution comprising amino acids; and
- converting the amino acids in the solution comprising amino acids to chiral amino acids.

41. A process according to claim 38, 39 or 40, wherein the enzyme is ω -aminotransferase.

42. A process for producing a cutinase reaction product from a solution comprising a substrate of cutinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is a cutinase;

- contacting the assembly with a solution comprising a substrate of the cutinase; and
- converting a substrate in the solution comprising the substrate of the cutinase to a reaction product of the cutinase.

43. A process for producing a cutinase reaction product from a solution comprising a substrate of cutinase, wherein the process comprises the steps of:

- providing an enzymatically active supramolecular assembly according to any one of claims 1 to 28, wherein the enzyme is a cutinase;
- contacting the assembly with a solution comprising a substrate selected from the group consisting of apple peel, tomato peel, orange peel, p-nitrophenyl ester, triolein, tricaprilin, fatty acids, geranyl diphosphate, butyl acetate, hexanol organophosphate malathion, Polycaprolone, and Dihexylphtalate; and
- converting a substrate in the solution comprising the substrate of the cutinase to a reaction product of the cutinase.

44. A method of forming an enzymatically active supramolecular assembly, the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii) contacting the solution containing a plurality of self-assembling enzymatic components with a buffer to form an enzymatically active supramolecular assembly.

45. A method according to claim 44, wherein the assembly is a particle.

46. A method according to claim 45, wherein the particle is spherical.

47. A method according to claim 46, wherein the particle has a diameter of about 100 nm, and wherein the buffer comprises about 50mM Tris-HCl at about pH 6.8.

48. A method according to claim 45, wherein the particle has a diameter of about 400 nm, and wherein the buffer comprises about 50mM Tris-HCl at about pH 6.5.

49. A method according to claim 45, wherein the particle has a diameter of about 1500 nm, and wherein the buffer comprises about 50mM Tris-HCl at about pH 5.6.

50. A method according to claim 45, wherein the particle has a diameter of about 1500 nm, and wherein the buffer comprises about 10 mM MgCl_2 at about pH 6.1.

51. A method according to claim 45, wherein the particle has a diameter of about 1000 nm, and wherein the buffer comprises about 25 mM MgCl_2 in about 10mM Tris at about pH 8.0.

52. A method according to claim 45, wherein the particle has a diameter of about 200 nm, and wherein the buffer comprises about 5 mM MgCl_2 in about 10mM Tris at about pH 8.0.

53. A method according to claim 45, wherein the particle has a diameter of about 400 nm, and wherein the buffer comprises about 50 mM MgCl_2 in about 10mM Tris at about pH 8.0.

54. A method according to claim 45, wherein the particle has a diameter of about 30 to about 40 nm or about 120 to about 200 nm, and wherein the buffer comprises about 50 mM NaNO_3 in about 10 mM Tris at about pH 6.8.

55. A method according to claim 45, wherein the particle has a diameter of about 30 to about 200 nm, and wherein the plurality of self-assembling enzymatic

components are present at a concentration of about 1.0 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

56. A method according to claim 45, wherein the particle has a diameter of about 600 nm, and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 3 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

57. A method according to claim 45, wherein the particle has a diameter of about 200 nm, and wherein the buffer comprises about 6 mM MgCl_2 at about pH 7.5 and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 0.5 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

58. A method according to claim 45, wherein the particle has a diameter of about 633 nm, and wherein the buffer comprises about 8 mM MgCl_2 at about pH 7.0 and wherein the plurality of self-assembling enzymatic components are present at a concentration of about 1 mg/ml in the solution containing the plurality of self-assembling enzymatic components.

59. A method of switching an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state, said method comprising contacting a solution comprising a supramolecular assembly according to any one of claims 1 to 28, wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of increased pH to unassemble the supramolecular assembly.

60. A method according to claim 59 wherein the buffer of increased pH is at pH 8.0.

61. A method of switching an enzymatically active supramolecular assembly between a supramolecular assembly state and an unassembled state, said method comprising contacting a solution comprising an unassembled supramolecular assembly of a supramolecular assembly according to any one of claims 1 to 28, wherein the self-assembling enzymatic component comprises a tandem repeat of self-assembly polypeptides, with a buffer of decreased pH to assemble the supramolecular assembly.

62. A method according to claim 61 wherein the buffer of decreased pH is at about pH 6.0

63. A method according to any one of claims 59 to 62 wherein the self-assembly polypeptide is a P₁₁ family polypeptide.

64. A method according to claim 63, wherein the self-assembly polypeptide is P₁₁₋₄

65. A method according to any one of claims 59 to 64 wherein the method further comprises the step of recovering the supramolecular assembly from solution.

66. A method of forming an enzymatically active supramolecular assembly having a diameter 'd', the method comprising the steps of (i) forming a solution containing a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, and (ii) contacting the solution containing a plurality of self-assembling enzymatic components with a buffer to form a solution having a plurality of self-assembling enzymatic components at a concentration 'c' wherein the solution formed has a pH 'a' and comprises MgCl₂ at a concentration 'd' to form an enzymatically active supramolecular assembly, wherein:

$$D \text{ (nm)} = (127.32 - 15.77a + 2.44c - 2.22d + 0.42 ad)^2 + 2.0 \dots\dots\dots(\text{Formula I}).$$

67. A method of modulating the size of an enzymatically active supramolecular assembly, said method comprising the steps of contacting a solution comprising a plurality of self-assembling enzymatic components, wherein each self-assembling enzymatic component comprises a self-assembly polypeptide bound to an enzyme, with a buffer to form an enzymatically active supramolecular assembly, wherein the buffer comprises a component to modulate the size of the enzymatically active supramolecular assembly formed.

68. A method according to claim 66 or 67 wherein the component to modulate the size of the enzymatically active supramolecular assembly formed is Mg^{2+} and/or a pH modulating compound.

69. A method according to any one of claims 66 to 68 wherein the enzyme is a bovine carbonic anhydrase.

70. A method according to any one of claims 66 or 68, wherein the self-assembly polypeptide is a P_{11} family polypeptide.

71. A method according to any one of claims 66 to 70, wherein the self-assembly polypeptide is P_{11-4} .

72. A method according to any one of claims 66 to 71, wherein a linker binds the self-assembly polypeptide to the enzyme.

73. A method according to any one of claims 66 to 72, wherein the linker covalently binds the self-assembly polypeptide to the enzyme.

74. A method according to any one of claims 66 to 73, wherein the linker comprises a polypeptide.

75. A method according to any one of claims 66 to 74, wherein at least 90% of the amino acids in the linked polypeptide are glycine or serine or a combination thereof.

76. A method according to any one of claims 66 to 75, wherein the linker is a glycine-serine (GS) linker.

77. A method according to any one of claims 66 to 76, wherein the GS linker comprises (SEQ ID NO: 2).

Figure 1

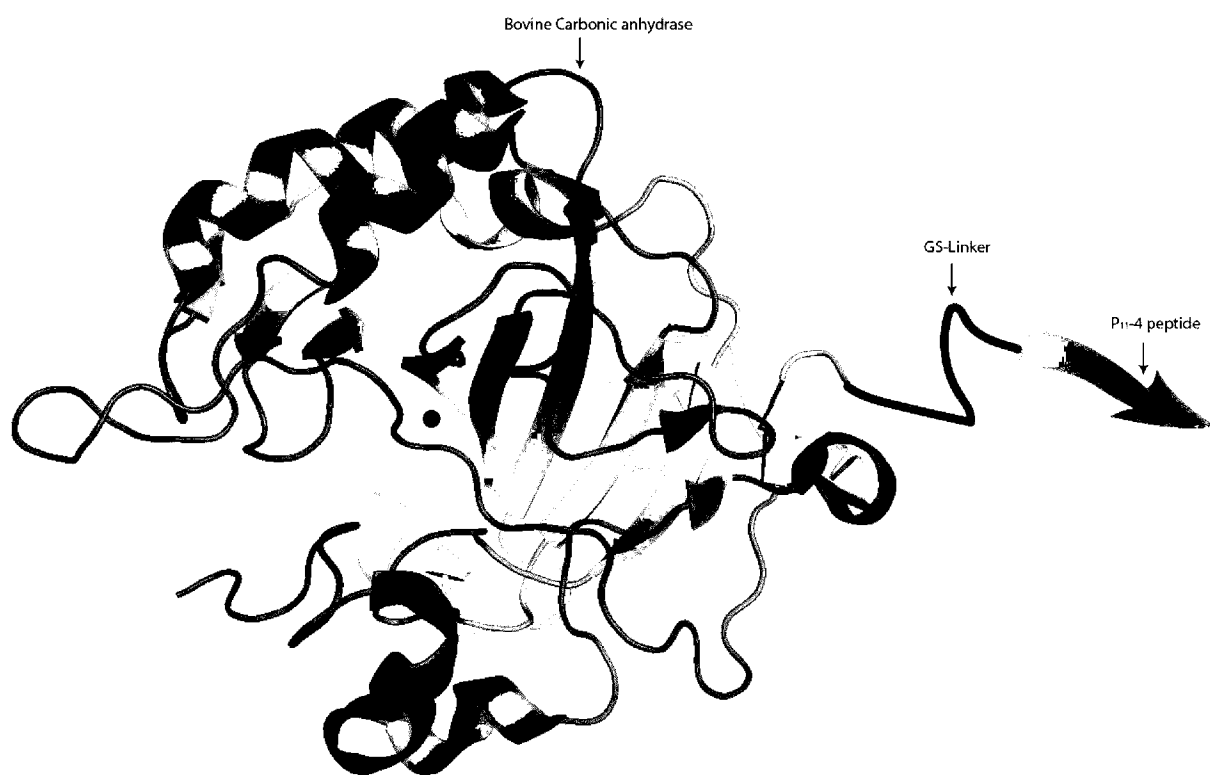
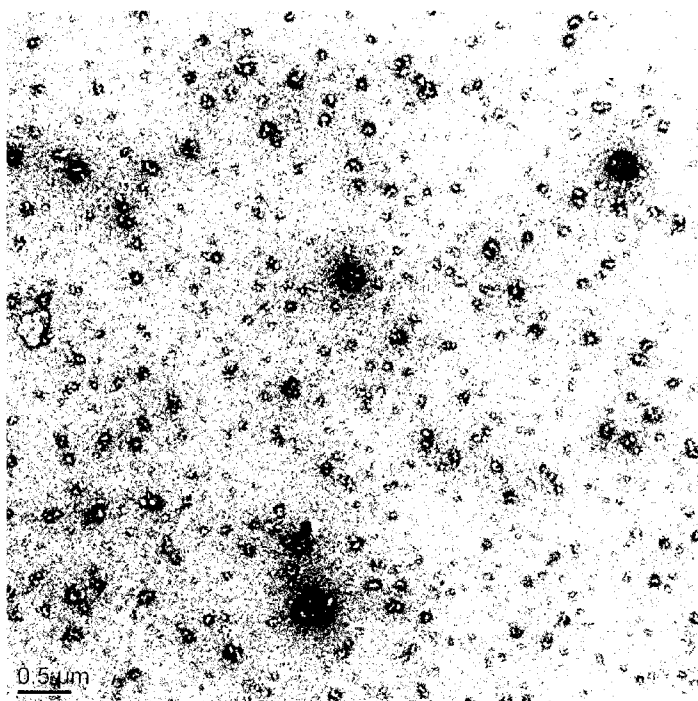


Figure 2

A



B

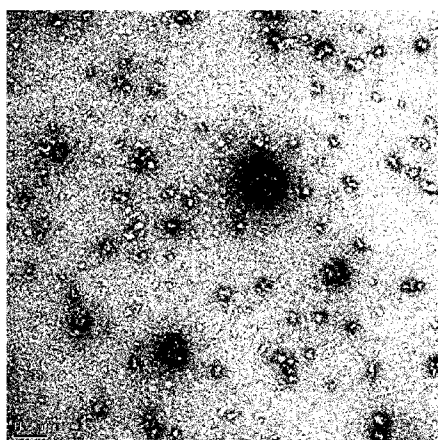


Figure 3

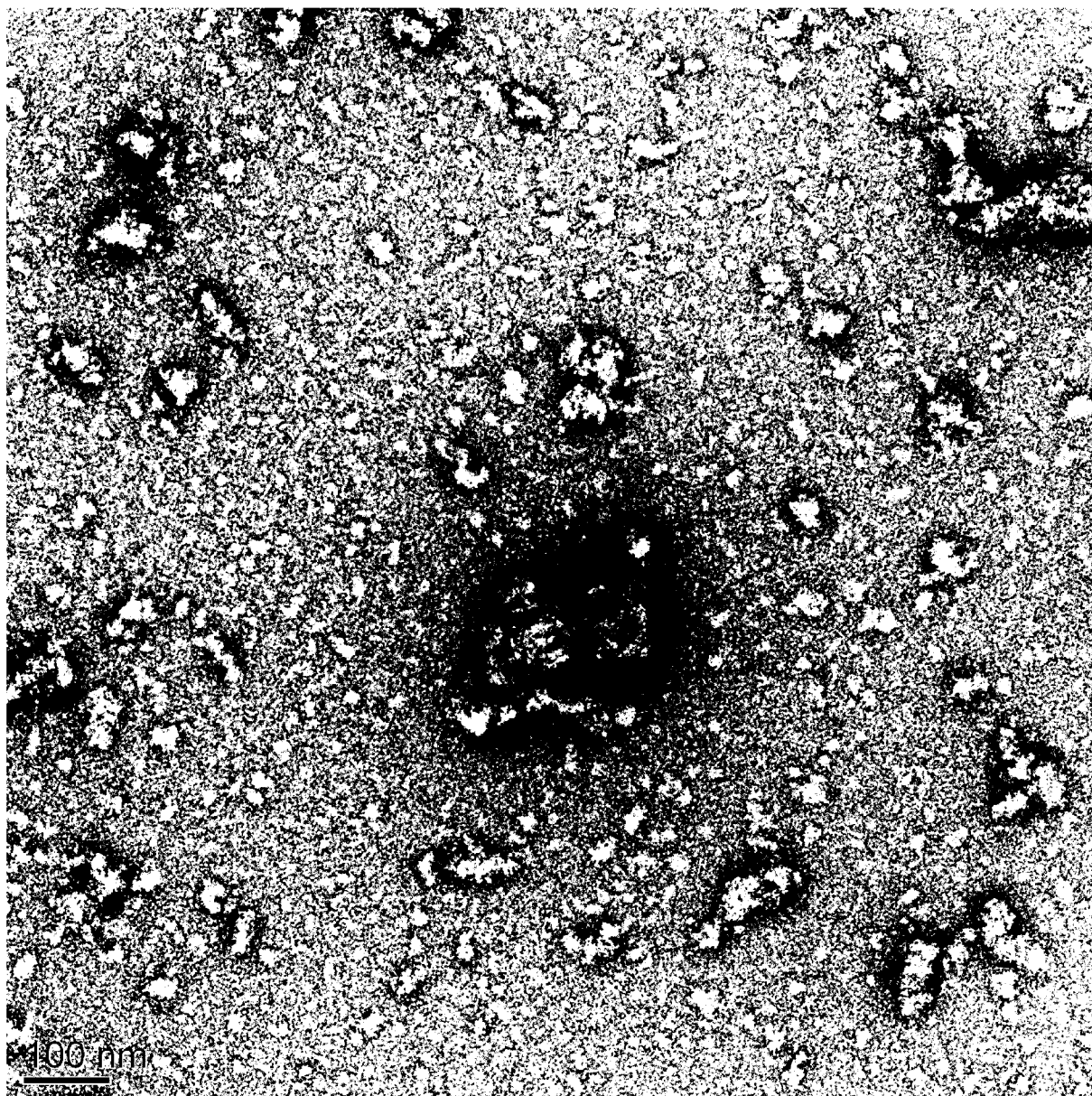
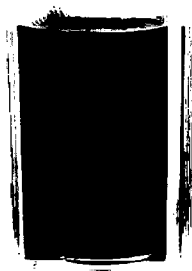


Figure 4

a)

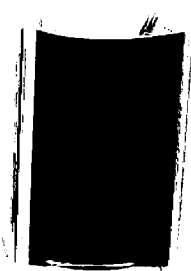
1



2

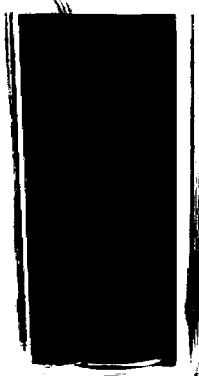


3



b)

1



2

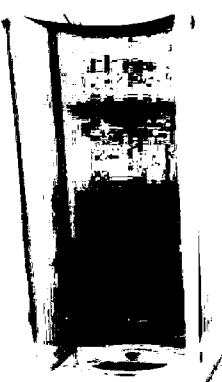


3

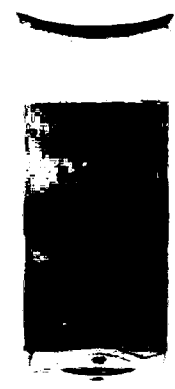


c)

