

**Functionalized compositions comprising an engineered phenylalanine
ammonia lyase (PAL)**

The field of the invention

5 The present invention relates to a composition comprising a solid carrier, an engineered phenylalanine ammonia lyase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase or a fragment thereof by embedding the engineered phenylalanine ammonia lyase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer,
10 wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group. The present invention also relates to methods of producing said composition and uses thereof.

Background of the invention

15 Phenylketonuria (PKU) is an inherited, autosomal recessive disease characterized by a deficiency in the intracellular liver enzyme phenylalanine hydroxylase (PAH). PAH catalyzes the conversion of the essential amino acid phenylalanine to tyrosine, and this enzymatic activity is facilitated by tetrahydrobiopterin (BH₄). PAH deficiency results in an abnormally
20 elevated concentrations of phenylalanine, which is toxic to the brain. High phenylalanine levels during infancy and early childhood cause profound cognitive and developmental defects, and poorly controlled blood phenylalanine levels in older children and adolescents are associated with learning disabilities, attention deficit hyperactivity disorder, and behavioral. Uncontrolled blood phenylalanine levels in adulthood are associated with executive
25 dysfunction, and a variety of behavioral and psychiatric problems.

The cornerstone of PKU treatment is a low phenylalanine diet in combination with phenylalanine-free L-amino acid. The deficiency of PAH in patients with blood phenylalanine levels greater than 600 micromol/L can be corrected by an enzyme substitution therapy with recombinant phenylalanine ammonia lyase (PAL) (Palynziq®), administered daily by up to 3
30 subcutaneous injections). However, hypersensitivity reactions are common and immune-mediated acute hypersensitivity reactions (Type III) have been reported in patients maintained on a pegylated derivative of the enzyme phenylalanine ammonia-lyase (pegvaliase). Thus,

there is a need to provide compatible and effective treatments for phenylketonuria.

Summary of the invention

The present invention provides a composition comprising a solid carrier, an engineered
5 phenylalanine ammonia lyase or a fragment thereof immobilized on the surface of the solid
carrier, a protective layer to protect the engineered phenylalanine ammonia lyase or a
fragment thereof by embedding the engineered phenylalanine ammonia lyase or a fragment
thereof, and a functional constituent immobilized on the surface of the protective layer,
wherein the functional constituent immobilized on the surface of the protective layer is a
10 polymer comprising repeat units wherein each repeat unit comprises at least one amino group
and/or at least one thiol group.

The present invention also provides a method of producing said composition comprising
a solid carrier, an engineered phenylalanine ammonia lyase or a fragment thereof immobilized
15 on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine
ammonia lyase or a fragment thereof by embedding the engineered phenylalanine ammonia
lyase or a fragment thereof, and a functional constituent immobilized on the surface of the
protective layer, wherein the functional constituent immobilized on the surface of the
protective layer is a polymer comprising repeat units wherein each repeat unit comprises at
20 least one amino group and/or at least one thiol group, the method comprising the following
steps:

- (a) providing a solid carrier;
- (b) immobilizing an engineered phenylalanine ammonia lyase or a fragment thereof on the
solid carrier;
- 25 (c) forming a protective layer on the surface of the solid carrier to protect the engineered
phenylalanine ammonia lyase or a fragment thereof immobilized on the solid carrier;
- (d) immobilizing a functional constituent on the surface of the protective layer, wherein the
functional constituent immobilized on the surface of the protective layer is a polymer
comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
30 least one thiol group.

It has been surprisingly found by the inventors of the present application that compositions as
provided by the present invention if applied therapeutically have an unexpected high in vivo

enzymatic activity, show low cytotoxicity, and do not disrupt the intestinal barrier if localized in the gastrointestinal tract, thus making them extremely promising for therapeutic use, in particular for prevention, delay of progression or treatment of phenylketonuria.

Brief description of the figures

Figure 1) shows a schematic representation of the process for the production of the composition of the invention: a) engineered PAL or fragment thereof is immobilized on the solid carrier; b) and c) a protective layer grows around the immobilized engineered PAL or fragment thereof embedding the immobilized engineered PAL or fragment thereof; and d) a functional constituent is immobilized on the surface of the protective layer.