1



2



3



5/55

Figure 5

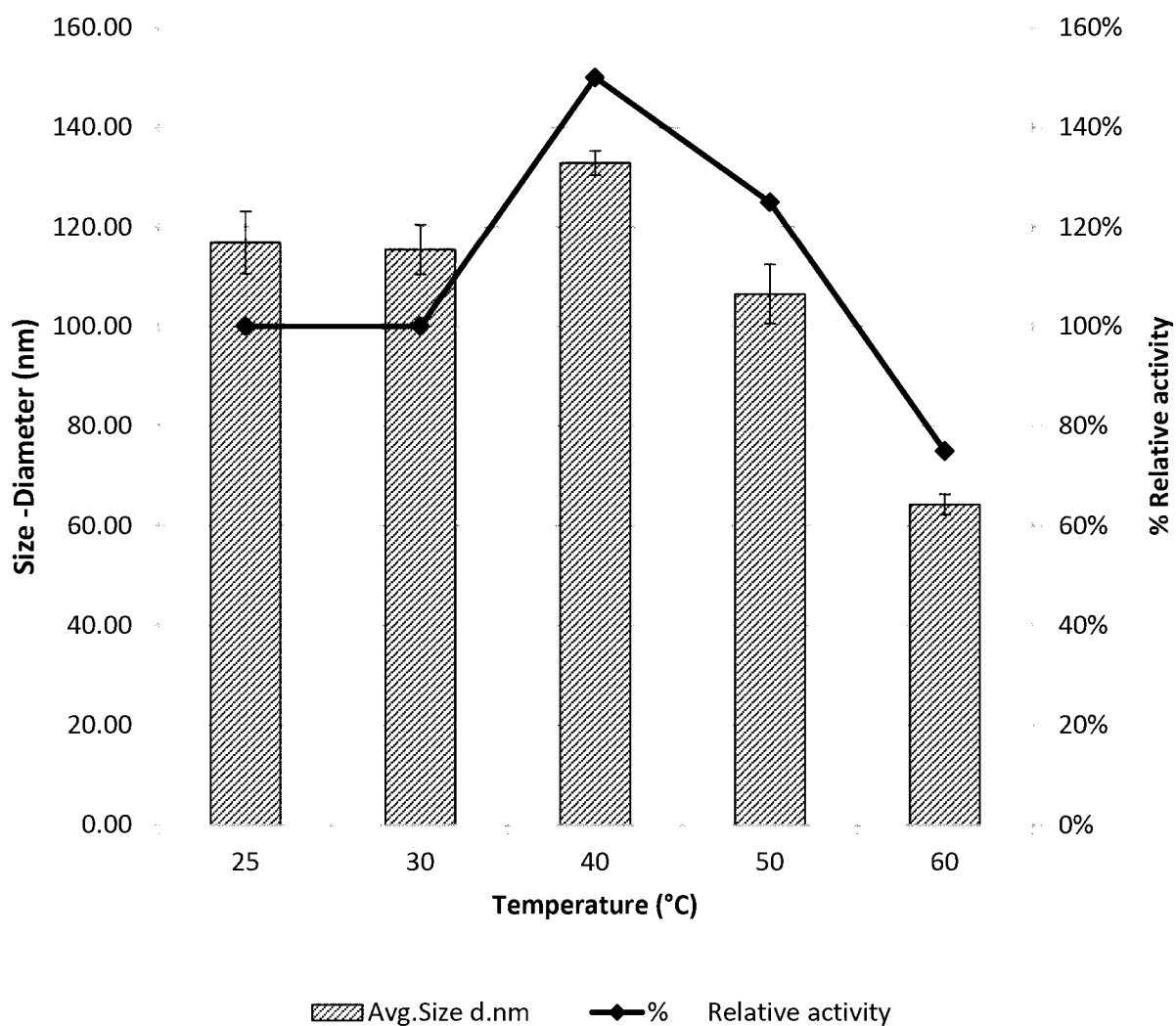


Figure 6

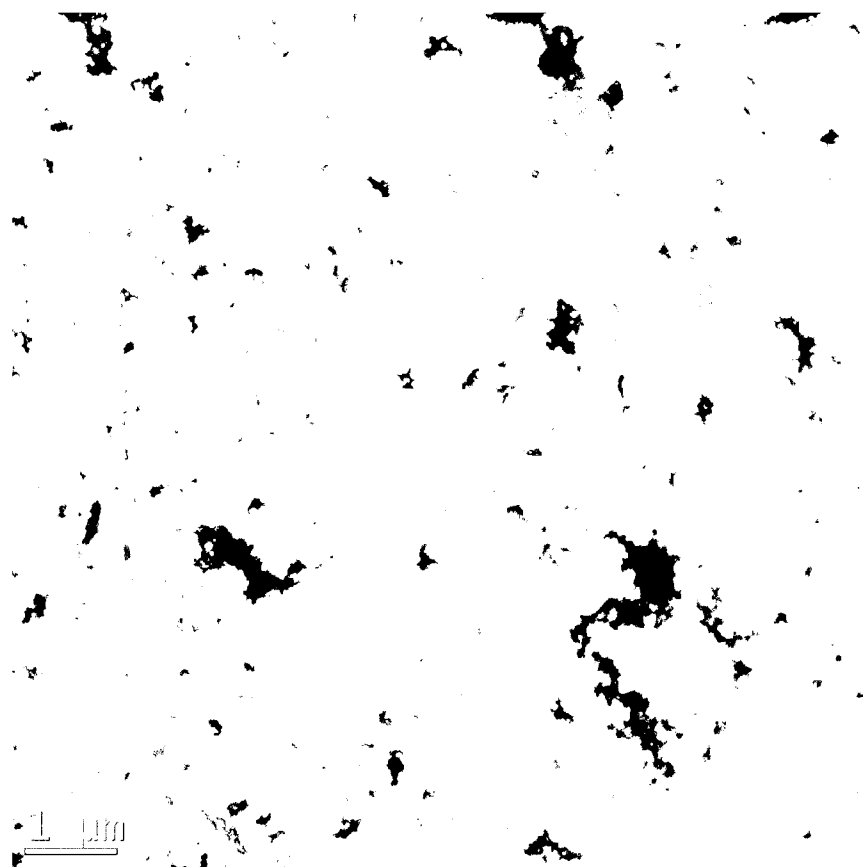


Figure 7

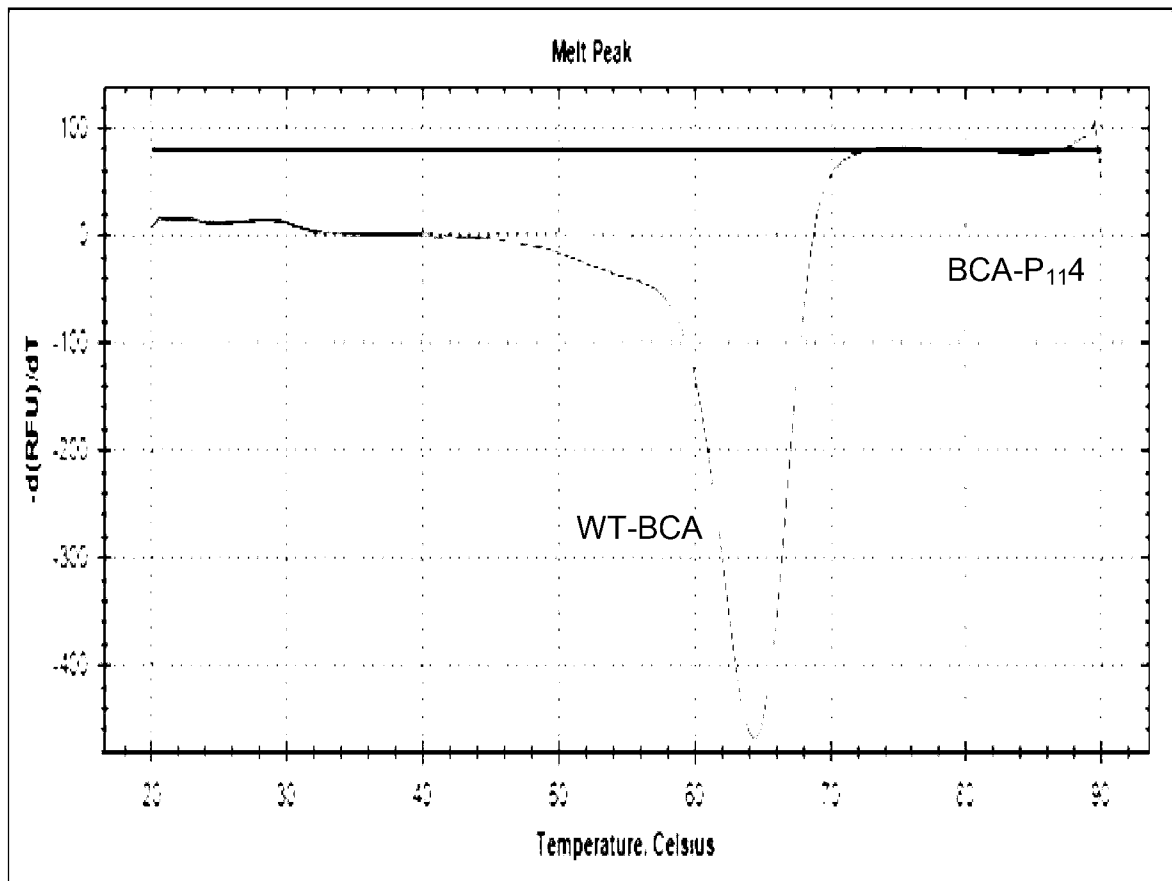


Figure 8

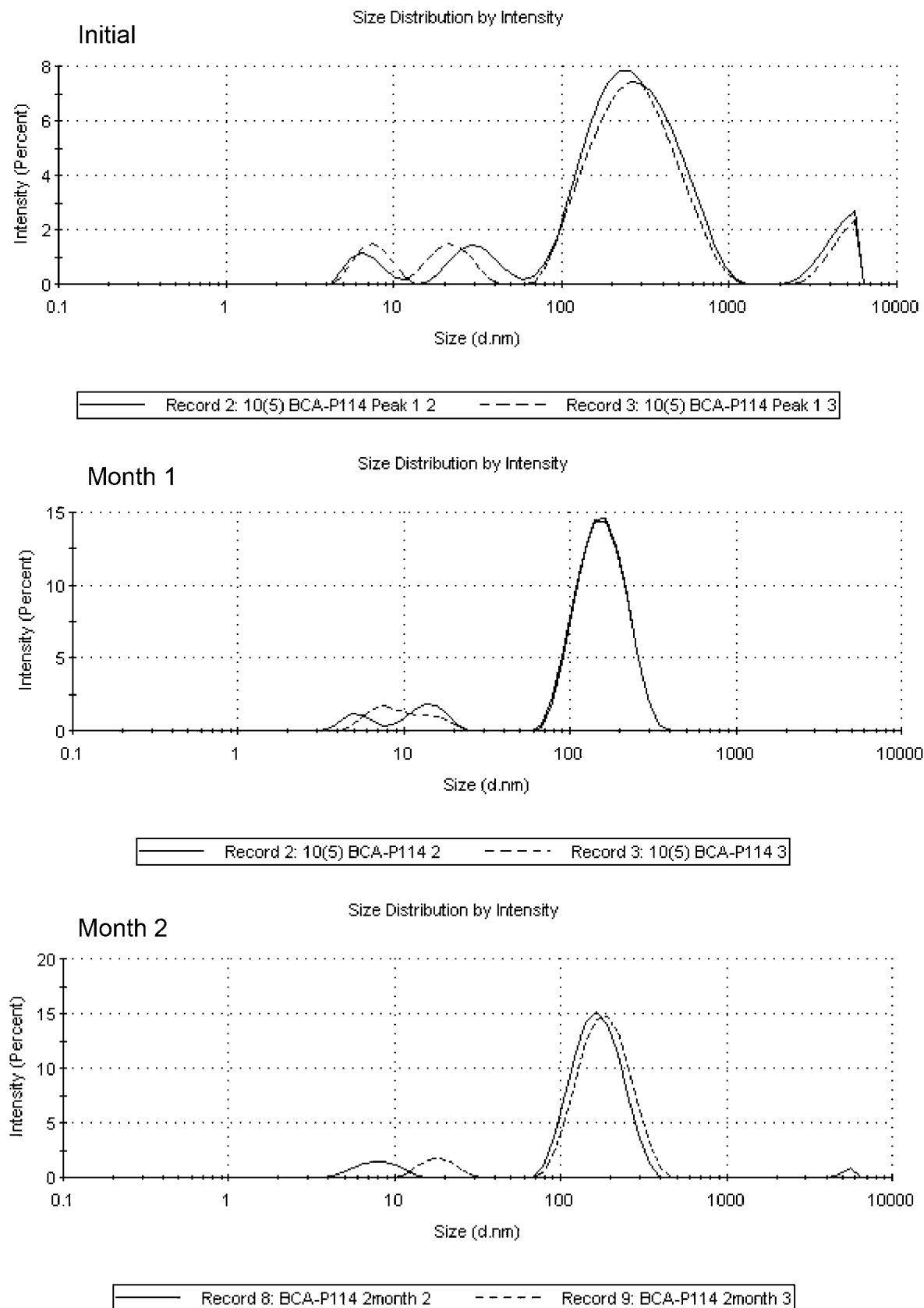


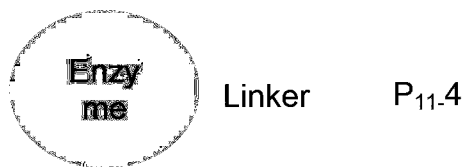
Figure 9

Particle size stability			
Time interval	Z.Avg. (d.nm)	Std.Dev (d.nm)	PdI
Initial	136.2	183.2	1
Month 1	104.5	52.6	0.45
Month 2	121.5	64.5	0.55

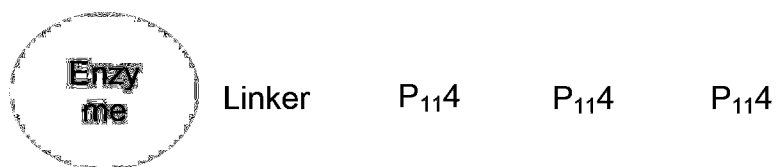
10/55

Figure 10

A



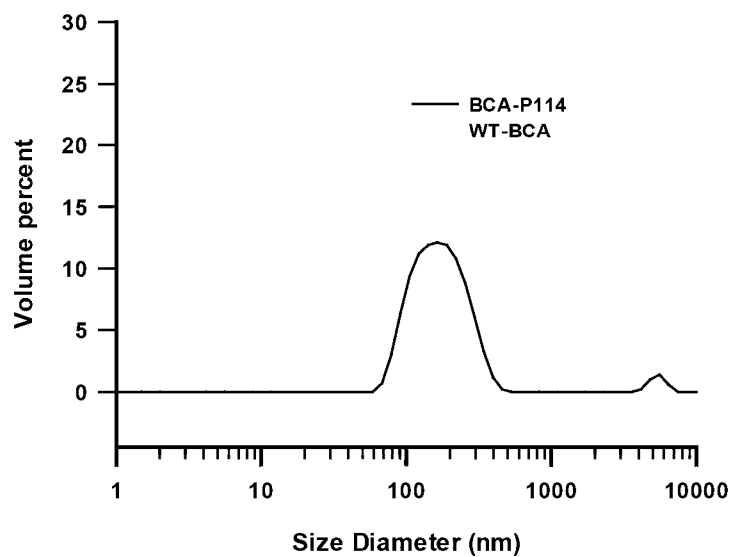
B



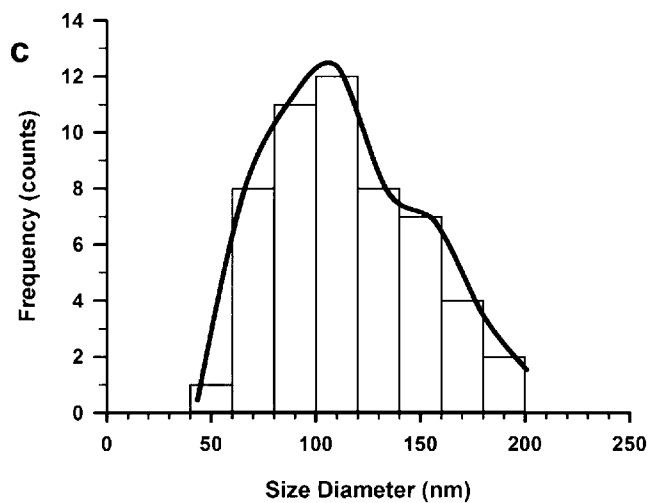
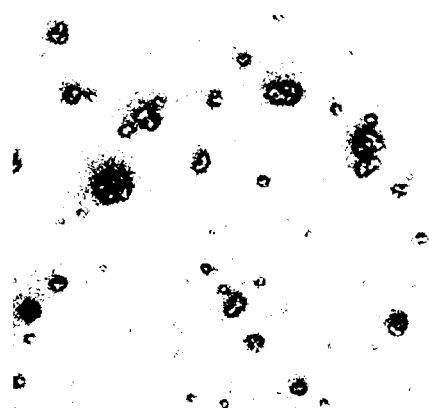
11/55

Figure 11

A



B



12/55

Figure 12

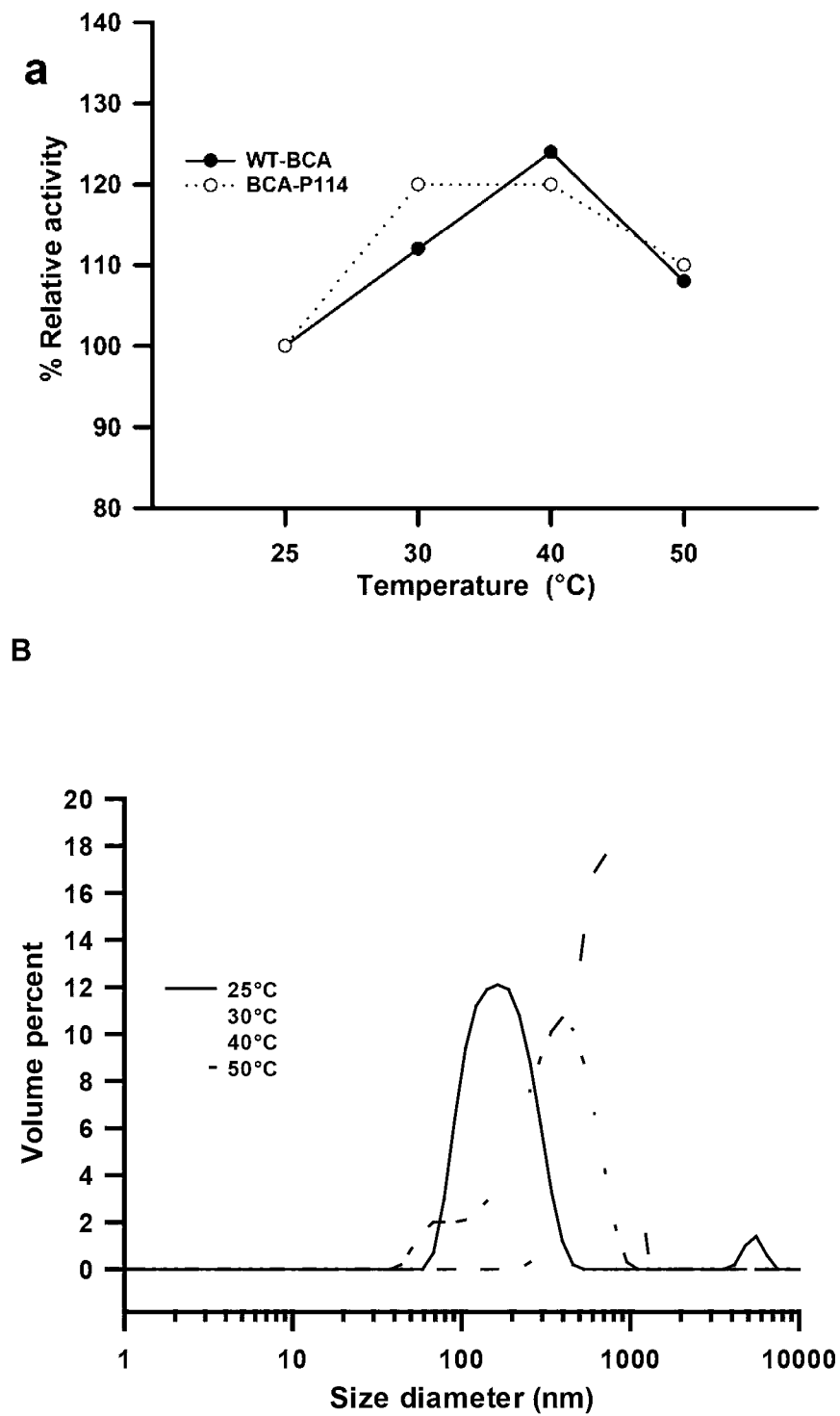


Figure 13

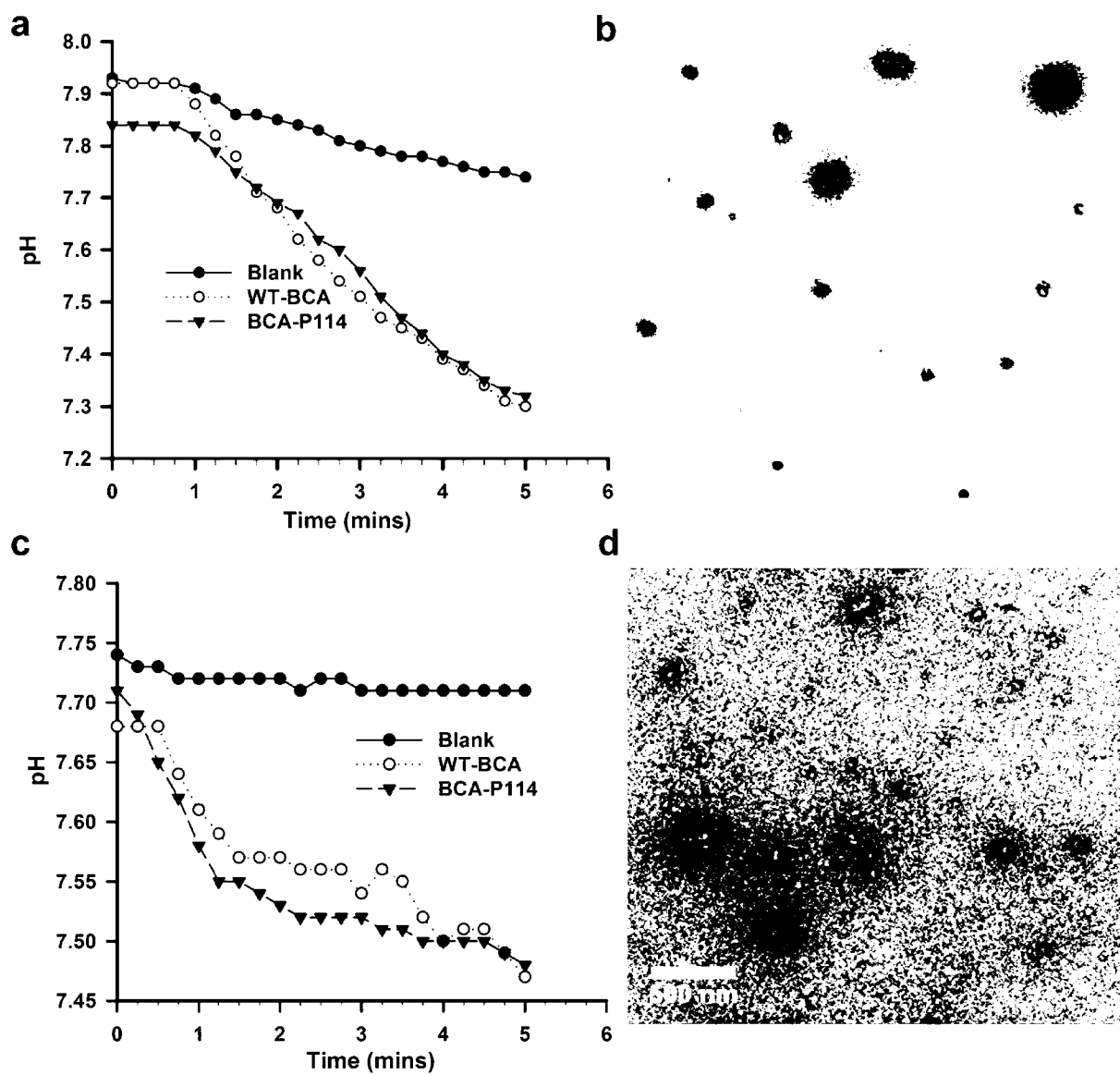
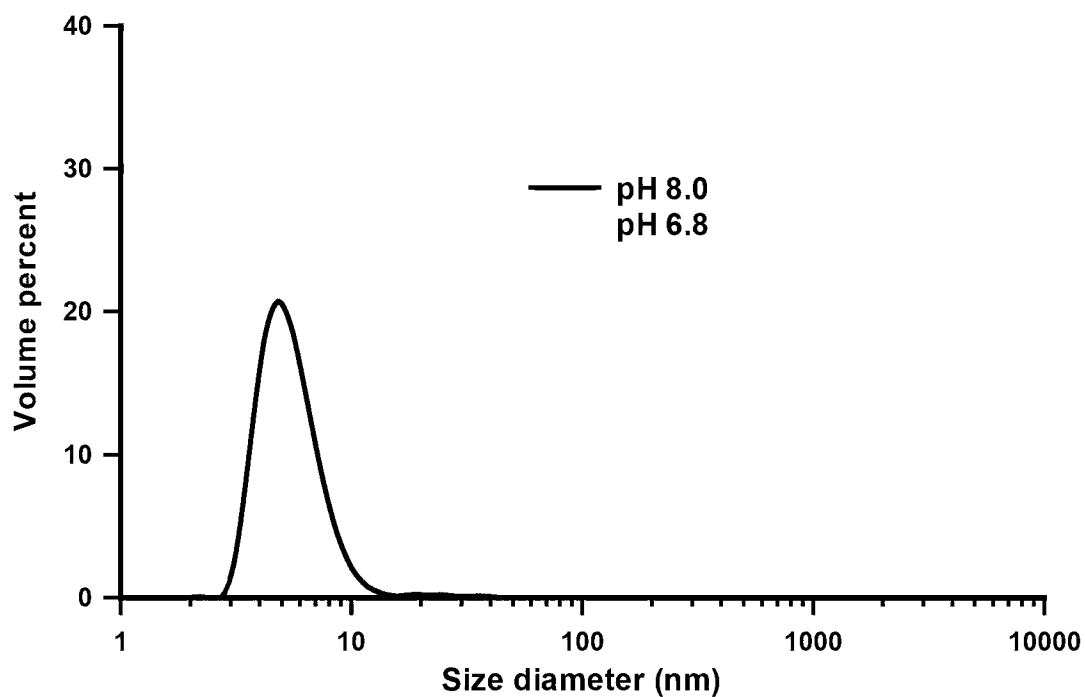


Figure 14

A



B

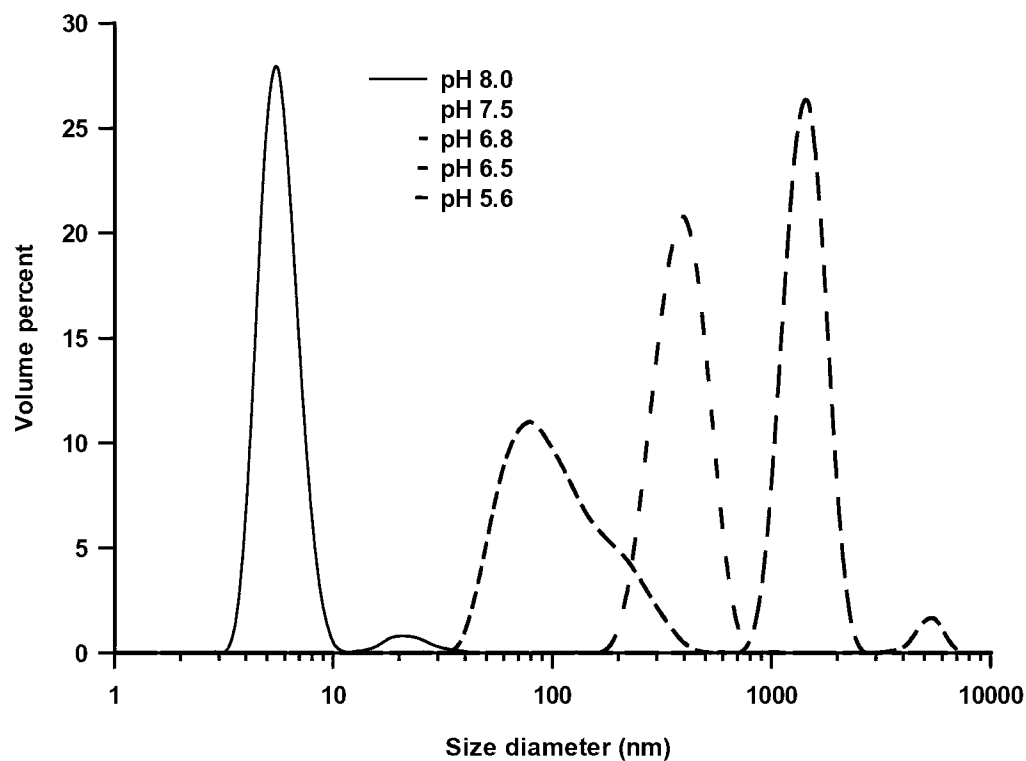
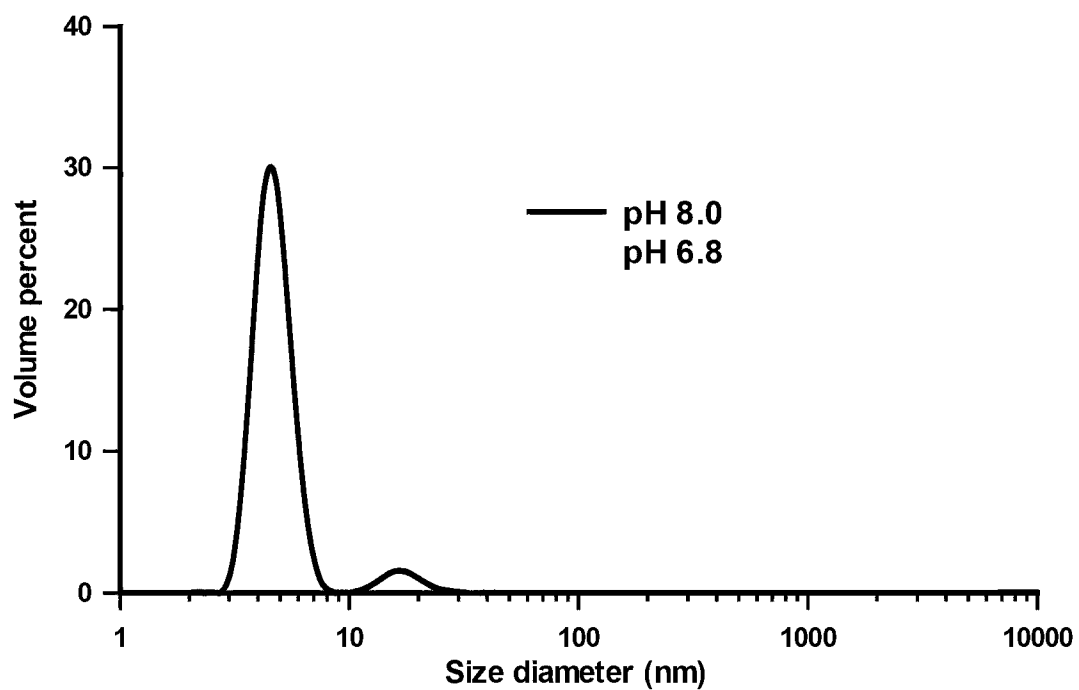


Figure 14 Cont

C



D

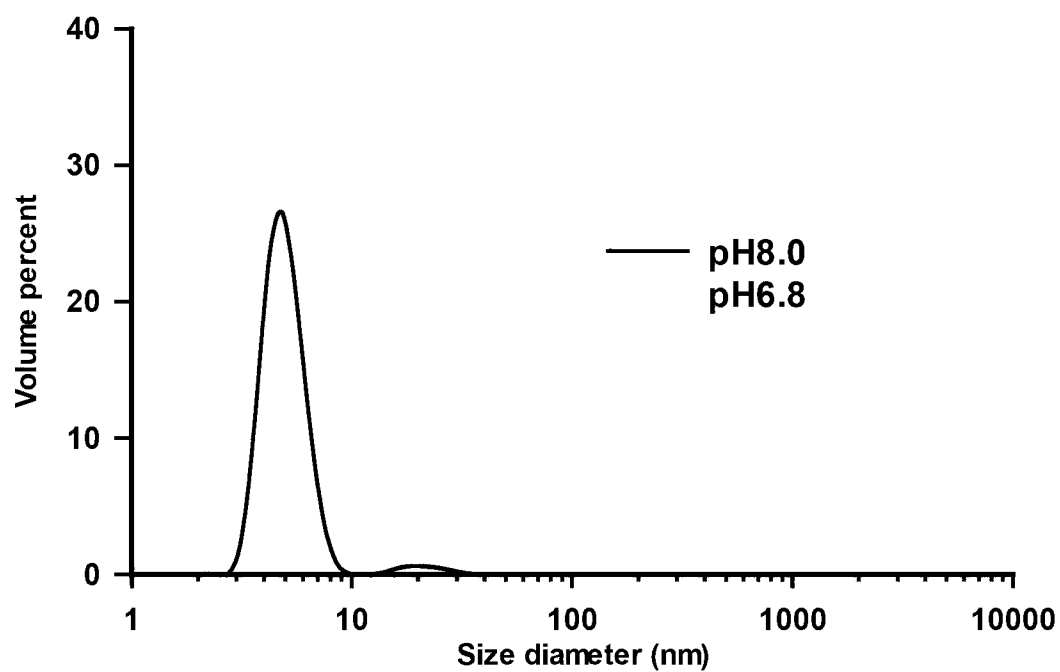
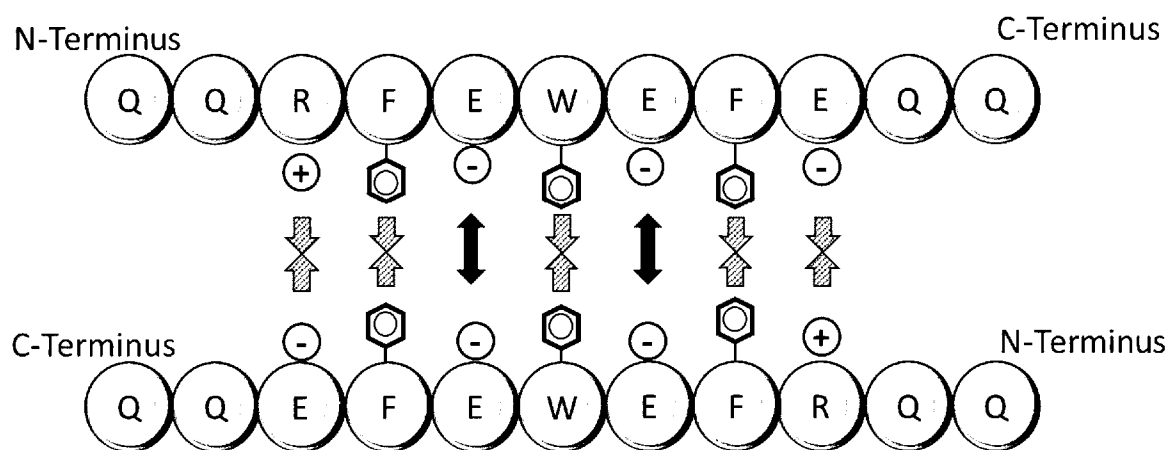


Figure 15

A



B

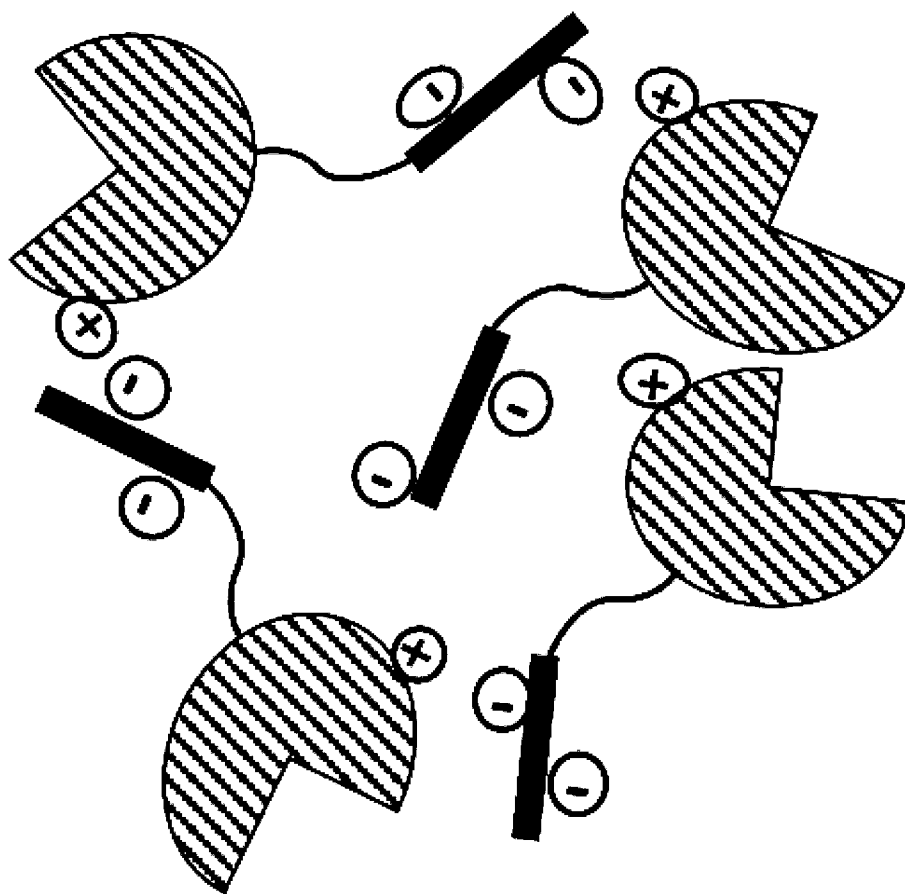


Figure 16

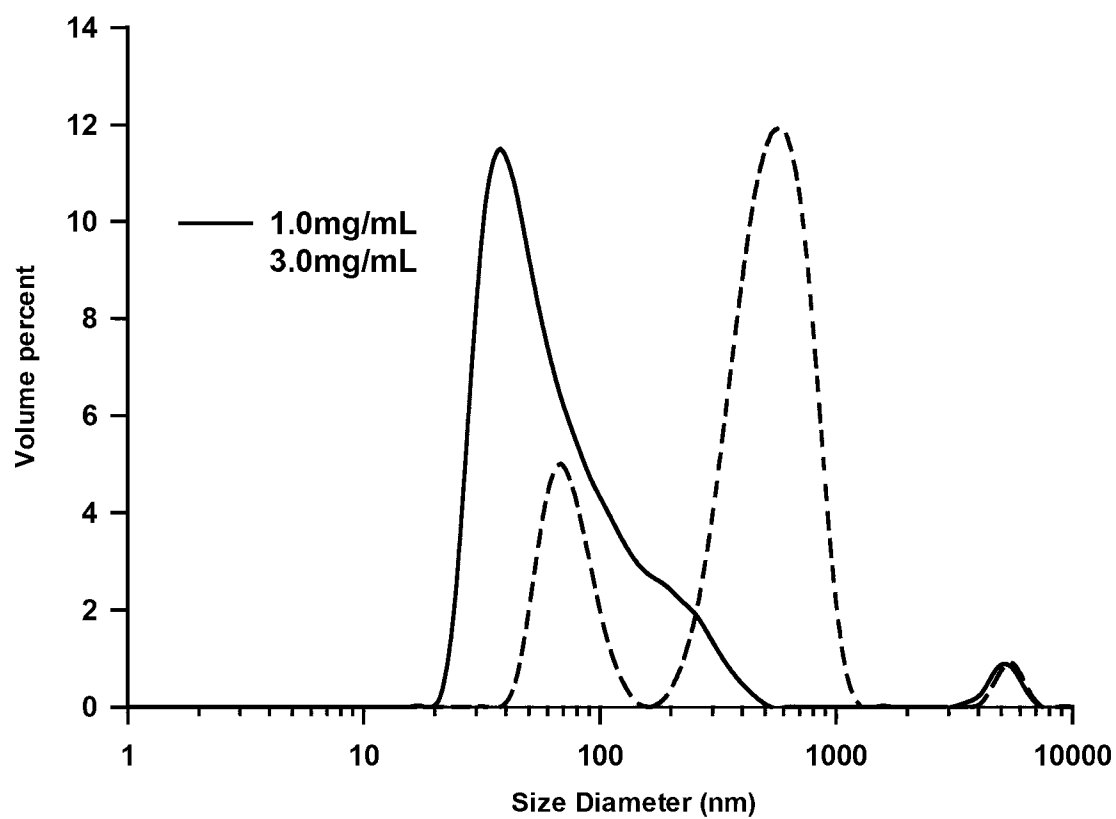


Figure 17

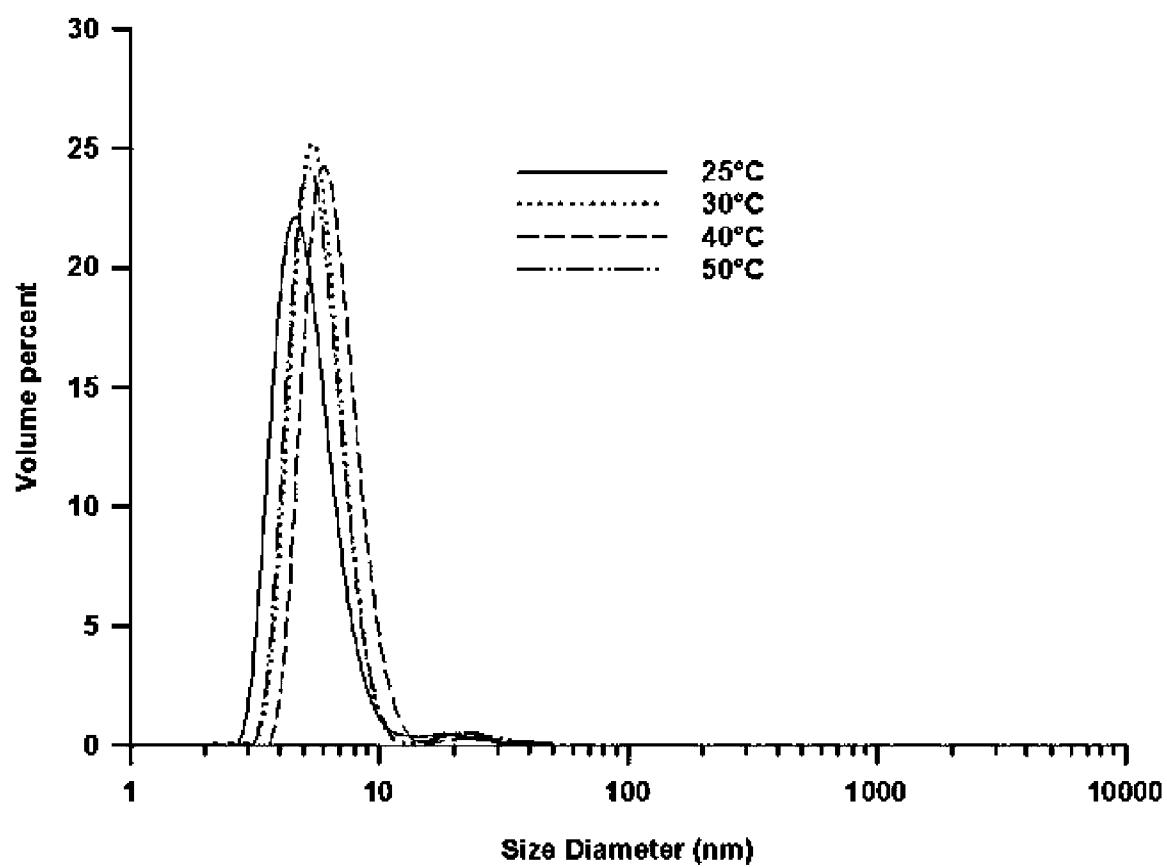
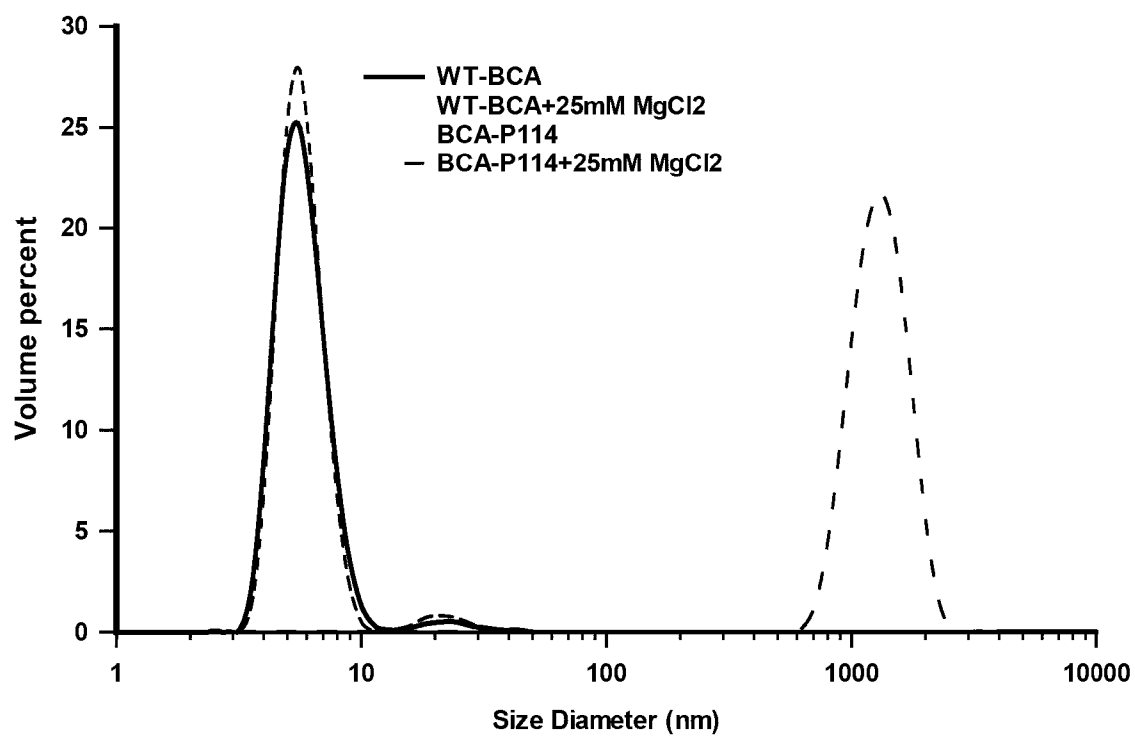


Figure 18

A



B

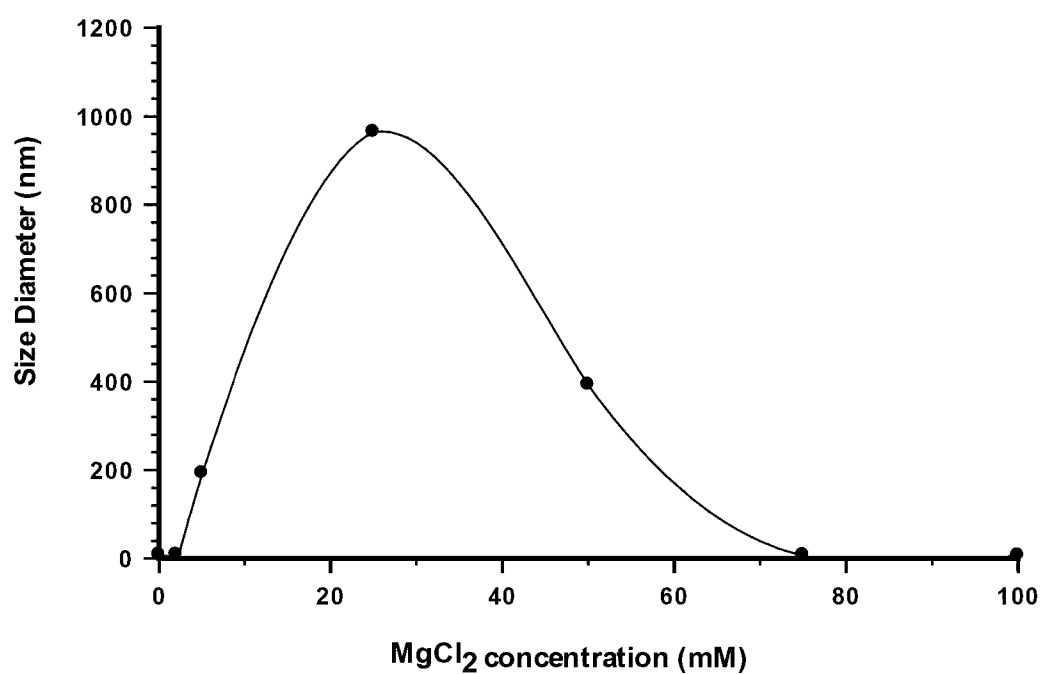
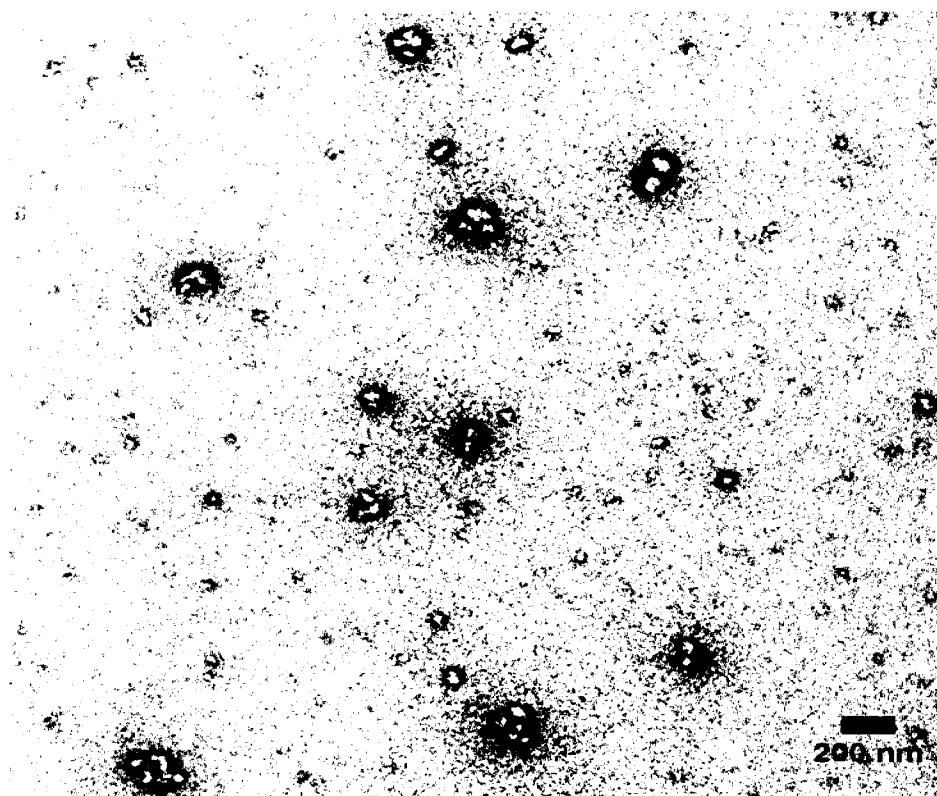


Figure 18 Cont

C



D

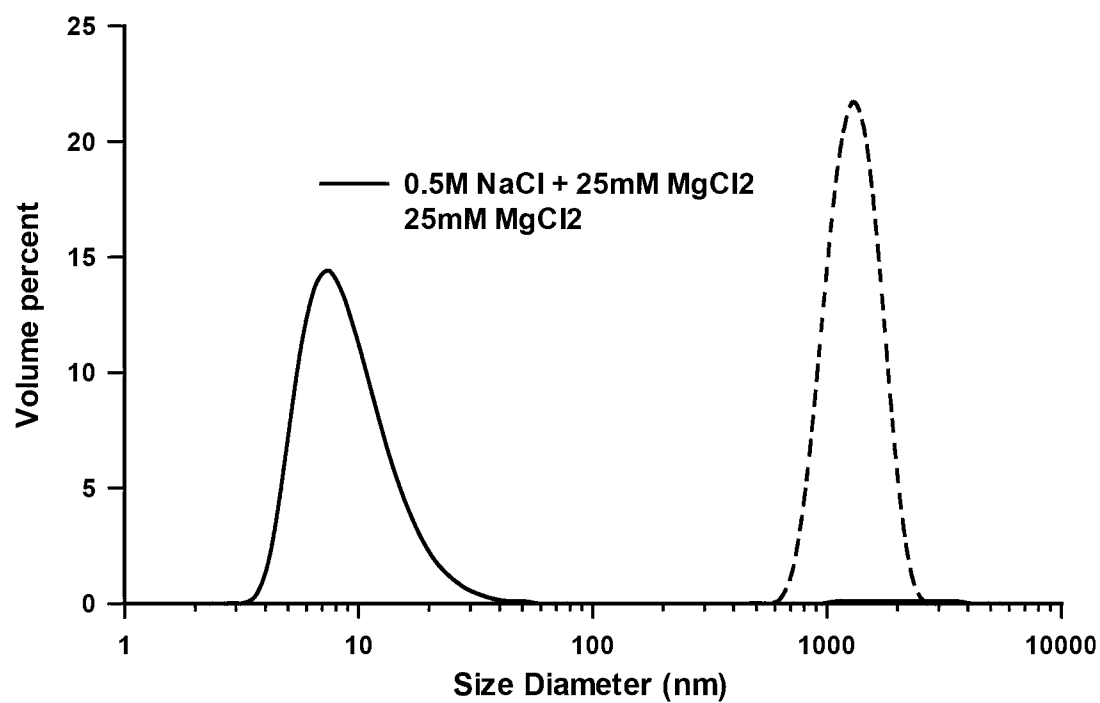
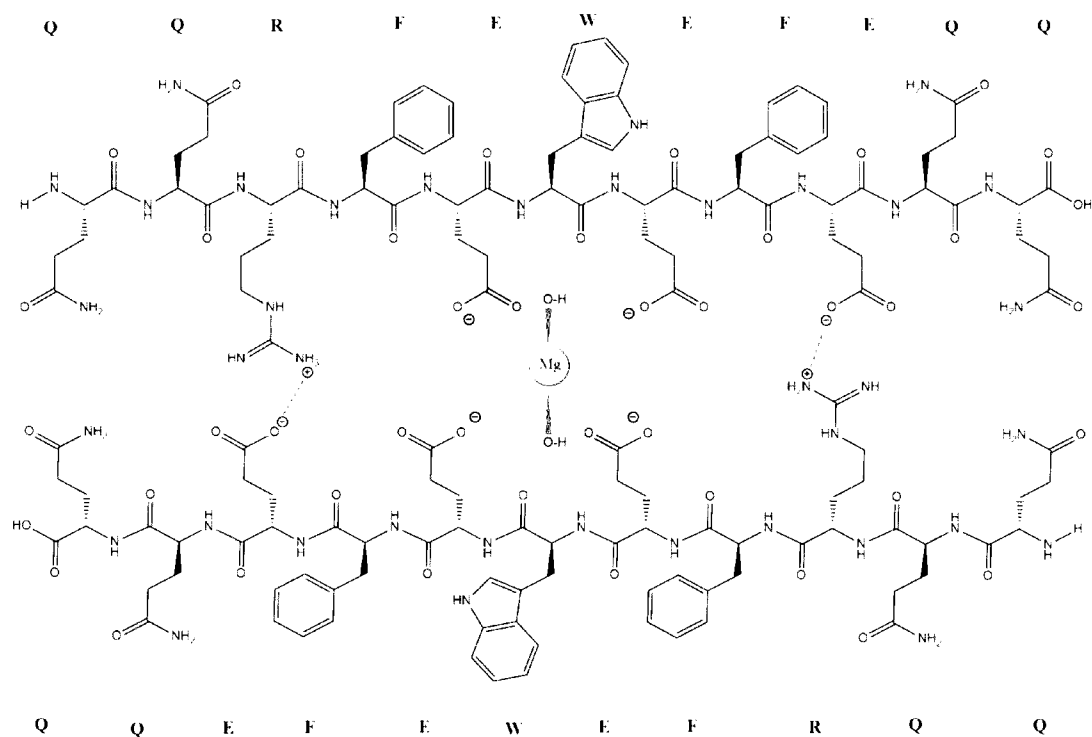