Figure 2) shows the added value of the covalent bonding of the enzyme surface to the protective layer. (A) Protein quantification performed on reaction supernatants of NP-1(1), NP-1(2) and NP-1. (B) PAL loading per dry weight of SNP. (C) SNP-specific activities expressed in U/g SNP. (D) PAL-specific activities expressed in U/g PAL.

Figure 3) shows the phenylalanine ammonia lyase (PAL) activity of the nanoparticles. The biocatalytic activity of engineered PAL immobilized and protected on the nanoparticles has been quantified (in U/g) after exposure to phenylalanine.

Figure 4) shows resistance to external stresses. NP-1 and engineered PAL were exposed to (A-B) acidic condition (pH4) or (C) to proteases and their stability was assessed by the measurement of the PAL enzymatic activity at different time points.

Figure 5) shows the *in vitro* biocompatibility and efficacy of NP-1 on model of intestinal barrier. (A) *In vitro* assessment of the integrity of the intestinal barrier by the measurement of the transepithelial electrical resistance (TEER). Differentiated Caco-2/HT29-MTX-E12 co-culture were exposed to NP-1 (9.7mU) in presence or not of pancreatin (30mU) or to pancreatin (30mU) alone for 6h. The graph represents the time course profile evolution of averaged normalized TEER data over 6h. The dash line represents the untreated condition. (B) *In vitro* metabolization of Phenylalanine (Phe) on a model of intestinal barrier. Differentiated Caco-2/HT29-MTX-E12 co-culture cultivated in cell culture medium containing 0.4mM of Phe were exposed to NP-1 (9.7mU) or engineered PAL (9.7mU) with or without pancreatin (30mU) for 6h at the apical side of the barrier. The metabolization of Phe was evaluated by the quantification of trans-cinnamic acid (TCA) in the basal side of the barrier. The graph shows the time course profile evolution of the accumulation of TCA over 6h.

Figure 6) shows the quantification of trans-cinnamic acid (TCA) in urine of rats. Wistar rats were dosed with NP-1 (n=5) or NP-2 (n=5) intraduodenally and simultaneously gavaged with d5-Phe. Urines were collected over a period of 24h post dosing and analysis by LC-MS. Graphs show the concentration of d5-hippuric acid in the urines. **p<0.01 by t-test.

5 **Figure 7)** shows the plasmatic concentration of Phe in BTBR-*Pah*^{enu2}/J mice. BTBR-*Pah*^{enu2}/J mice having *ad libitum* access to drinking water containing L-Phe were dosed intraduodenally with NP-1 (0.581U; 7mg), NP-2 (7mg) or engineered PAL (0.581U) twice per day over a period of 12 days. Blood samples were taken at days 0, 4, 6, 8, 10 and 12 for plasma extraction and analysis by LC-MS. (A) Graph shows the plasmatic concentration of Phe in BTBR-*Pah*^{enu2}/J mice. (B) Graph shows the normalized plasmatic concentration of Phe in BTBR-*Pah*^{enu2}/J mice.

10 **Figure 8)** shows absorbance of nanoparticles NP-1, NP-1(1) and NP-1(2) at 460 nm.

Detailed description of the invention

The present invention relates to a composition comprising a solid carrier, an engineered
15 phenylalanine ammonia lyase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase or a fragment thereof by embedding the engineered phenylalanine ammonia lyase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a
20 polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

For the purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and *vice versa*. It
25 is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Features, integers, characteristics, compounds described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any
30 other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are

mutually exclusive. The invention is not restricted to the details of any foregoing embodiments.

The term “comprise” and variations thereof, such as, “comprises” and “comprising” is
5 generally used in the sense of include, that is, as “including, but not limited to”, that is to say permitting the presence of one or more features or components.

The singular forms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise.

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The term "about" refers to a range of values $\pm 10\%$ of a specified value. For example, the phrase "about 200" includes $\pm 10\%$ of 200, or from 180 to 220.

The term “solid carrier” as used herein refers usually to a particle. Preferably the solid carrier
15 is a monodisperse particle or a polydisperse particle, more preferably a monodisperse particle. The solid carrier usually comprises organic particles, inorganic particles, organic-inorganic particles, self-assembling organic particles, silica particles, gold particles, titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP). The particle size of the solid carrier is usually between and 1 nm and 1000 μm , preferably between 10 nm
20 and 100 μm , particularly about 50 nm.