Figure 19

A



B

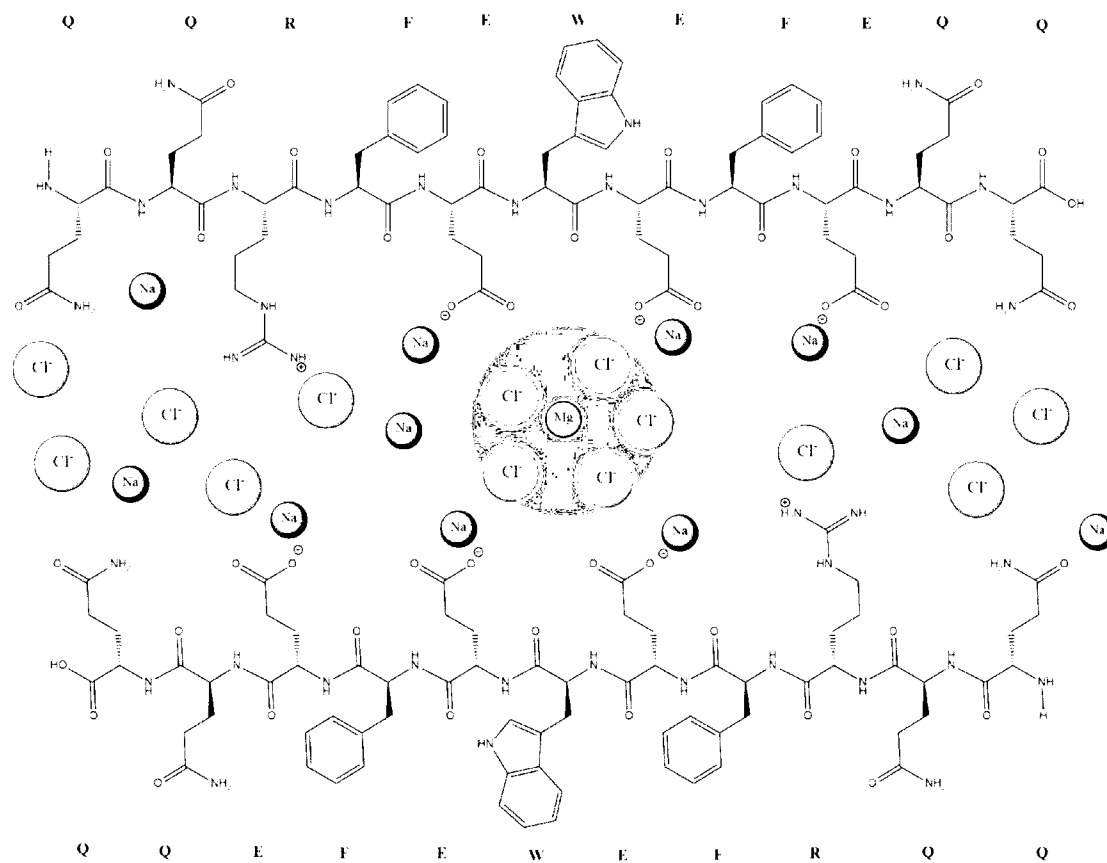


Figure 20

A

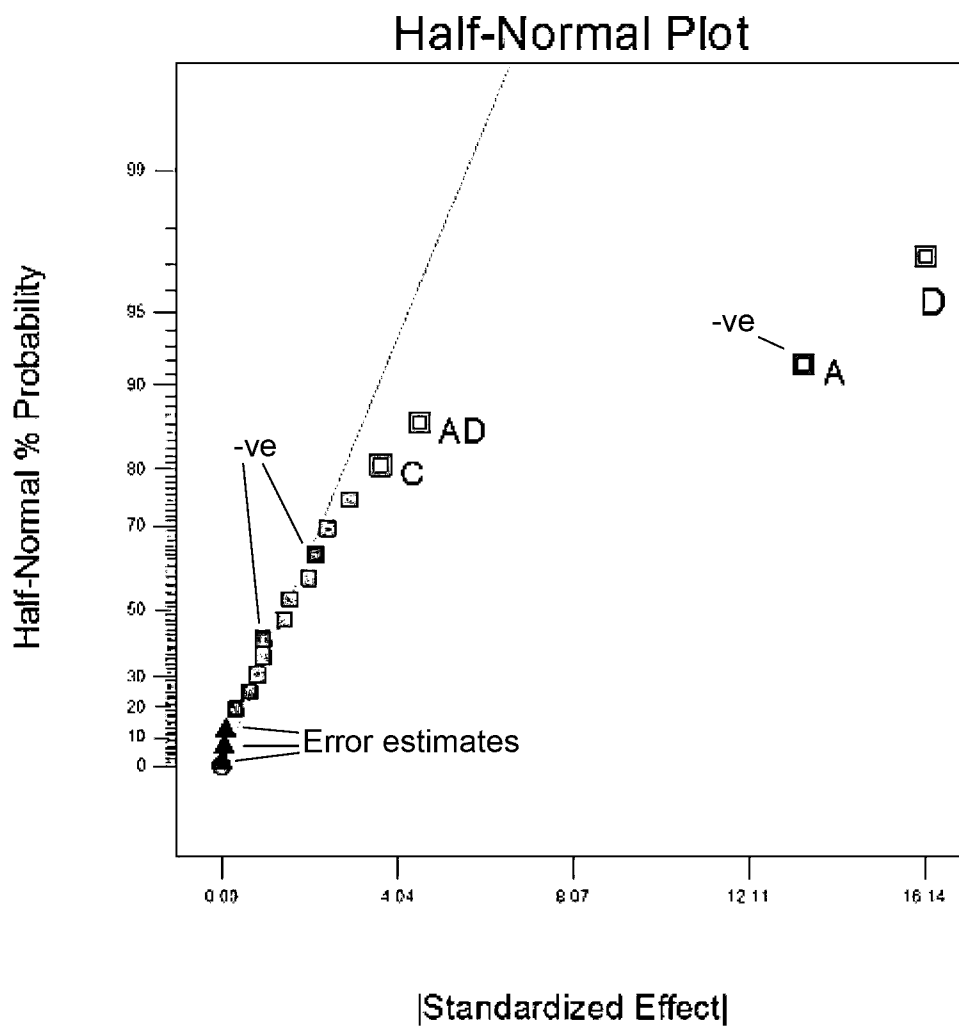
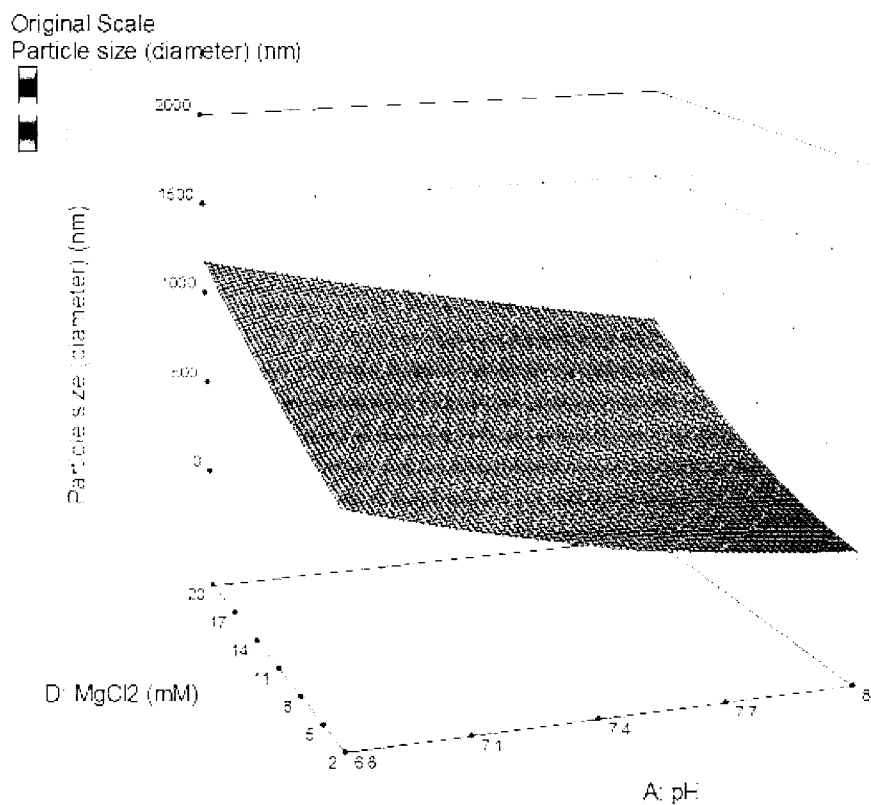


Figure 20

B



C

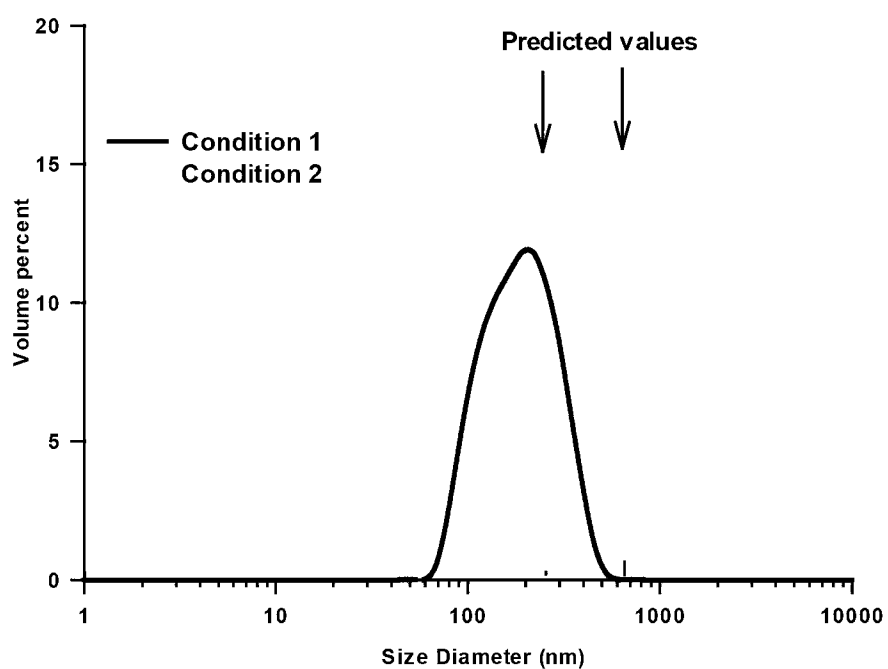


Figure 21

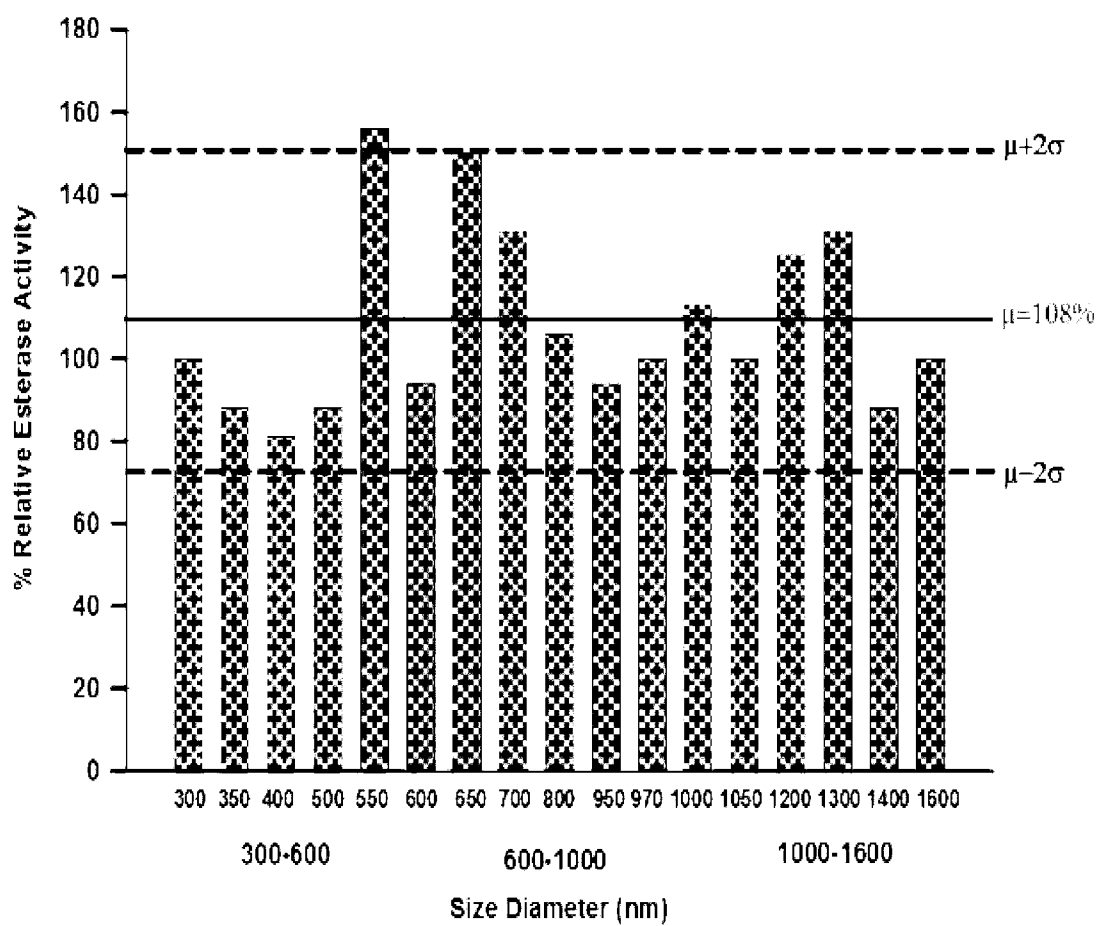
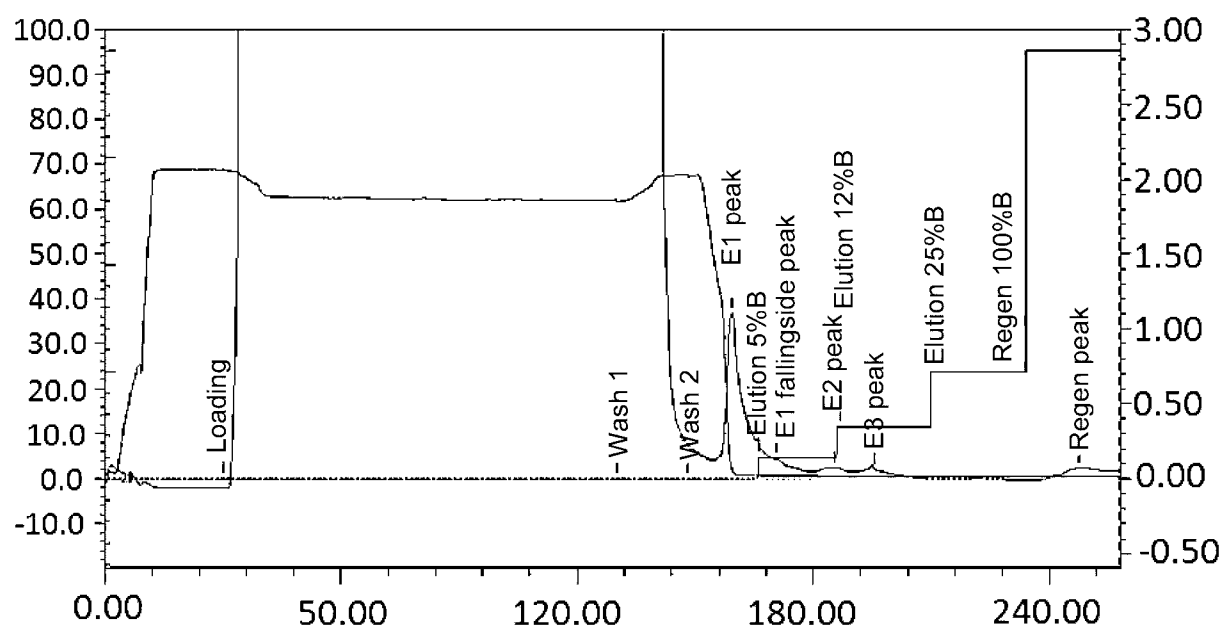


Figure 22

A



B

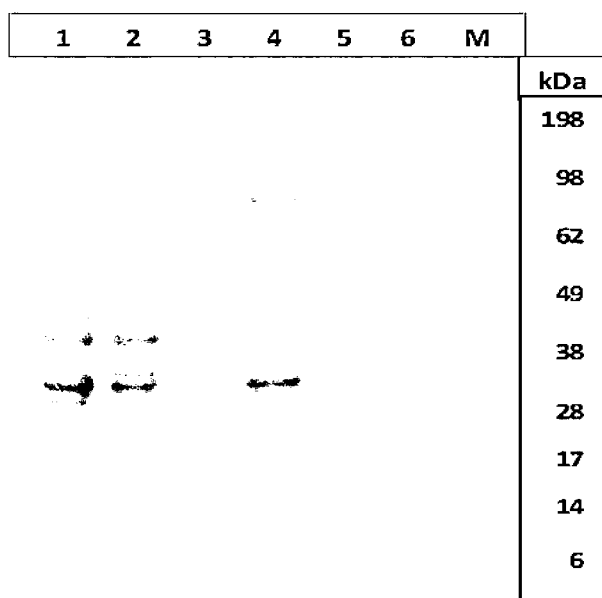
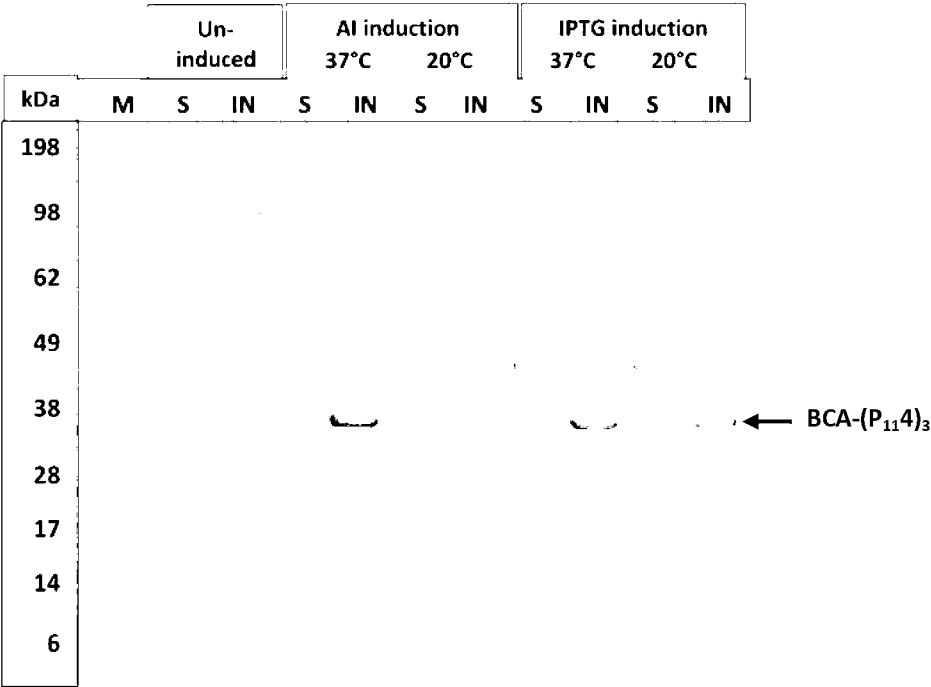


Figure 23

A



B

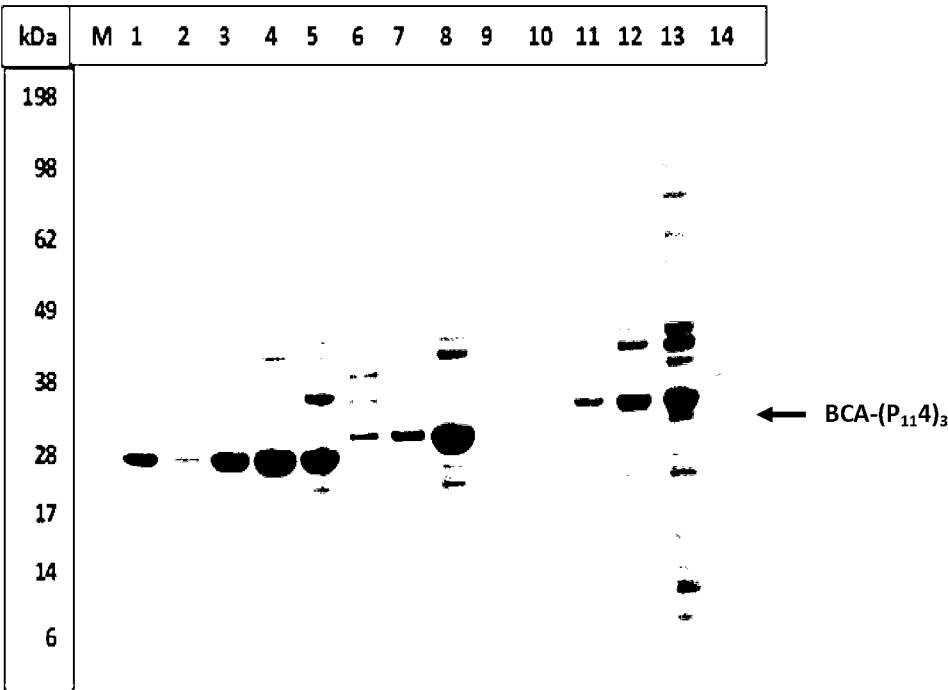


Figure 24

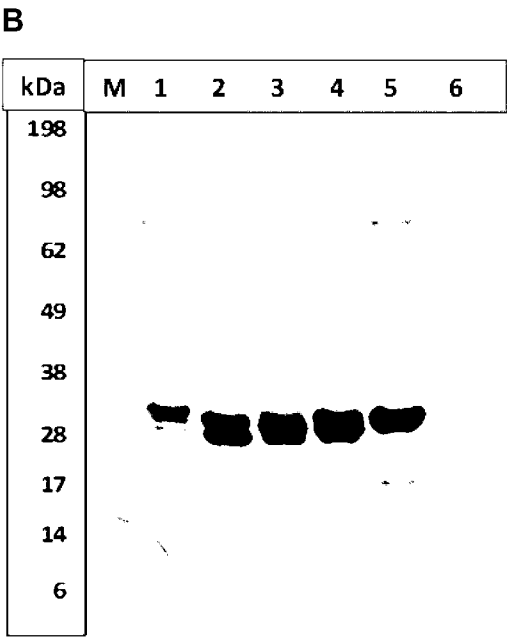
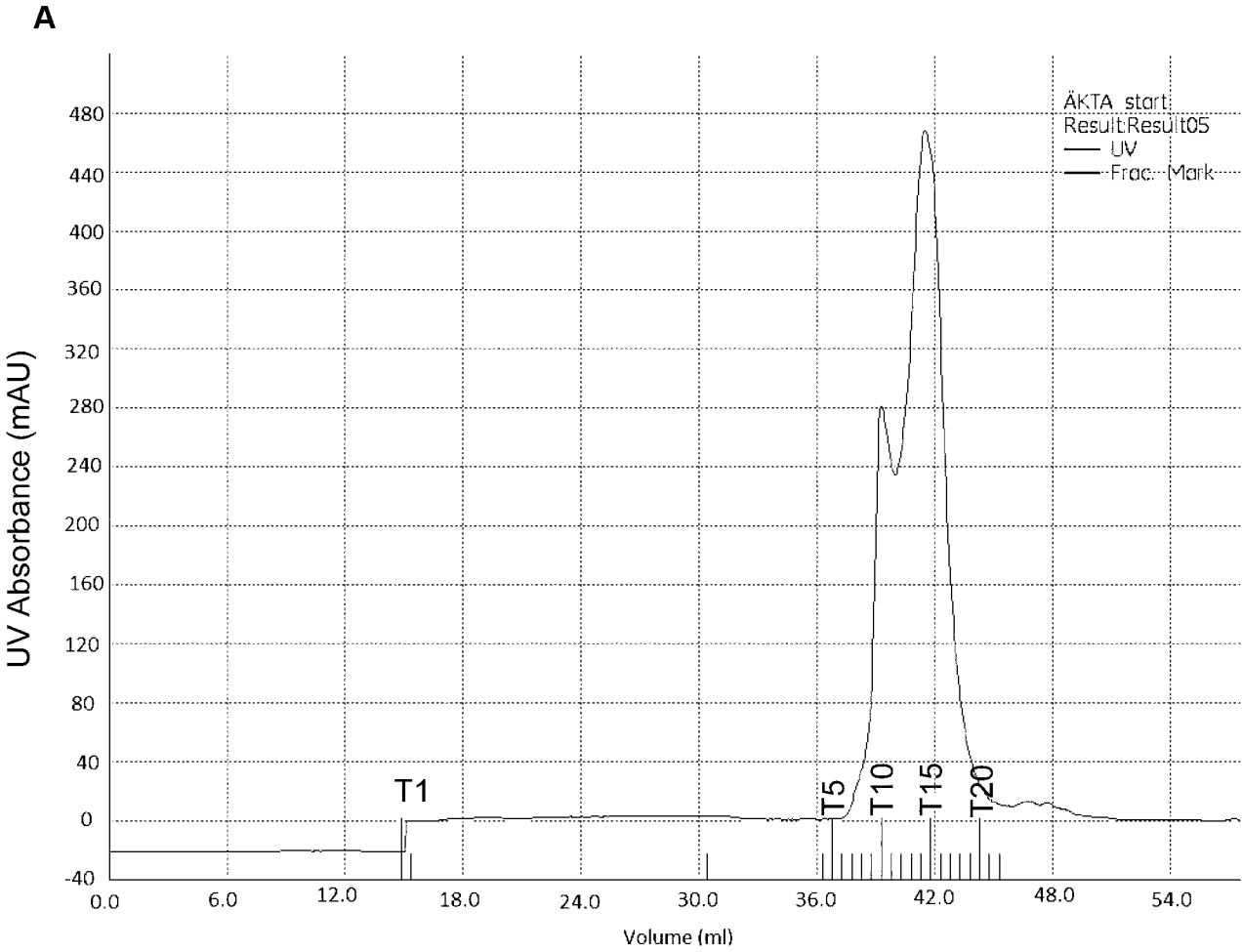
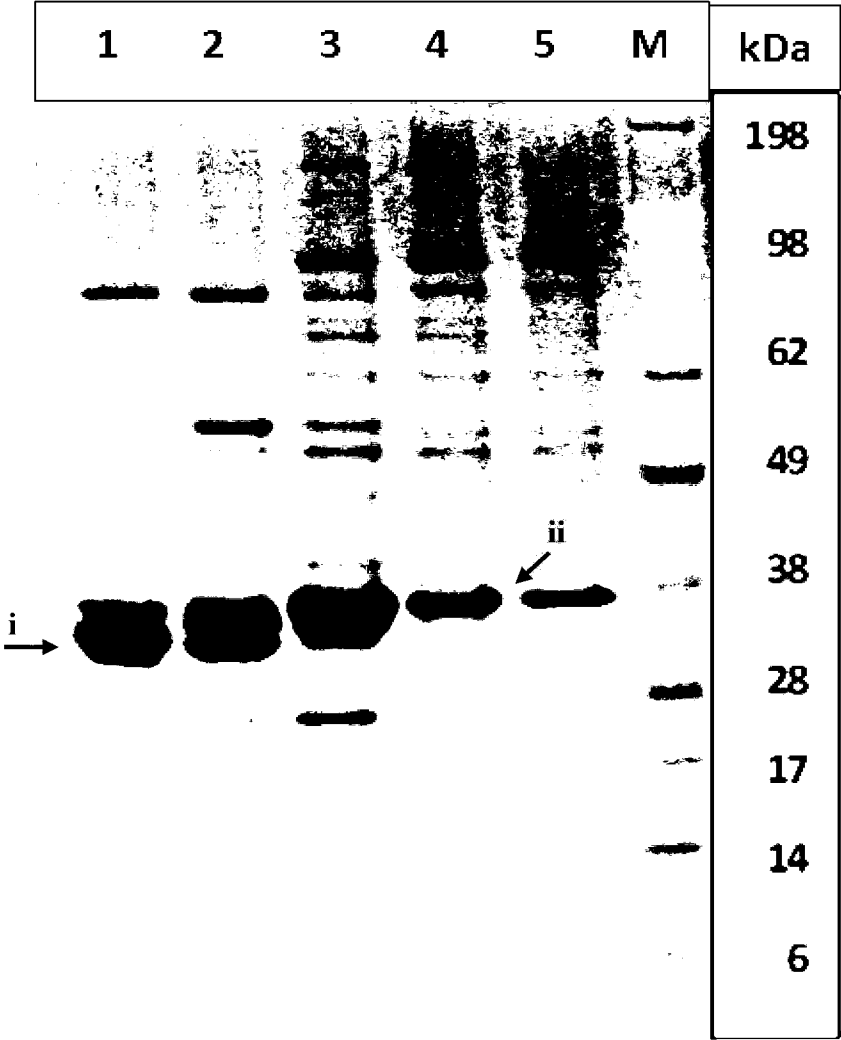


Figure 24

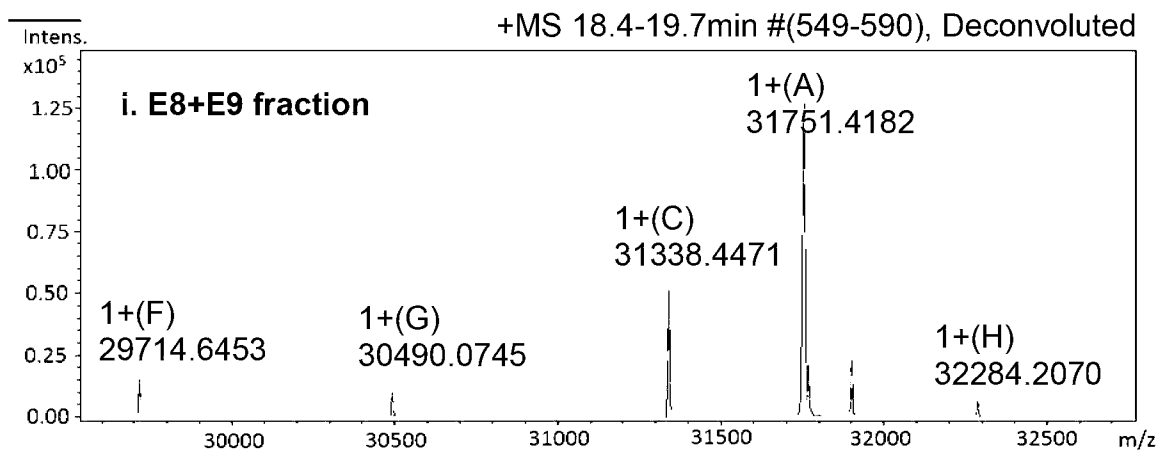
C



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Figure 25

A



B

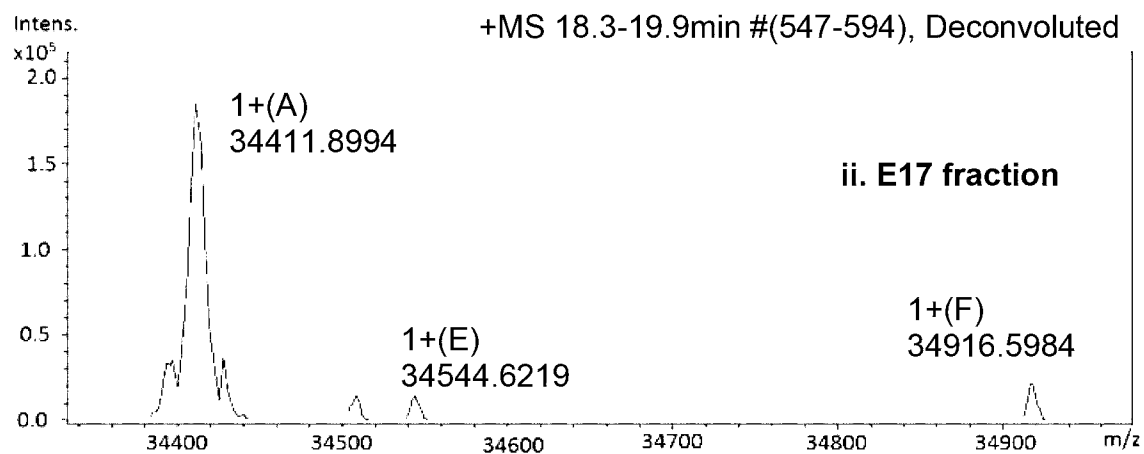
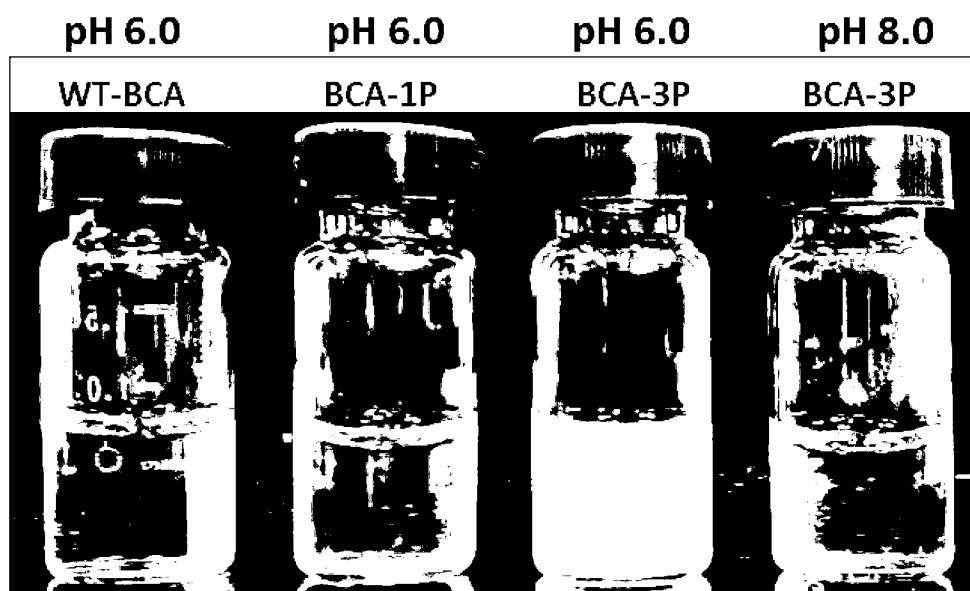


Figure 26

A



B

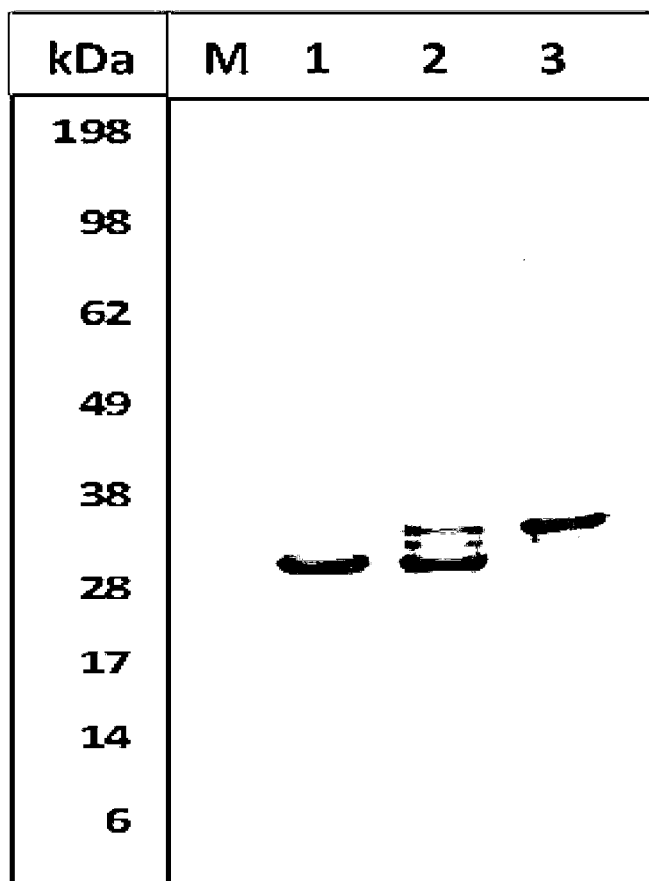


Figure 27

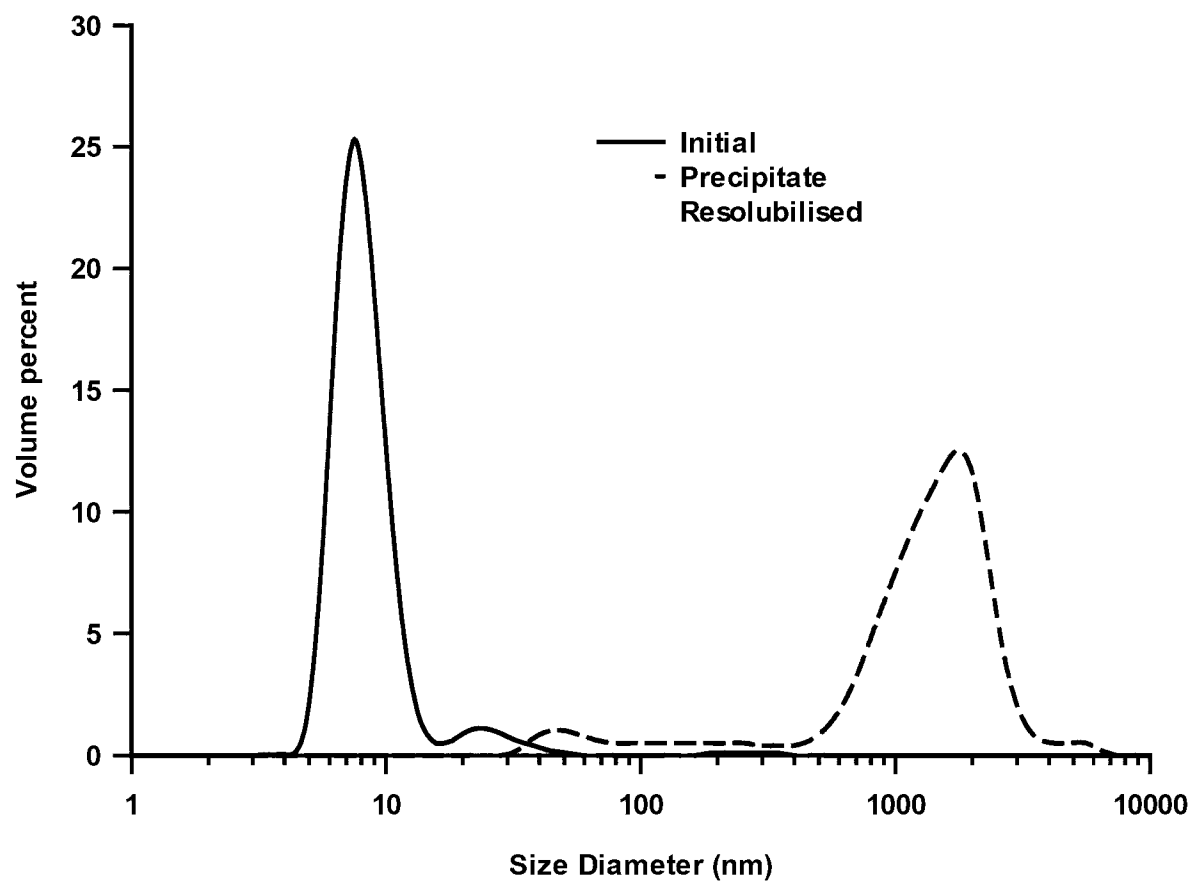


Figure 28

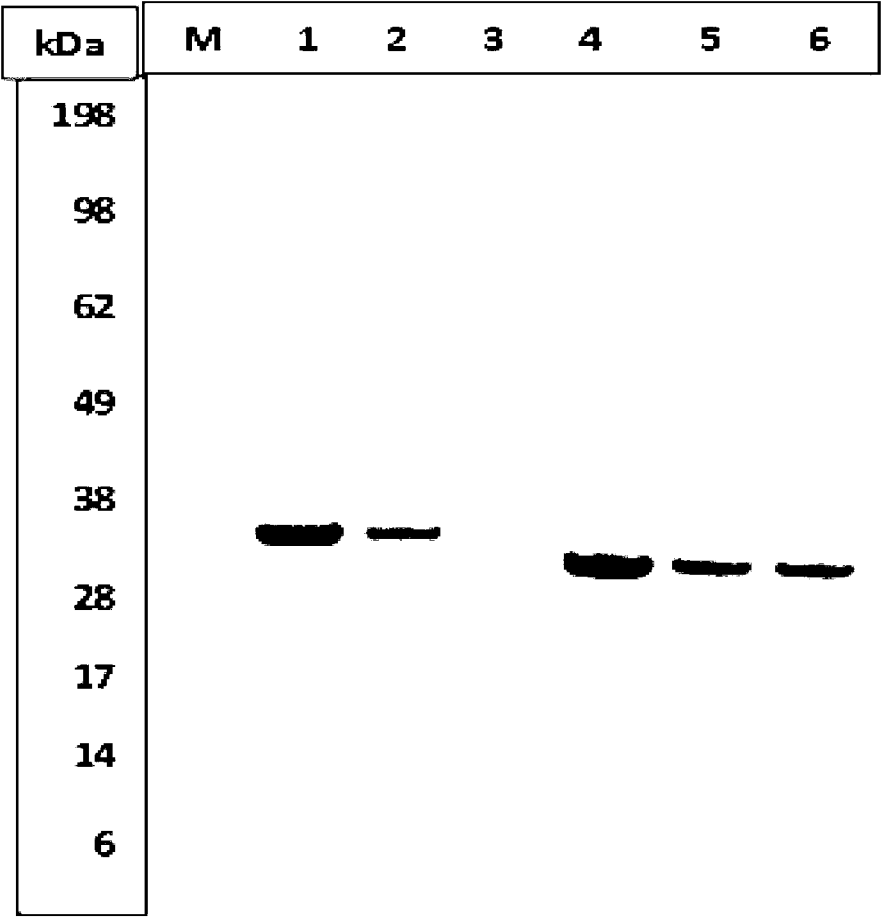
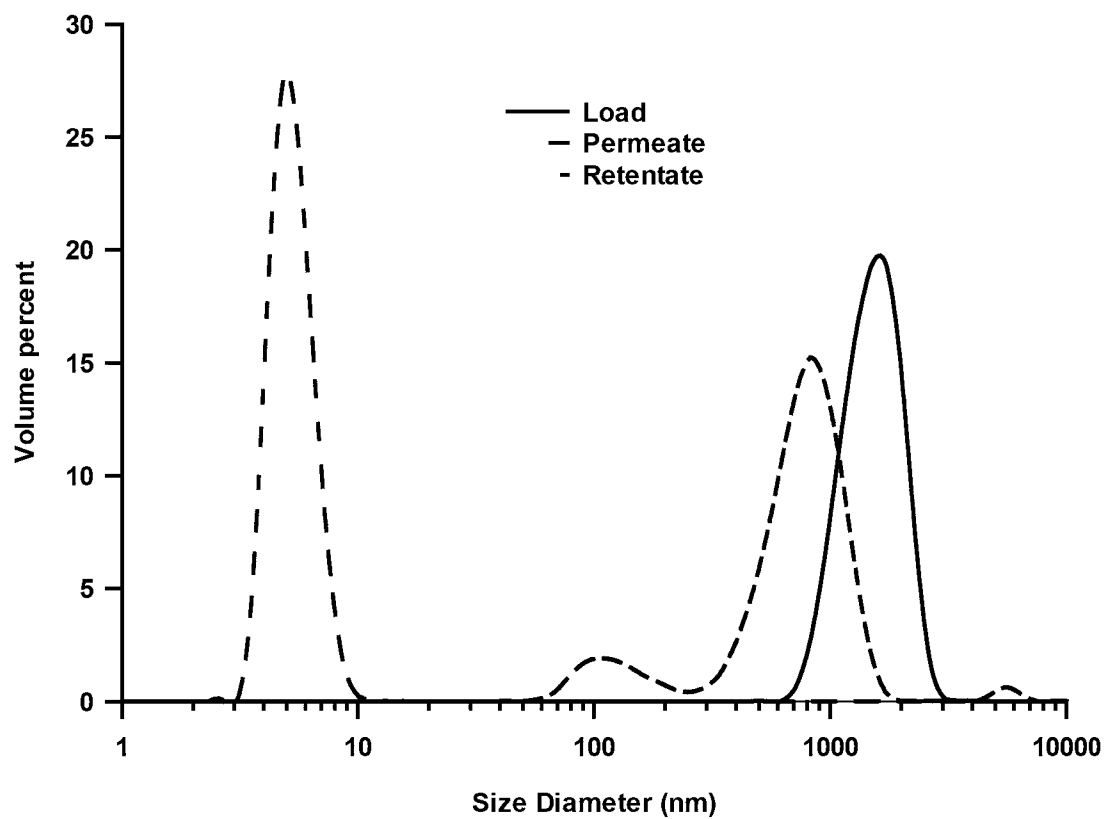


Figure 29

A



B

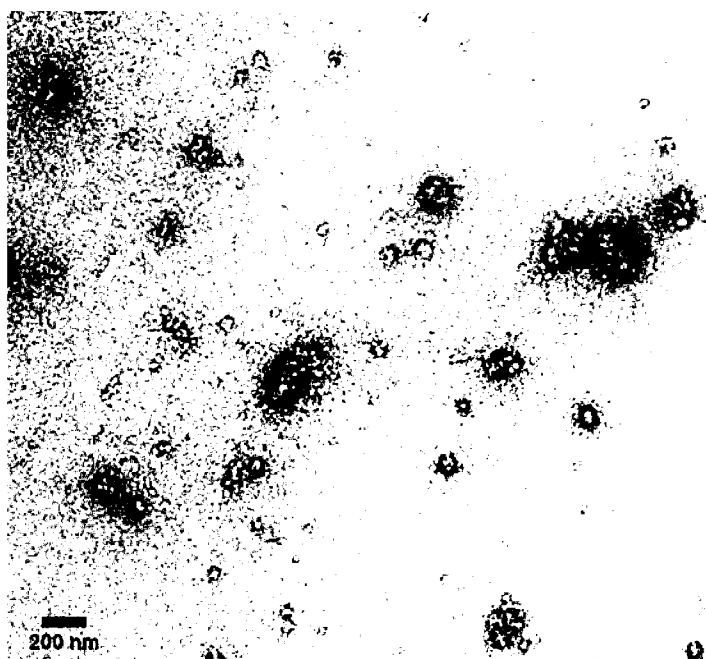


Figure 30

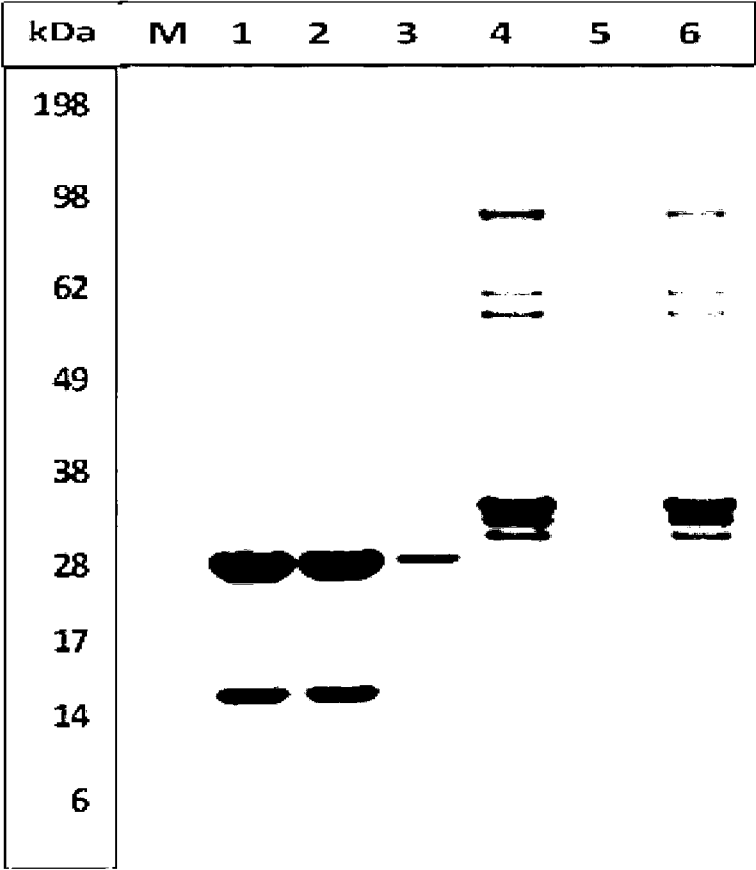


Figure 31

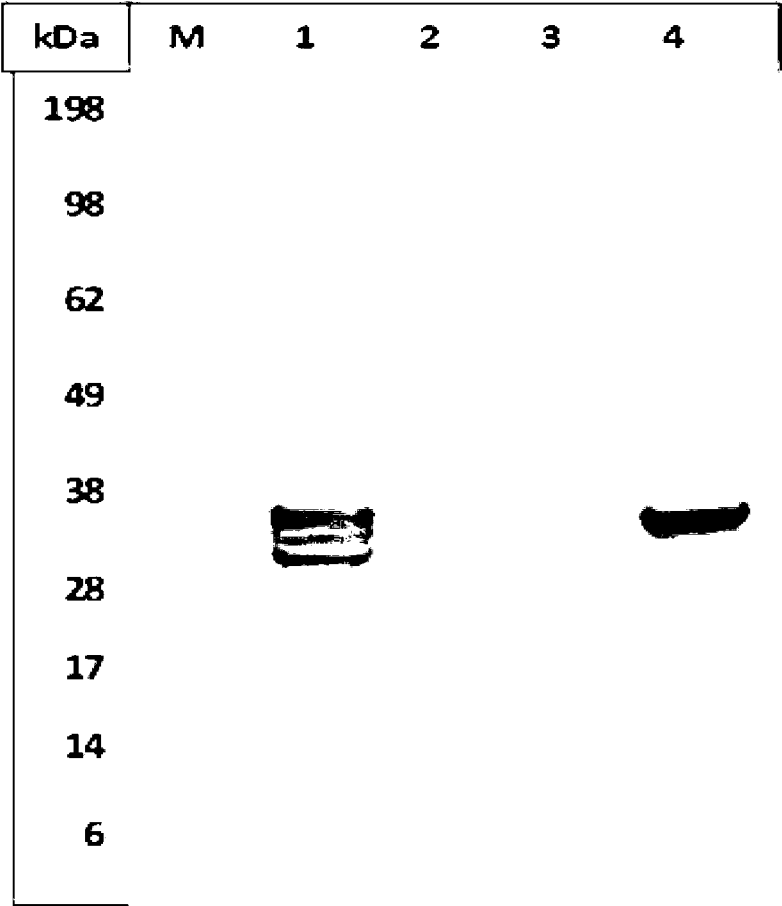
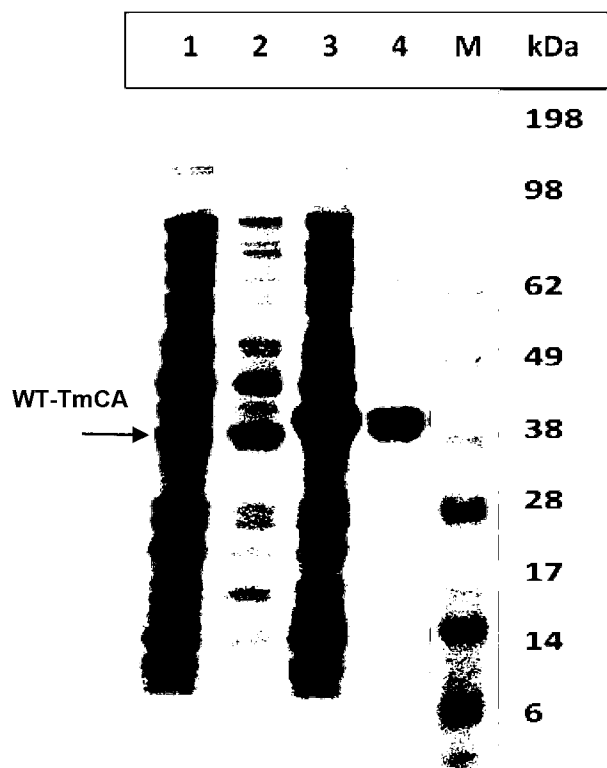


Figure 32

A



B

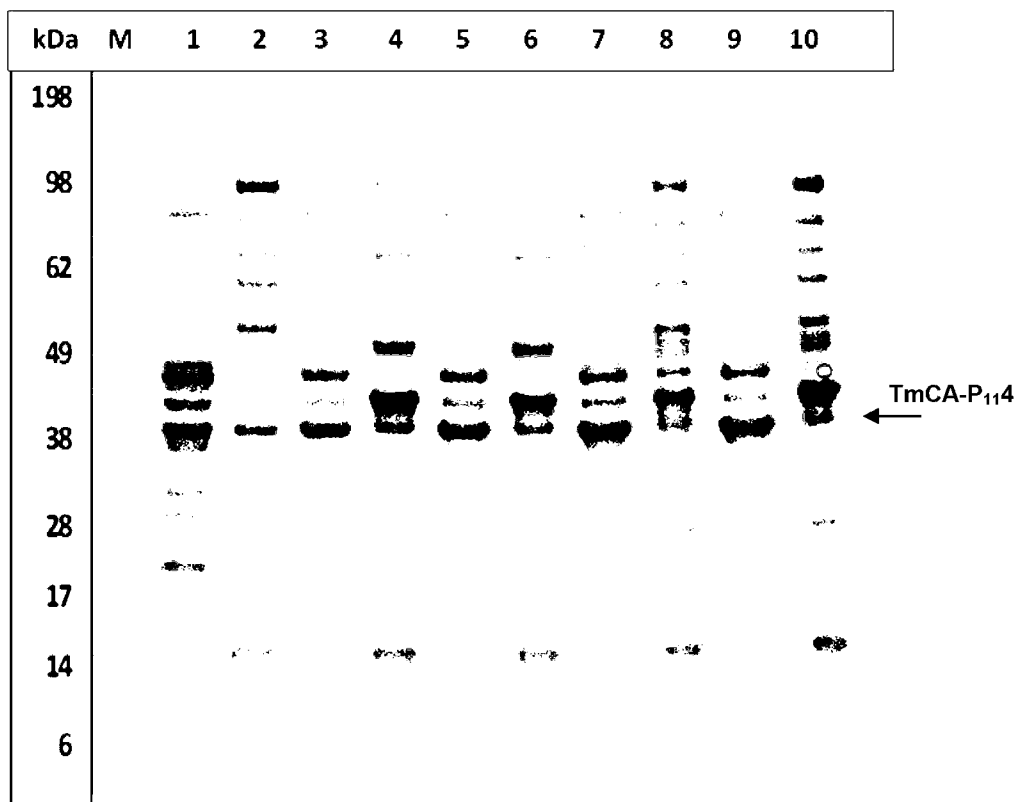
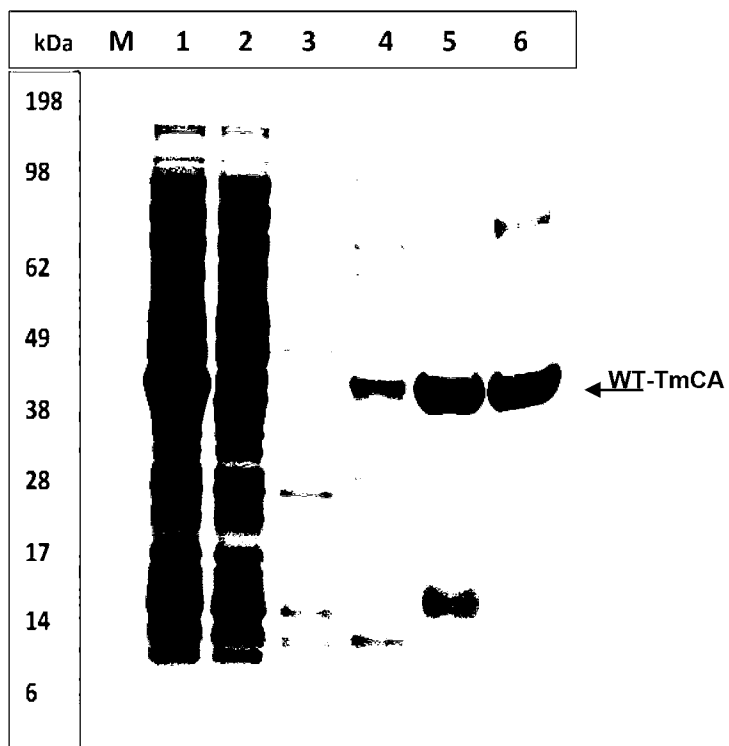
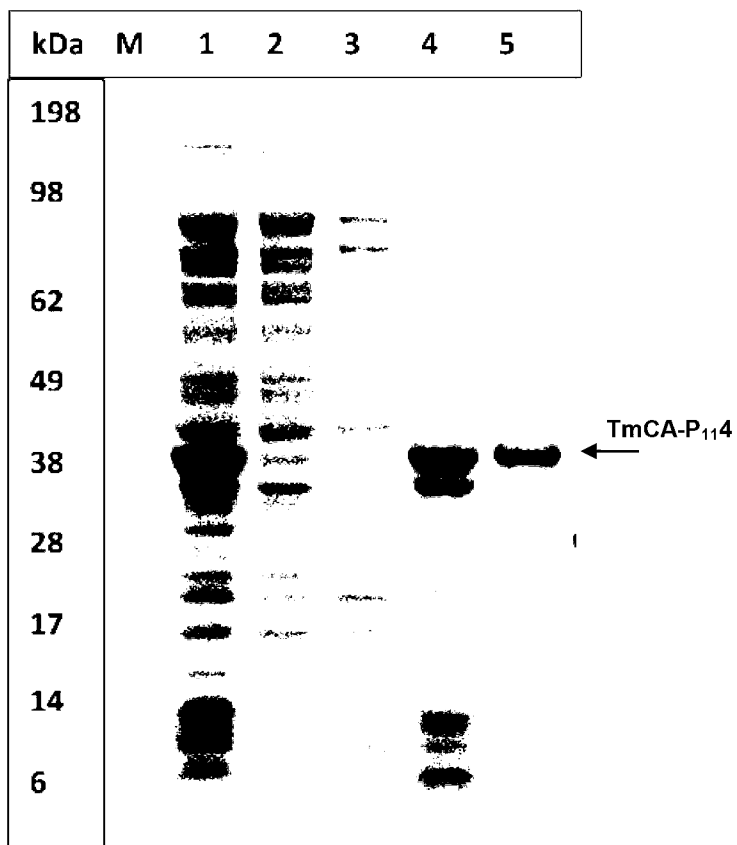


Figure 33

A



B



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Figure 34

A – WT-TmCA-

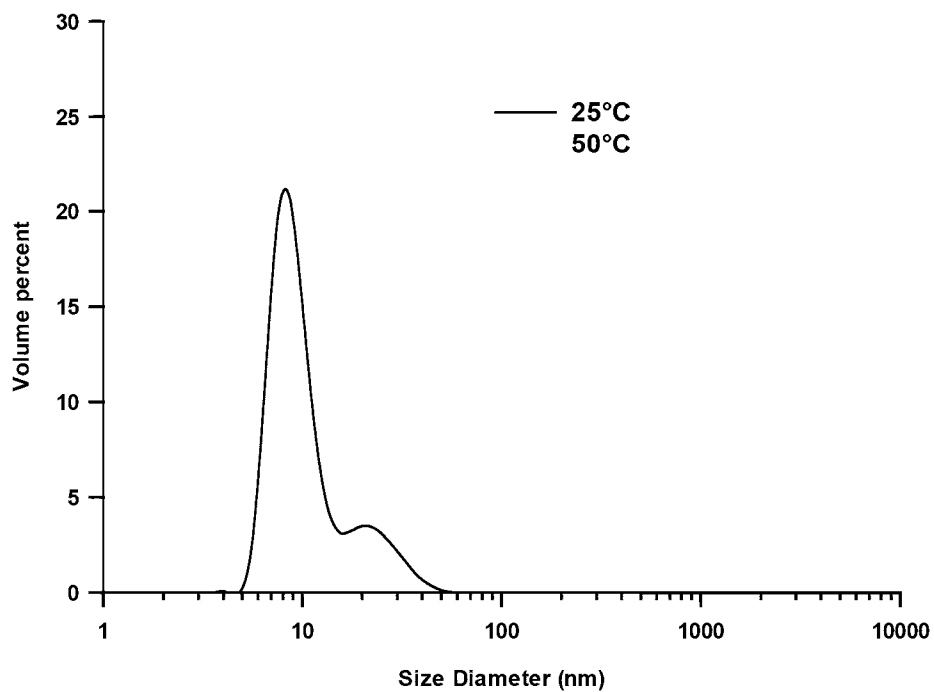
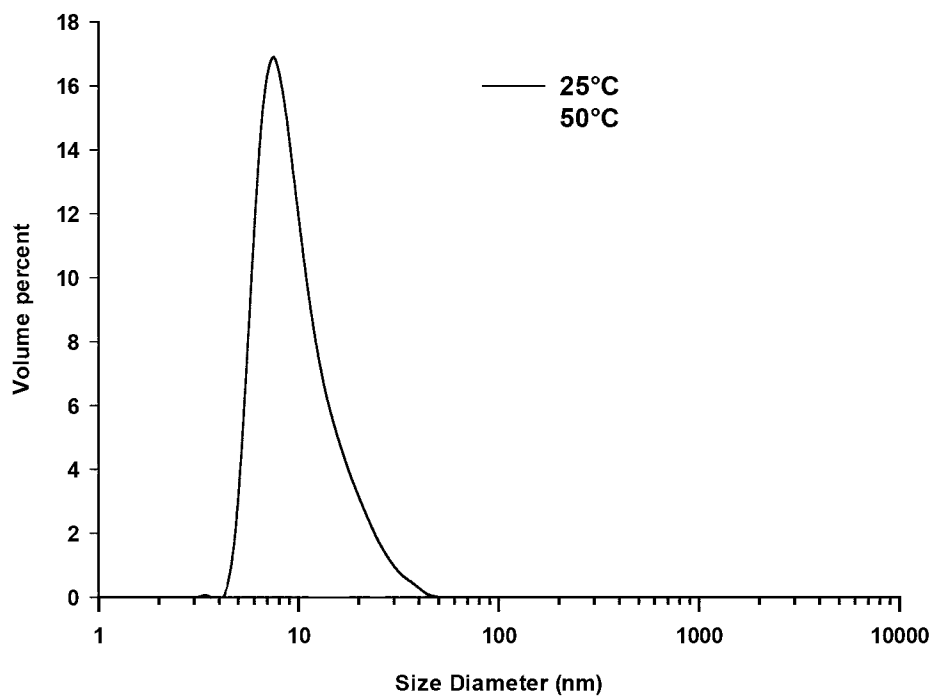
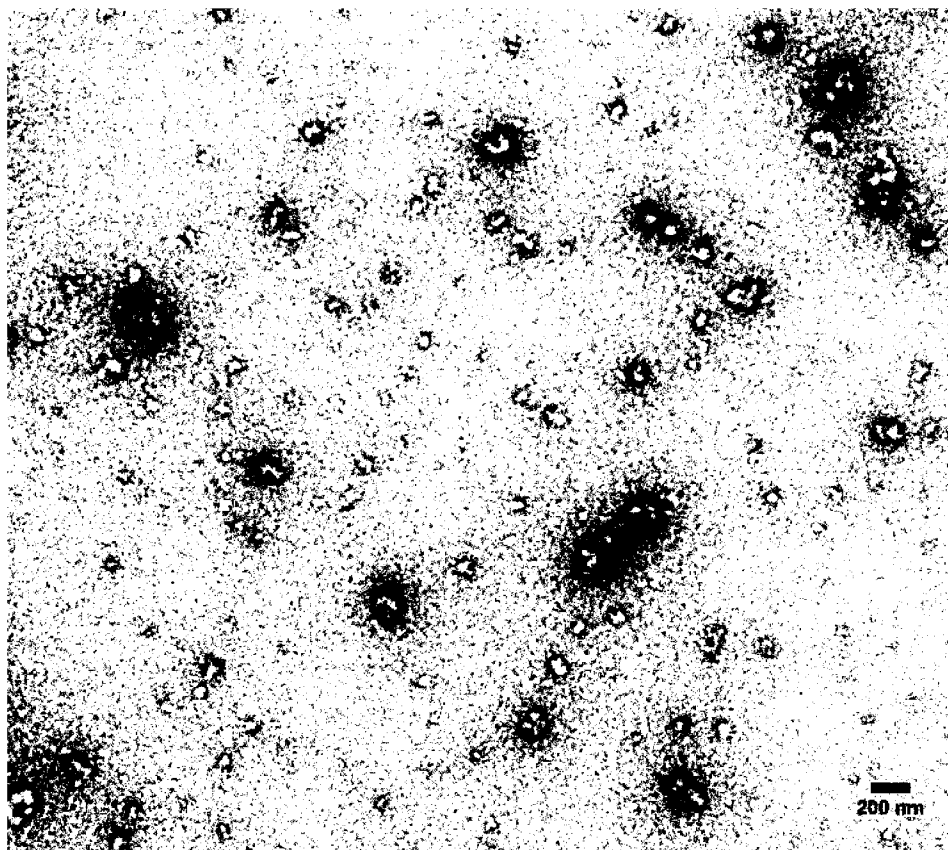
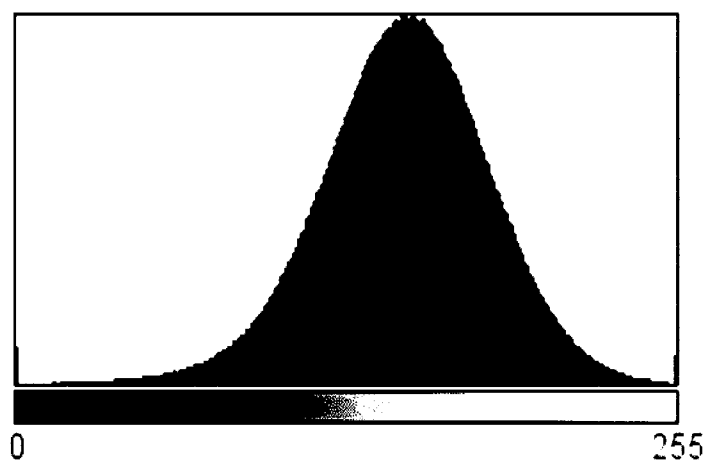
B – TmCA-P₁₁4

Figure 35

A



B



Count: 3234690

Min: 0

Mean: 149.646

Max: 255

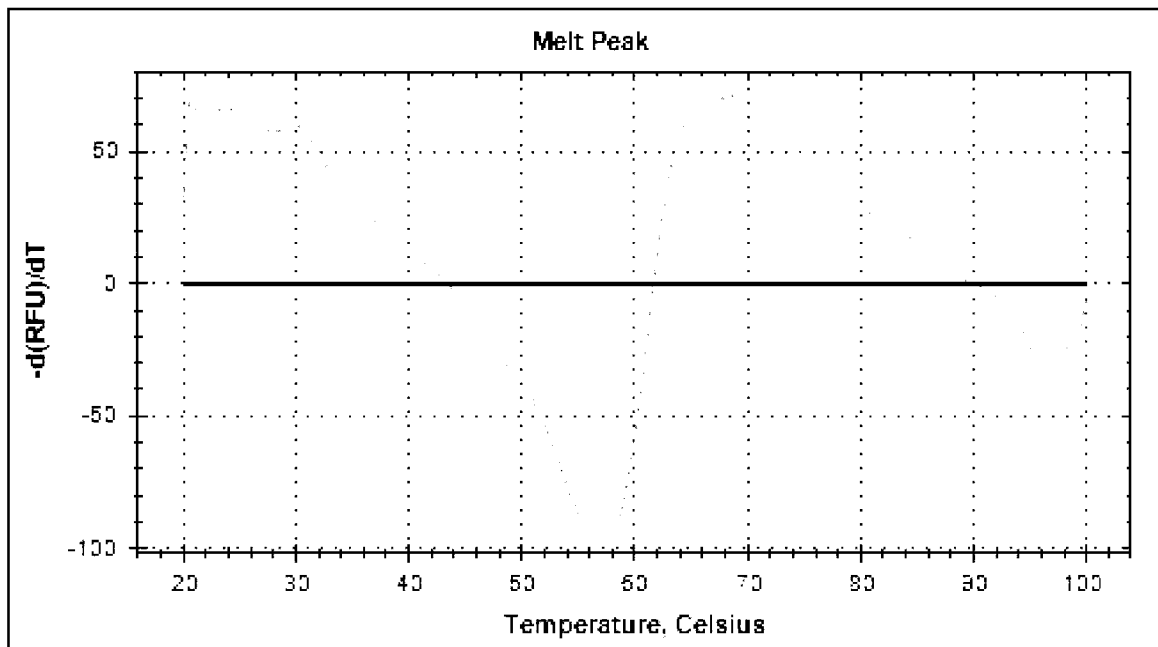
StdDev: 34.281

Mode: 153 (40816)

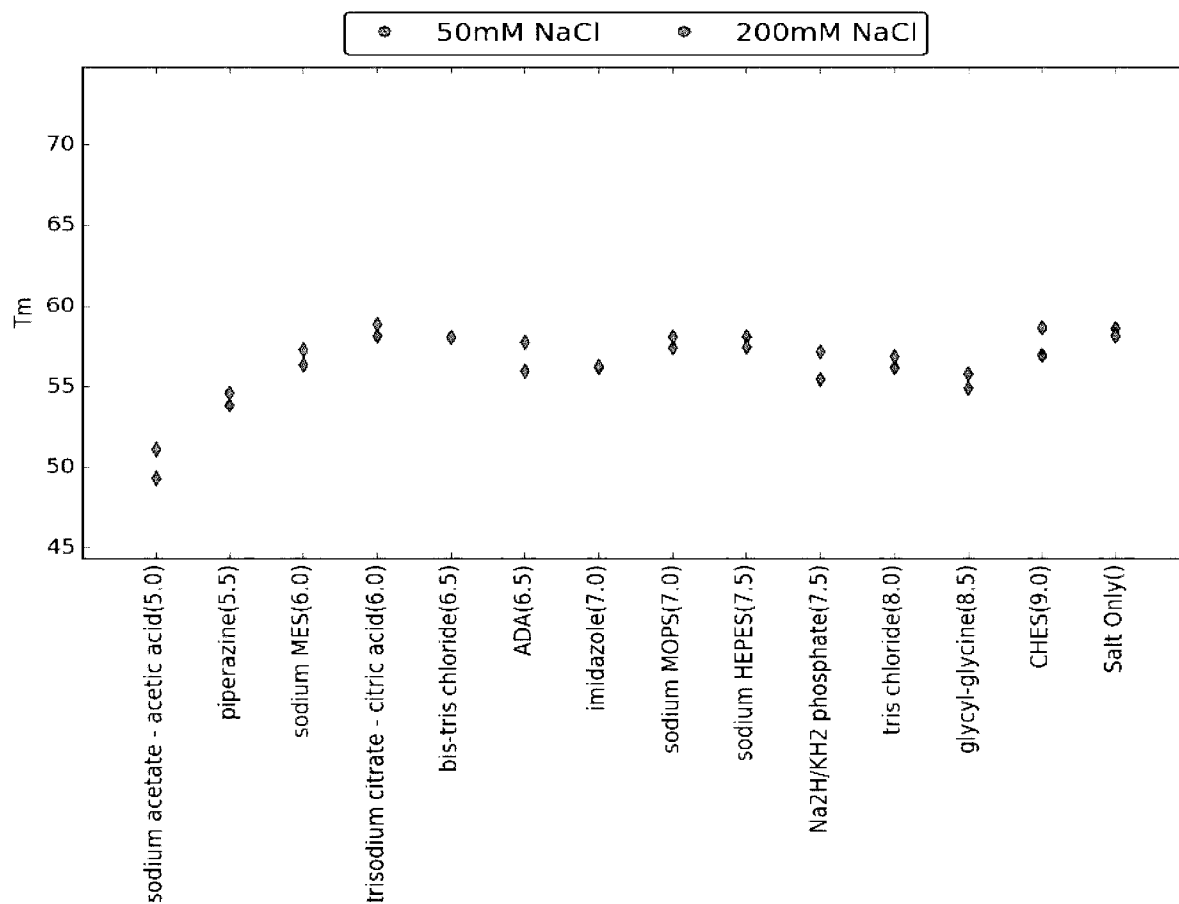
40/55

Figure 36

A - WT-TmCA



B - WT-TmCA



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Figure 36

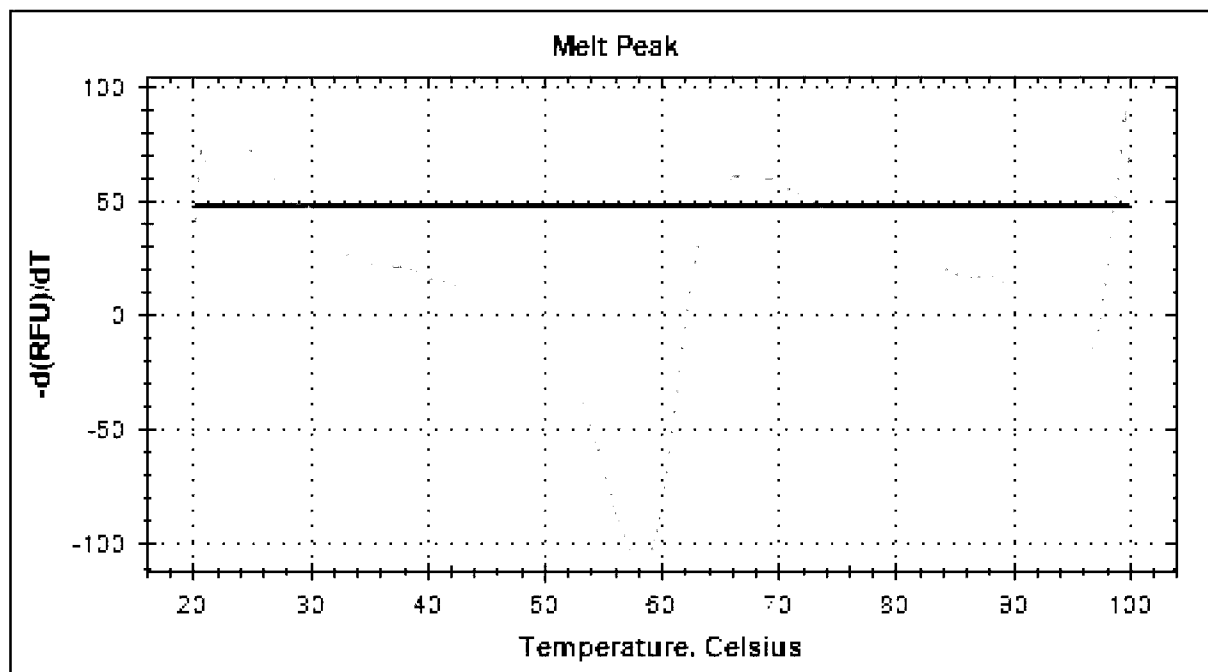
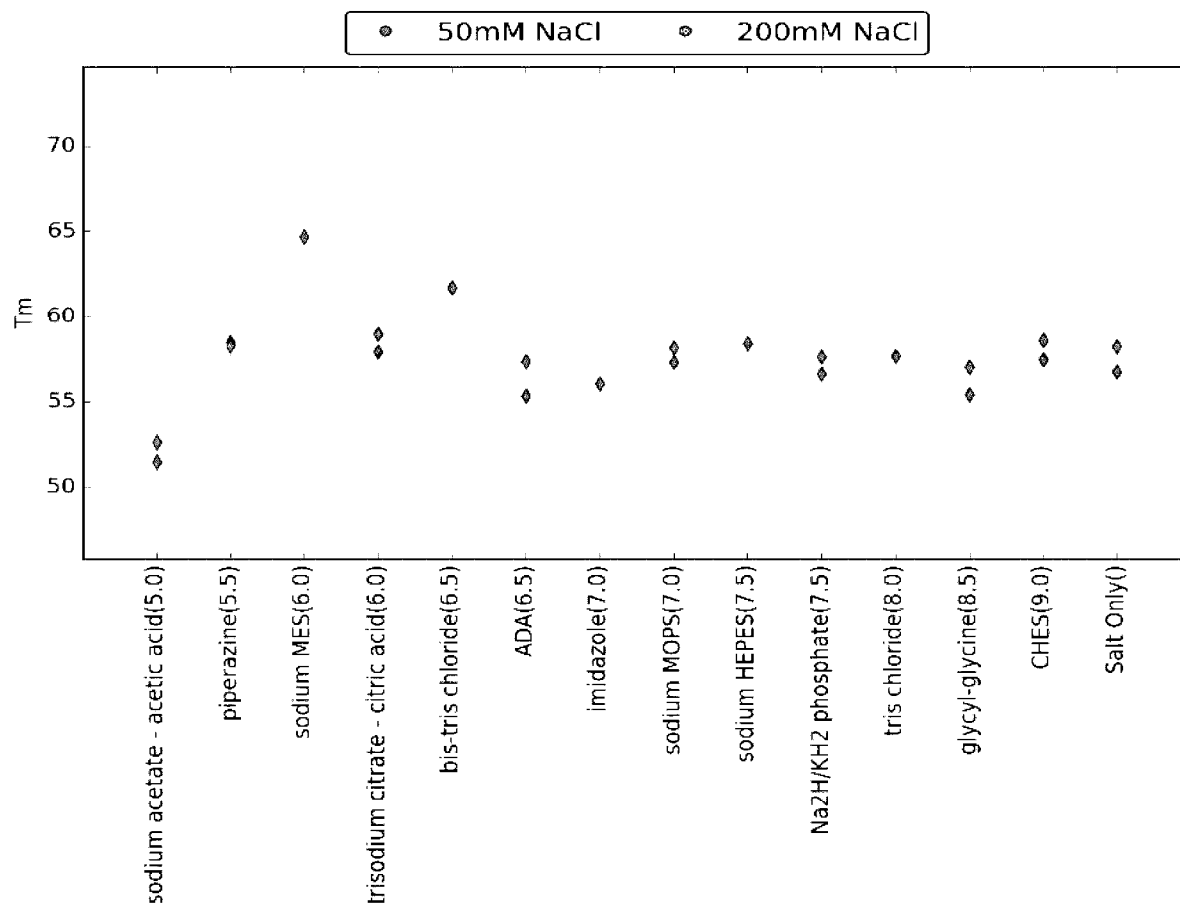
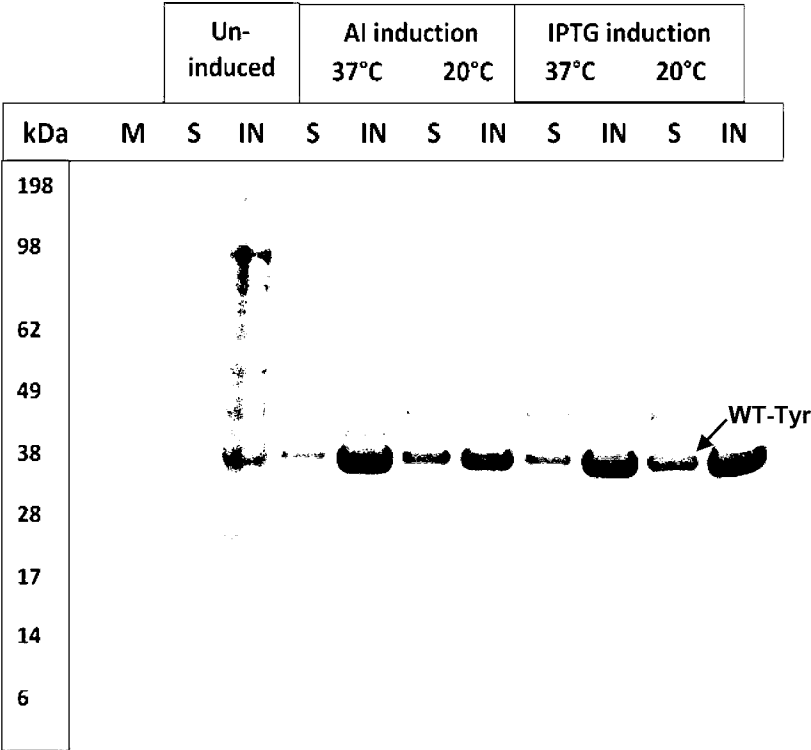
C - TmCA-P₁₁4D - TmCA-P₁₁4

Figure 37

A



B

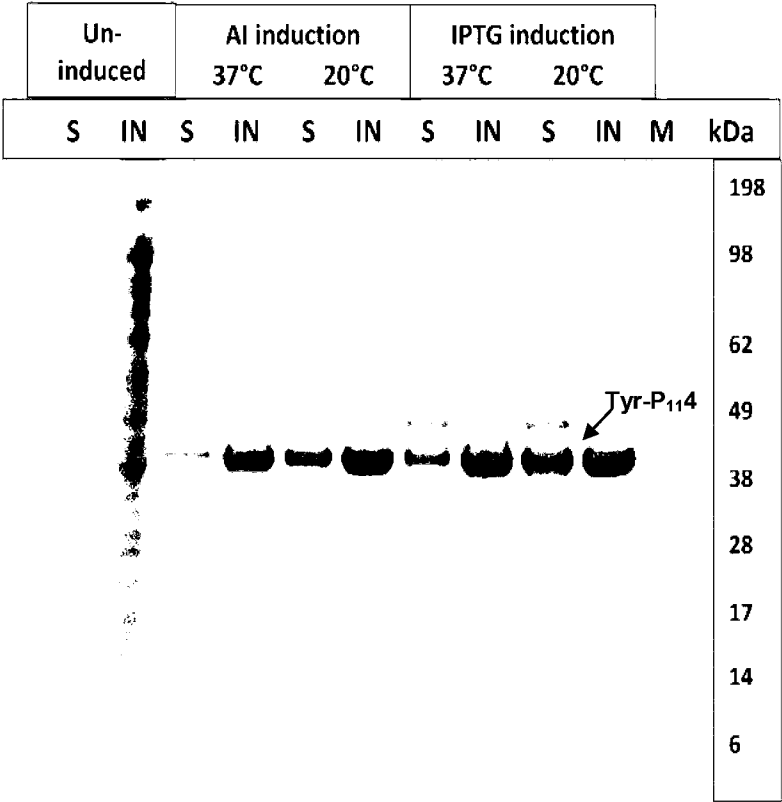
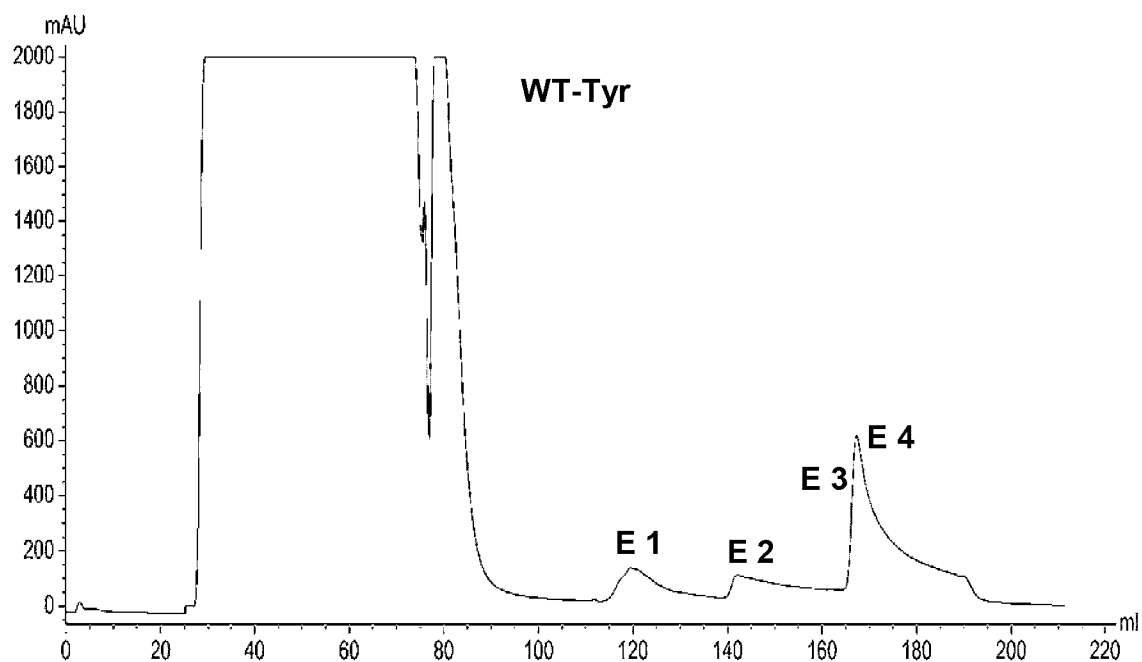


Figure 38

A



B

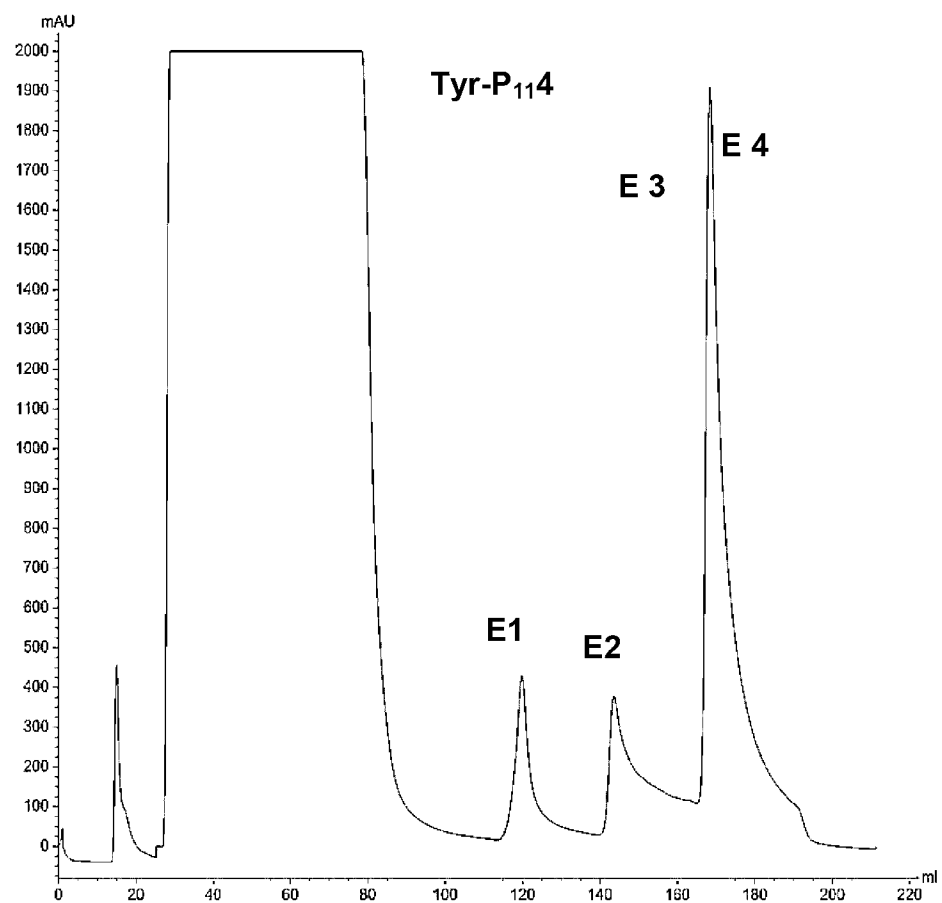
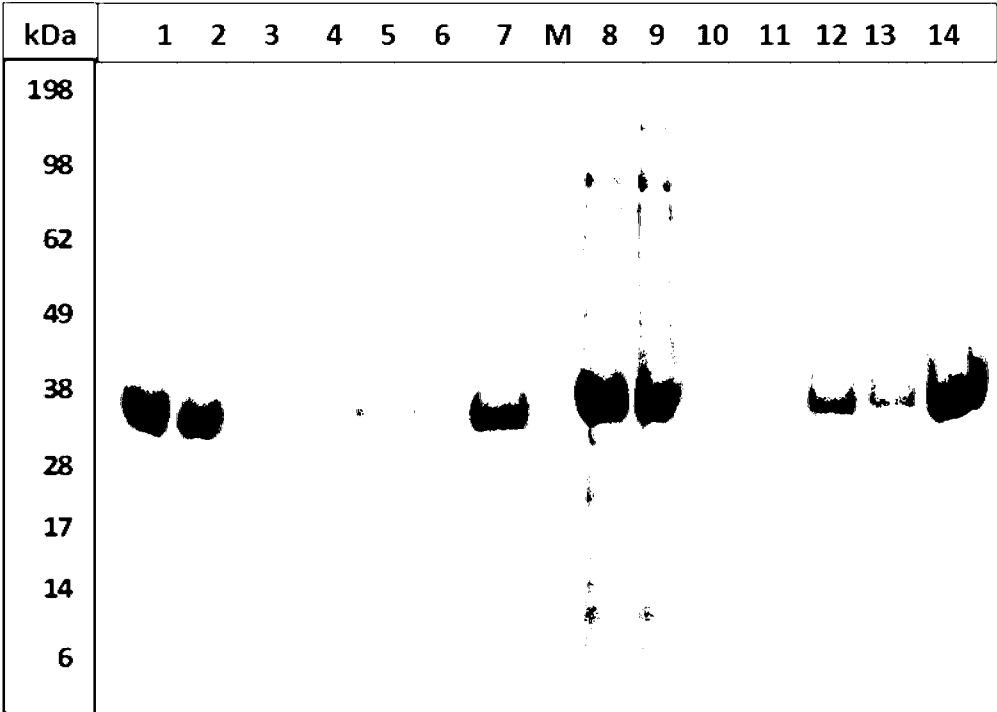


Figure 38

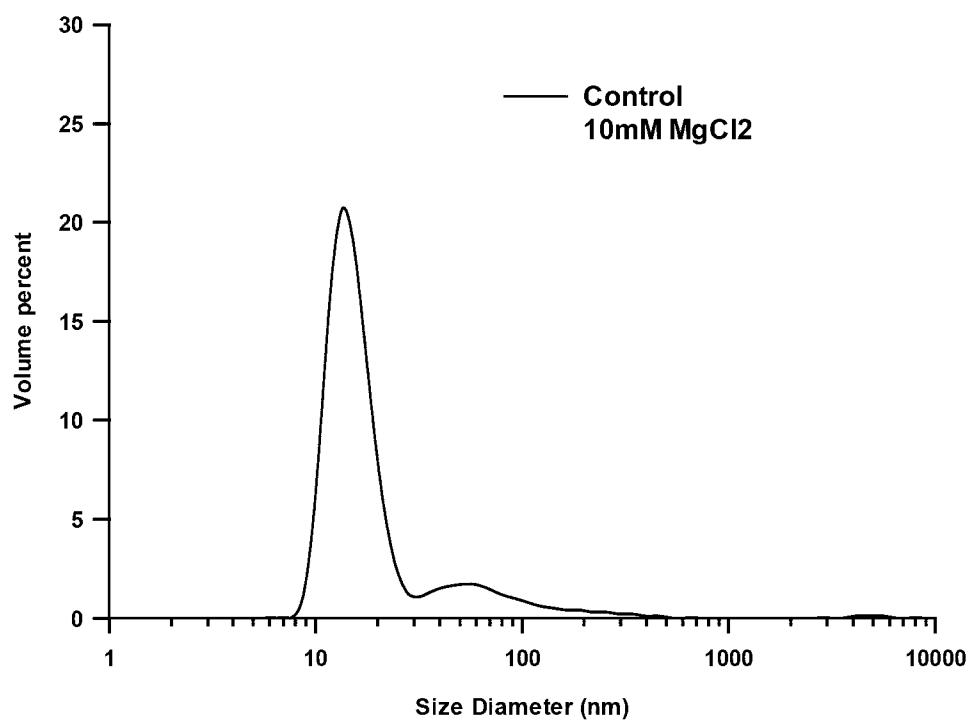
C



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Figure 39

A



B

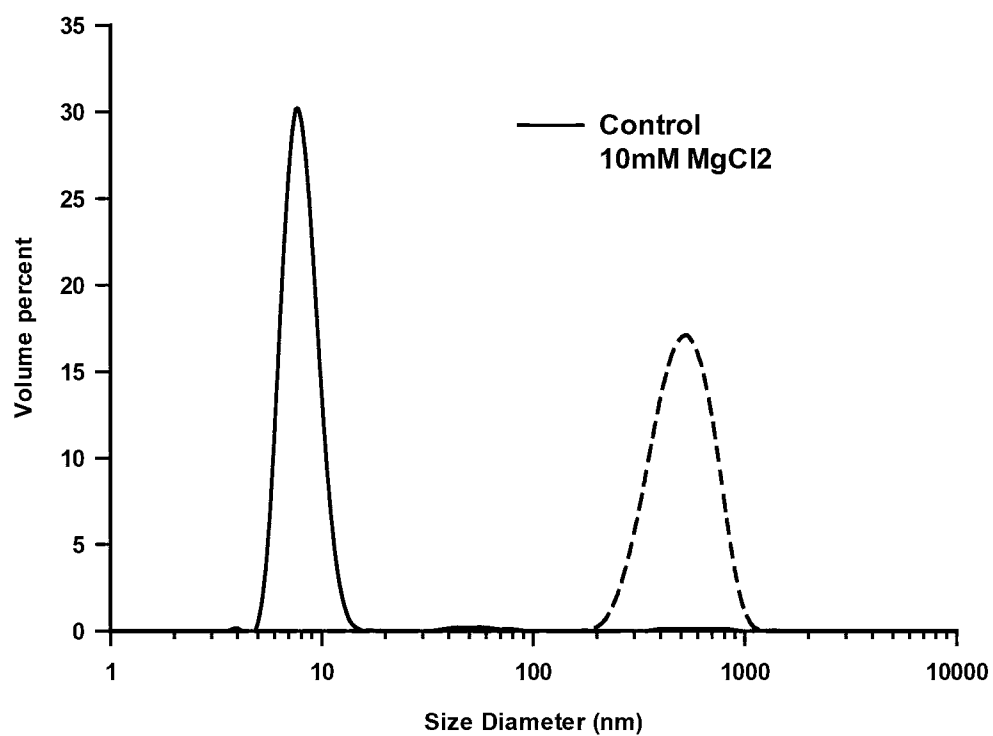
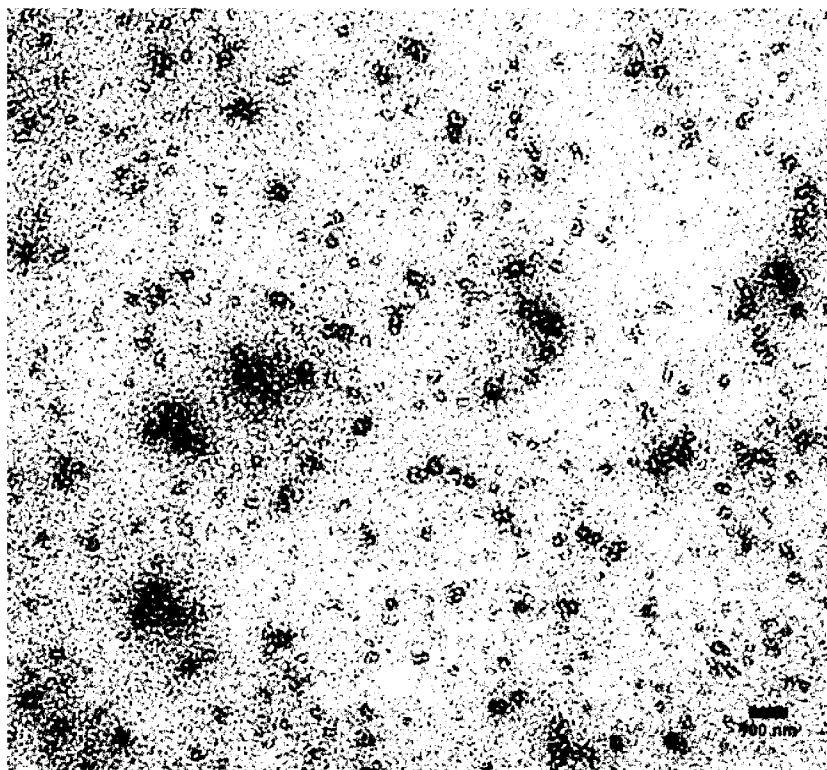


Figure 40

A



B

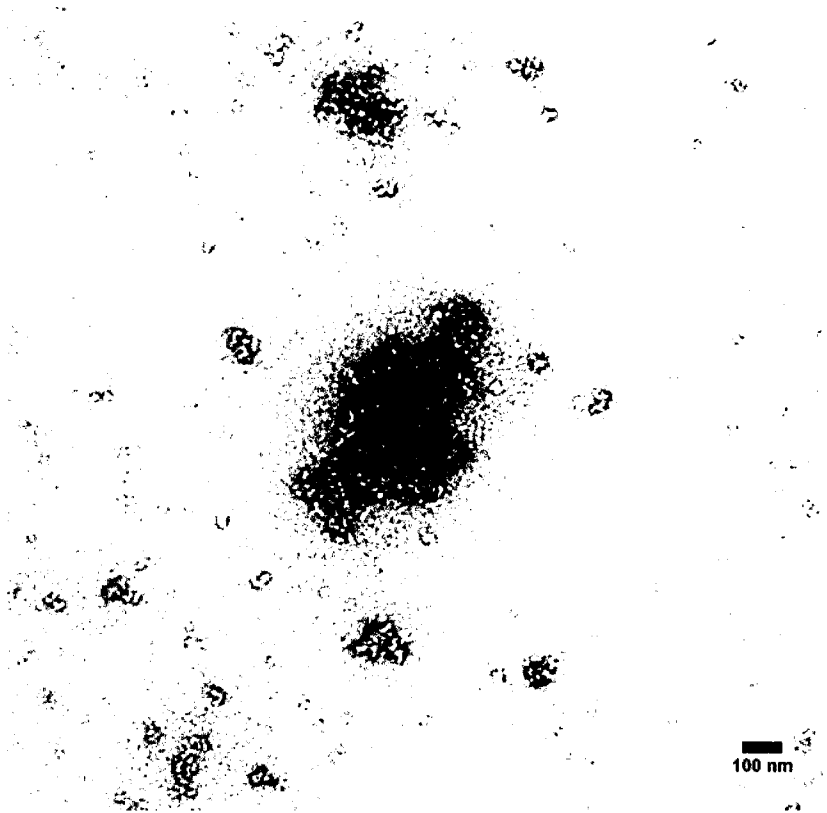
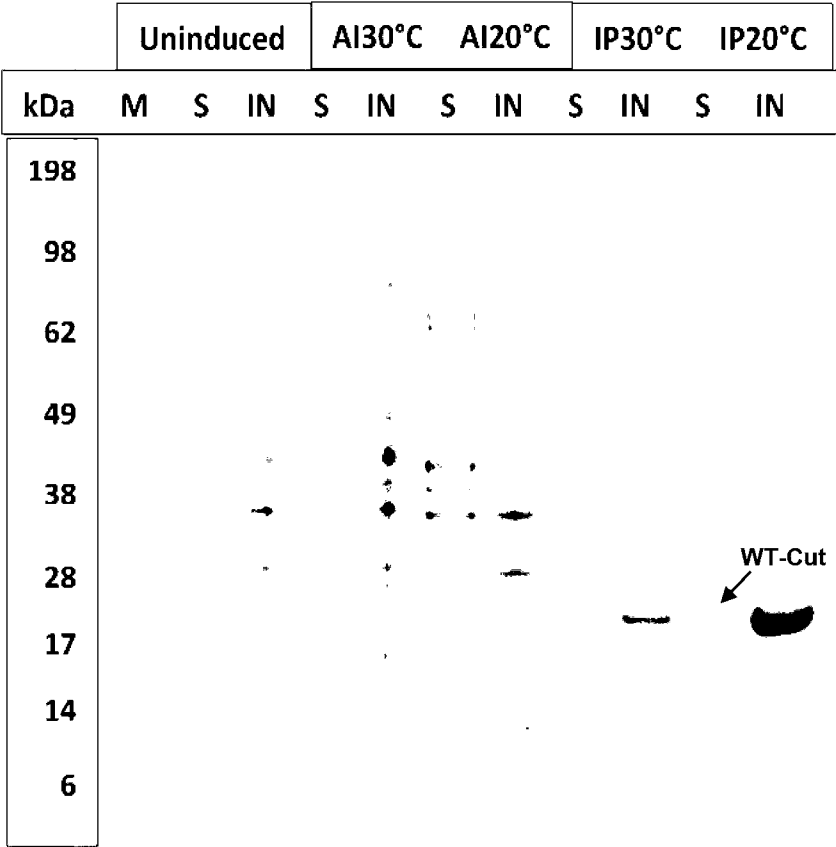


Figure 41

A



B

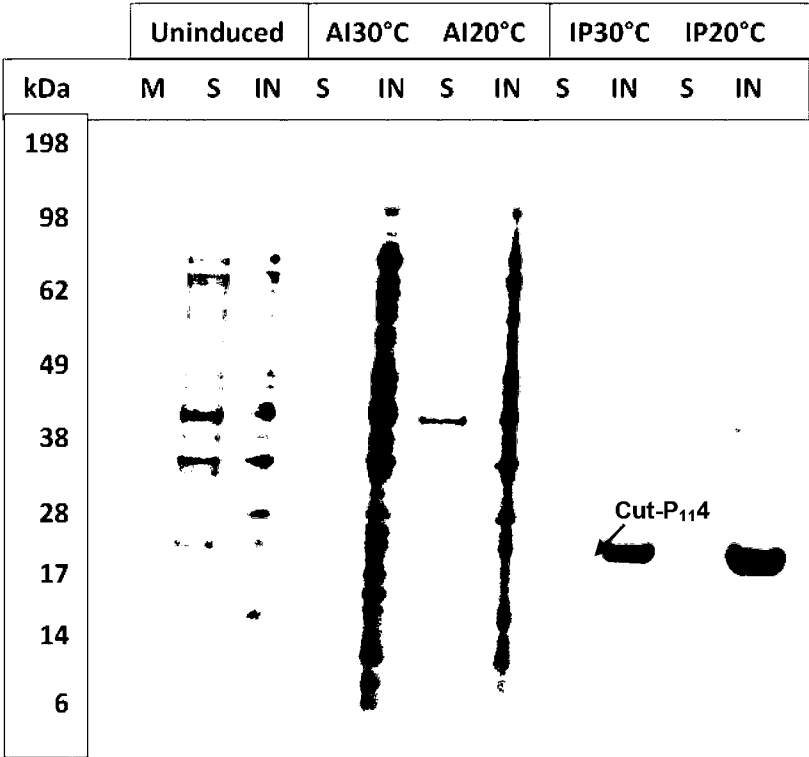


Figure 42

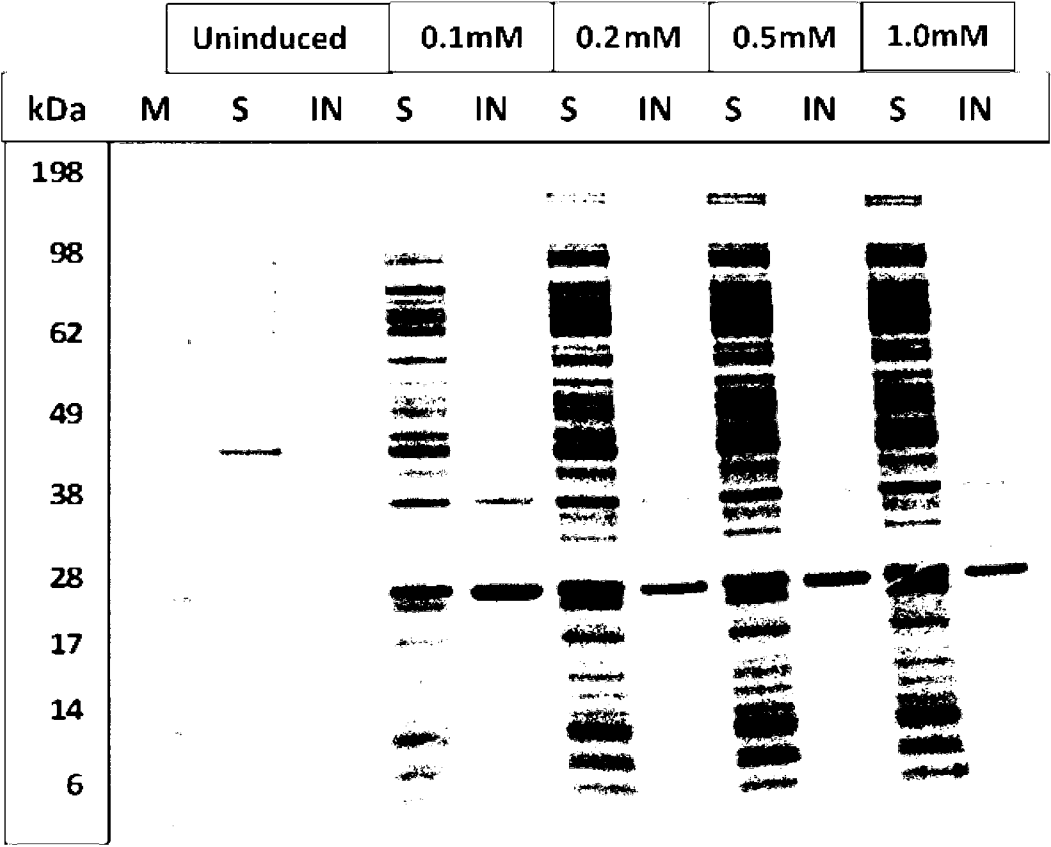
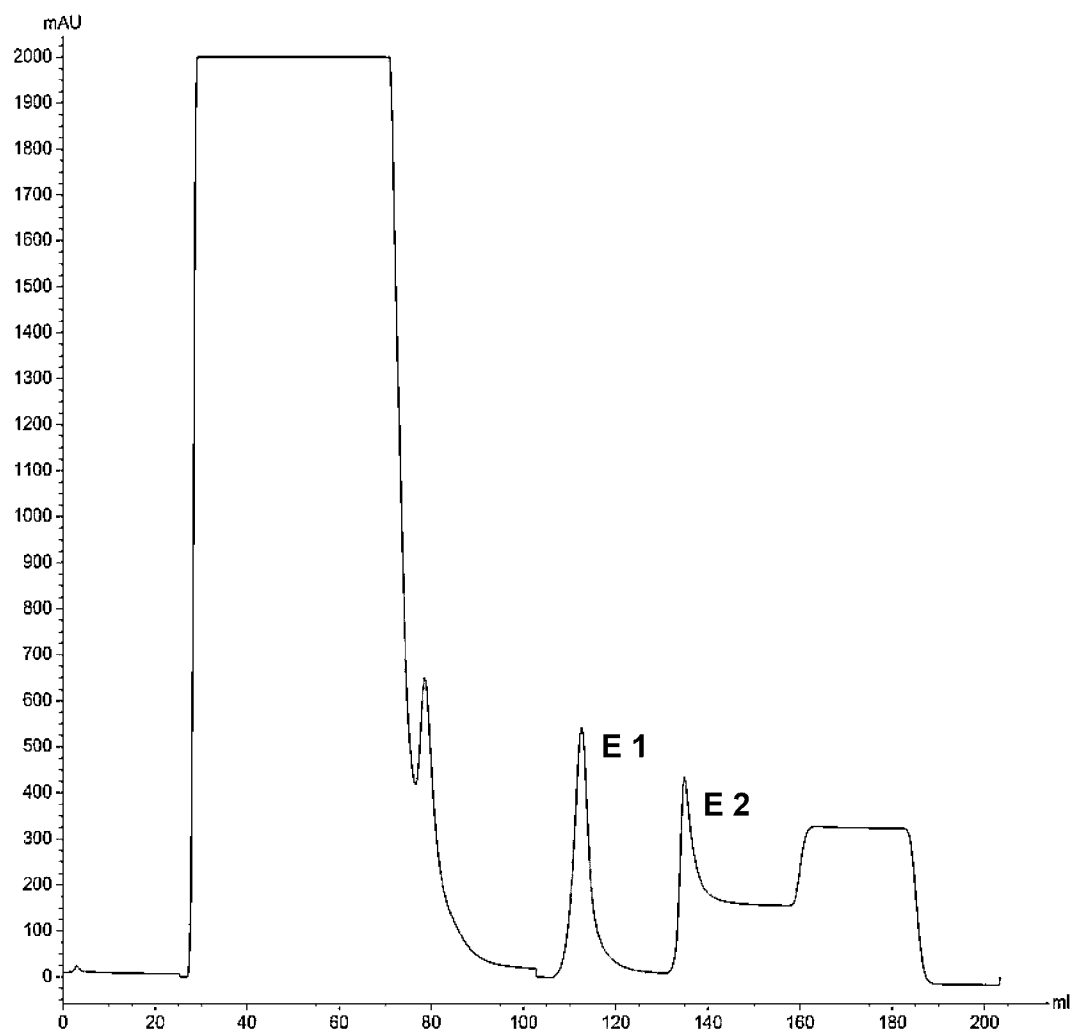
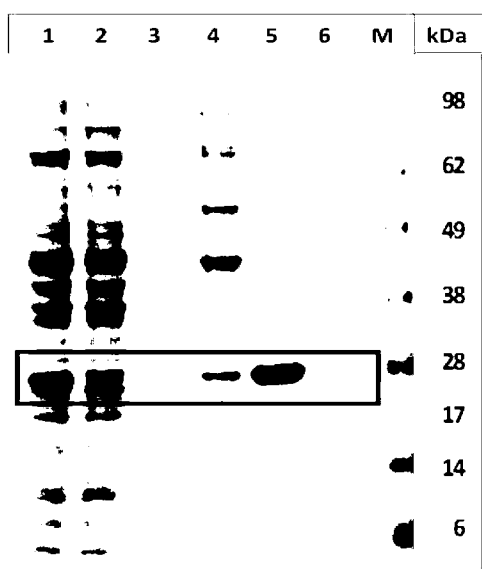


Figure 43

A



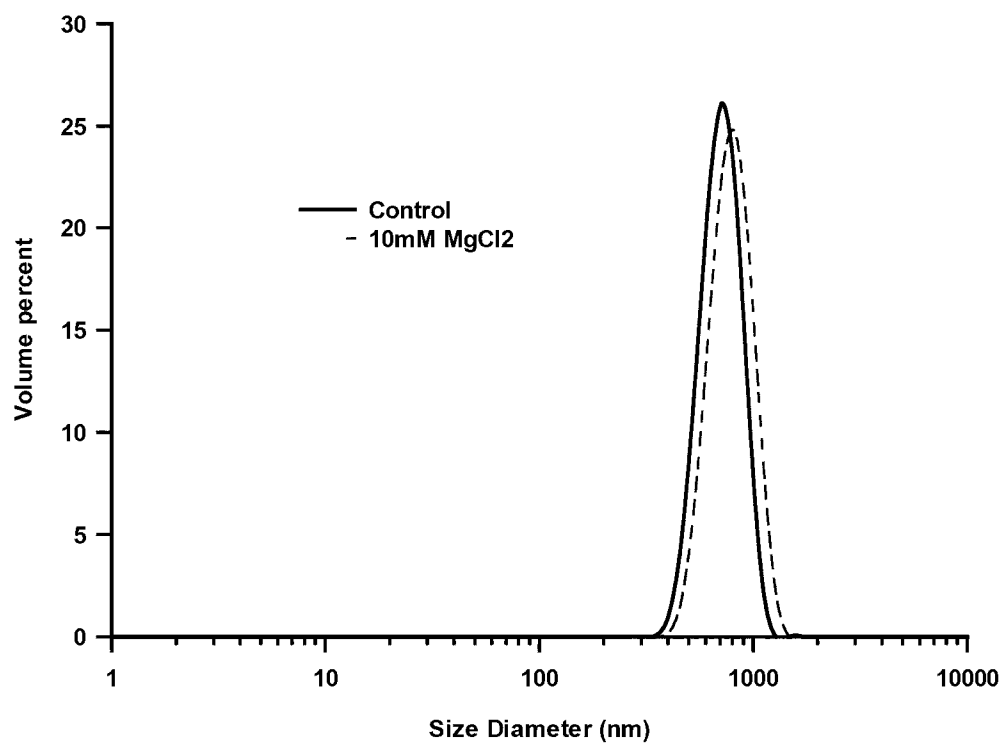
B



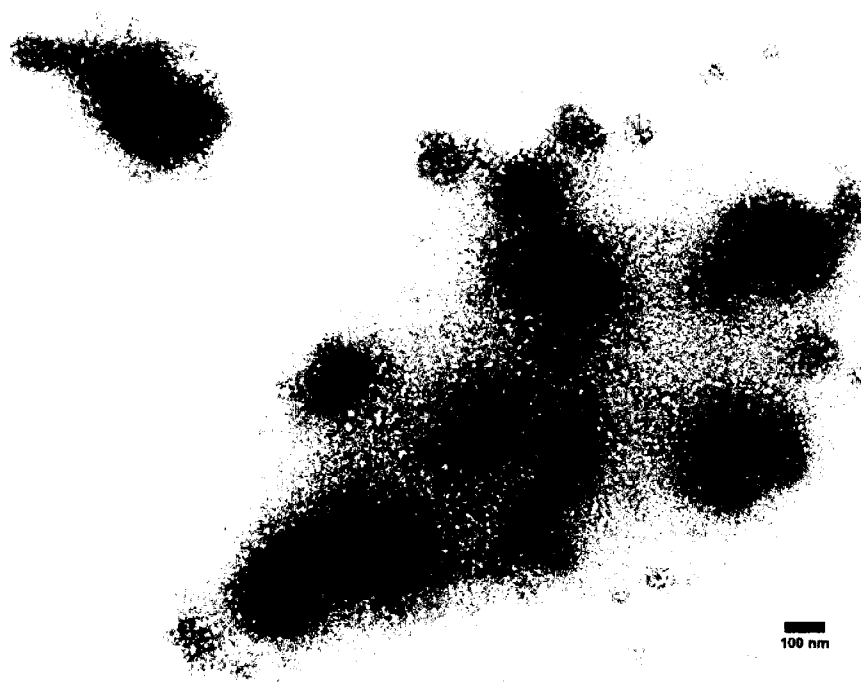
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Figure 44

A



B



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Figure 45

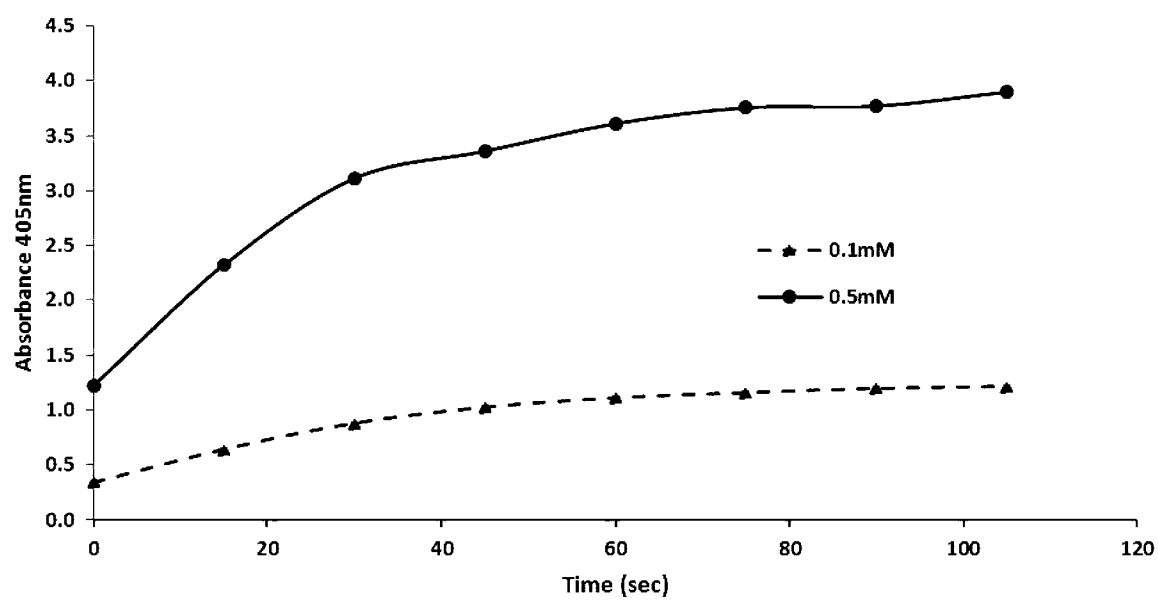
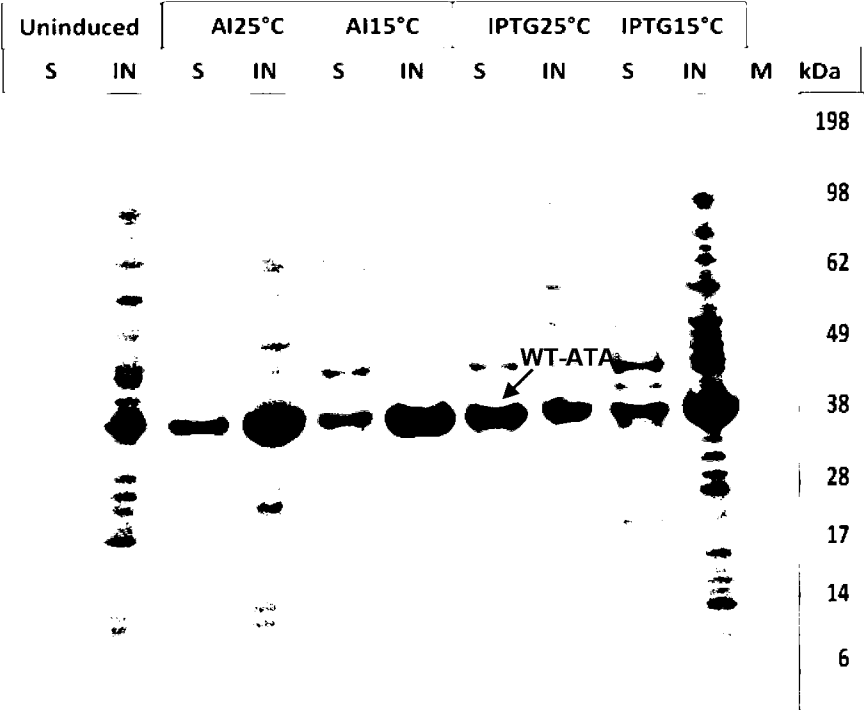
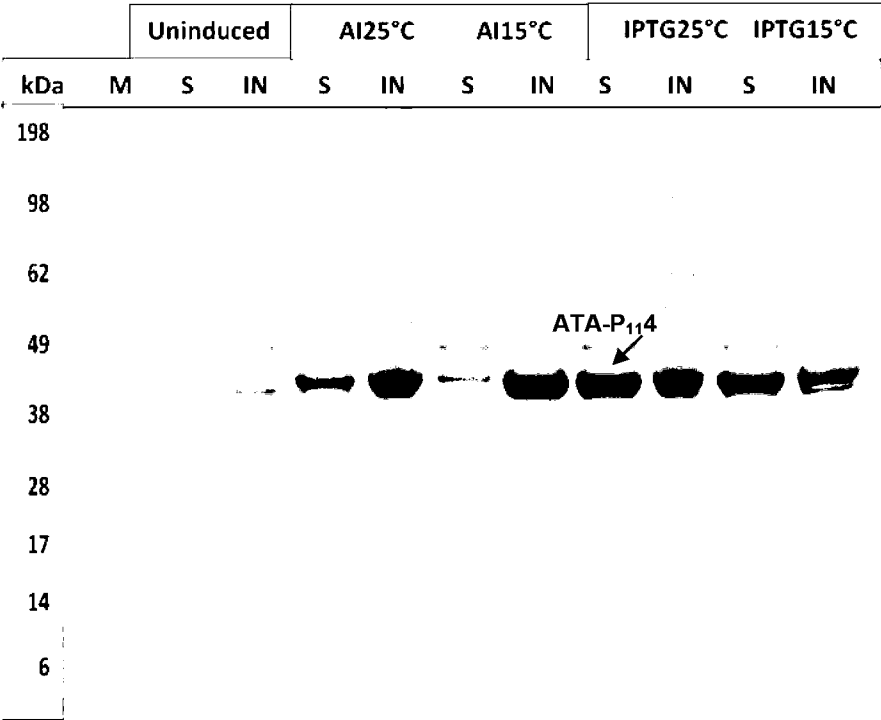


Figure 46

A



B



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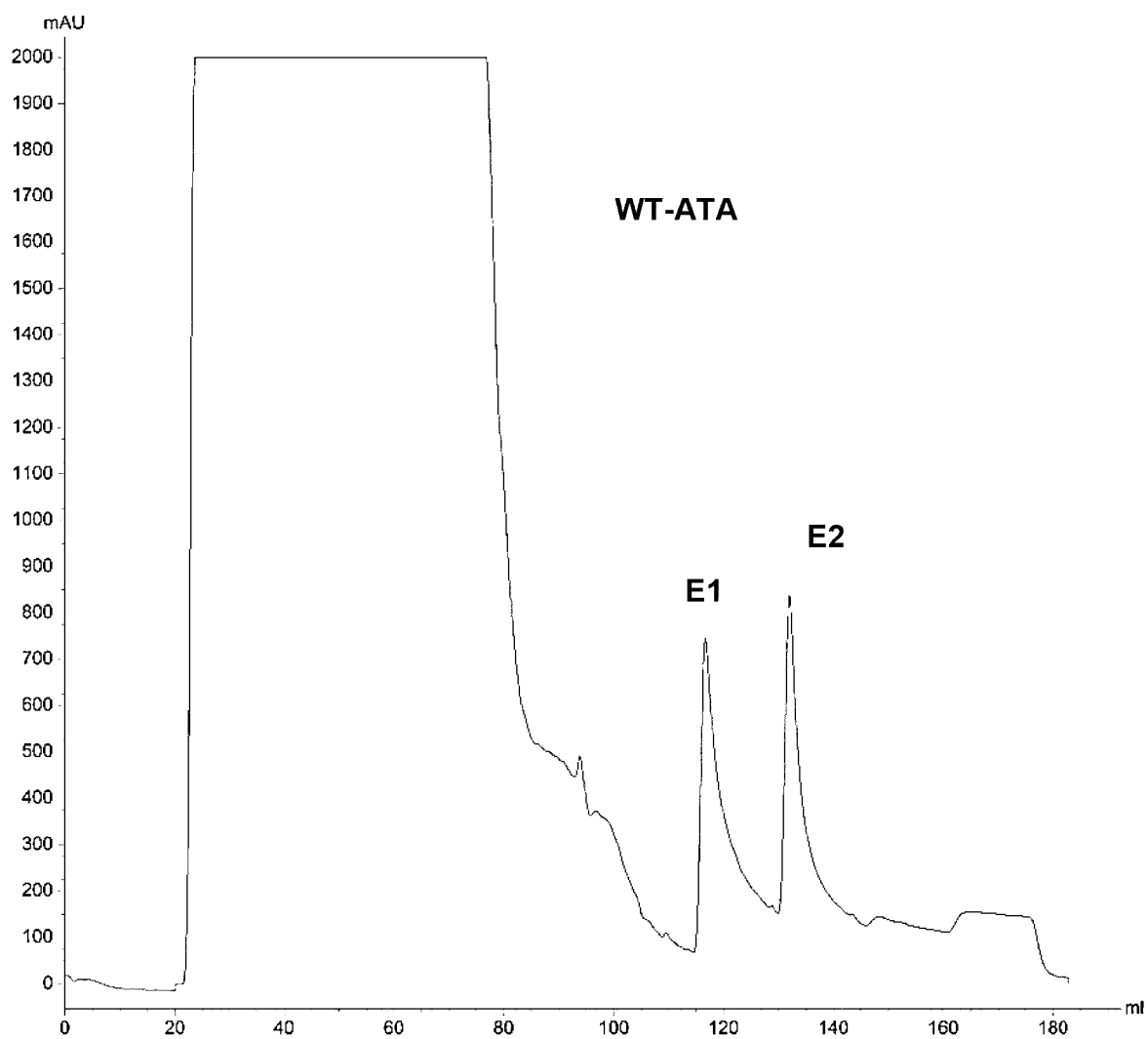
Figure 47**A**

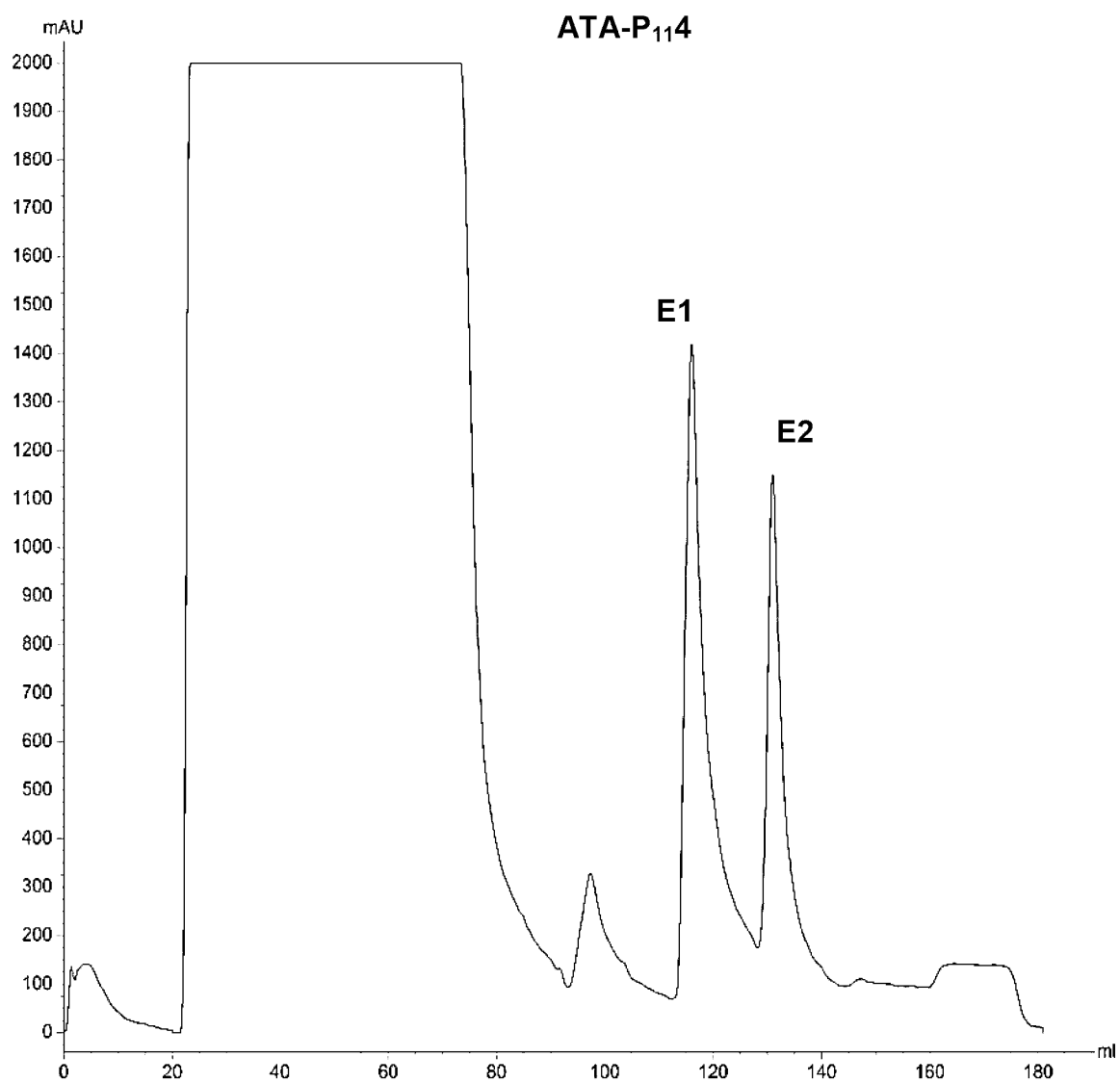
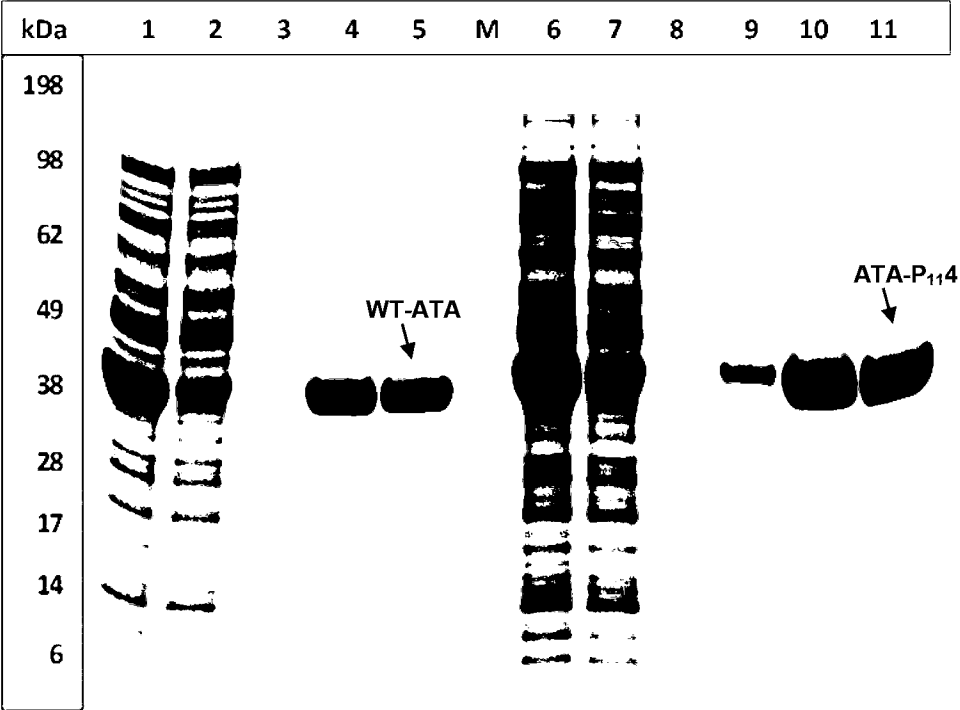
Figure 47**B**

Figure 47

C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/051293

A. CLASSIFICATION OF SUBJECT MATTER

B82Y 5/00 (2011.01) C08G 83/00 (2006.01) C12N 9/96 (2006.01) C12N 11/18 (2006.01)

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)

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)

WPIAP, EPODOC, MEDLINE, CAPLUS, BIOSIS: C08G 83/00, B82Y 5/00, self-assembly, P11-4, QQRFEWEFEQQ, P11, enzyme, carbonic, tyrosinase, aminotransferase, cutinase, supramolecular, nano and similar terms.

GOOGLE, ESP@CE: Applicant/Inventor Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 6 April 2017		Date of mailing of the international search report 06 April 2017	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Anita Cochrane AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832118	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2016/051293
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HICKLING C et al, "Nanofibrillar Peptide Hydrogels for the Immobilization of Biocatalysts for Chemical Transformations", Macromolecular Rapid Communications, 2014, 35: 868-874 Abstract, Scheme 1 & 2, pages 869 & 870	1-4, 10-20, 23-44, 59-62 & 65
X	ZHOU X-M et al, "Self-Assembly of Amyloid Fibrils that Display Active Enzymes", ChemCatChem, 2014, 6: 1961-1968 Abstract, pages 1962 & 1963	1, 5, 10, 14-16, 18-19, 29, 35 & 44
X	FERNER-OTNER-BLECKMANN J et al, "Multitechnique study on recombinantly produced <i>Bacillus halodurans</i> laccase and an S-layer/laccase fusion protein", Biointerphases, 2011, 6: 63-72 Abstract, pages 64 & 67	1, 3-5, 14-16, 18-20, 23-29, 35, & 44
X	WHEELDON IR et al, "A chimeric fusion Protein Engineered with Disparate Functionalities - Enzymatic Activity and Self-Assembly", J Mol Biol, 2009, 392: 129-142 Abstract, pages 130, 135, 136 & 139, Figures 1 & 5	1-4, 10, 14-20, 23-29, 35, 44, 59-62, 65, 67-68 & 72-77
P,X	SHANBHAG BK et al, "Self-Assembled enzyme Nanoparticles for Carbon Dioxide Capture", Nano Letters, 25 April 2016, 16: 3379-3384. Abstract, Figure 1, page 3380	1-47, 55 & 59-65
A	AGGELI A et al, "pH as a Trigger of Peptide B-sheet Self-Assembly and Reversible Switching between Nematic and Isotropic Phases", J Am Chem Soc, 2003, 125: 9619-9628 Abstract	

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
☐ on paper
☒ in electronic form
 - b. (time)
☐ in the international application as filed
☒ together with the international application in electronic form
☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:
Although there was a sequence listing originally filed it was not used for the purposes of this search and examination.