The term “linker” or “cross-linker” which are used synonymously herein refers to any linking reagents containing groups, which are capable of binding to specific functional groups (e.g. primary amines, sulfhydryls, etc.). A linker in the context of the present invention usually
25 connects the surface of the solid carrier with the engineered phenylalanine ammonia lyase. For example, a linker may be immobilized on the surface of the solid carrier e.g. on the silica surface as a carrier material and then the engineered phenylalanine ammonia lyase may be bound to an unoccupied binding-site of the linker. Alternatively, the linker may firstly bind to the engineered phenylalanine ammonia lyase and then the linker bound to the engineered
30 phenylalanine ammonia lyase may bind with its unoccupied binding-site to the solid carrier. Various types of linkers are known in the art, including but not limited to straight or branched-chain carbon linkers, heterocyclic carbon linkers, peptide linkers, polyether linkers,

and linkers that are known in the art as tags.

The term "protective layer" as used herein refers to a layer for protecting the functional properties of the engineered phenylalanine ammonia lyase or fragment immobilized on the surface of the solid carrier. The protective layer of the present invention is usually built with building blocks at least part of which are monomers capable of interacting with both each other usually by covalent binding and the immobilized engineered phenylalanine ammonia lyase usually by non-covalent binding. The protective layer is formed on the surface of the solid carrier to protect the engineered phenylalanine ammonia lyase or the fragment thereof immobilized on the solid carrier. The protective layers are usually homogeneous layers where at least 50%, preferably at least 70%, more preferably at least 90% of the engineered phenylalanine ammonia lyase or fragment thereof are embedded in the protective layer.

The term, "phenylalanine ammonia lyase or a fragment thereof" or "PAL or a fragment thereof" as used herein, refers to a class of enzymes within the aromatic amino acid lyase family (EC 4.3.1.23, EC 4.3.1.24 and EC4.3.1.25) which also includes histidine ammonia lyase, and tyrosine ammonia lyase. PALs are also sometimes referred to as phenylalanine/tyrosine ammonia lyases because some PALs may use tyrosine as well as phenylalanine as a substrate. PAL catalyze the conversion of L- phenylalanine to trans-cinnamic acid and ammonia. PAL activity refers to the enzymatic activity of PAL polypeptides. PAL may also contain the cofactor 3,5- dihydro-5- methylidene-4H-imidazol-4-one (MIO). This cofactor maybe required for catalytic activity and is formed by cyclization and dehydration of a conserved active site Ala167-Ser168-Gly169 tripeptide segment.

The term "engineered" and "non-naturally occurring" when used with reference to a phenylalanine ammonia lyase or a fragment thereof as used herein refers to a phenylalanine ammonia lyase or a fragment thereof corresponding to the natural or native form of the phenylalanine ammonia lyase or a fragment thereof that has been modified in a manner that would not otherwise exist in nature. The term "engineered phenylalanine ammonia lyase or a fragment thereof" does not include or encompass "wild-type" and "naturally-occurring" phenylalanine ammonia lyases or fragments thereof. As used herein, "wild-type" and "naturally-occurring" refer to the form of phenylalanine ammonia lyases or fragments thereof found in nature. For example a wild-type phenylalanine ammonia lyase or a fragments thereof

is a polypeptide present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation. Engineered PALs or fragments thereof are e.g. variants or functionally active fragments of the engineered phenylalanine ammonia lyase. The terms “fragment of the engineered phenylalanine ammonia lyase”,
5 “fragment thereof” in relation to the engineered phenylalanine ammonia lyase and “functionally active fragment of the engineered phenylalanine ammonia lyase” are thus used synonymously herein. By “variants or functionally active fragments thereof” in relation to the engineered phenylalanine ammonia lyase of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant not existing in nature) is capable of
10 exercising the same or improved physiological function as the wild-type phenylalanine ammonia lyase. Additions, deletions, substitutions and derivatizations of one or more of the amino acids are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. A fragment of a PAL comprises the homotetrameric enzyme wherein at least one monomer, preferably all four monomers of the homotetrameric
15 enzyme contains usually between 100 and 550 amino acids, preferably between 200 and 500 amino acids, more preferably between 300 and 450 amino acids. “Improved physiological function” or “Improved enzyme property” refers to an engineered PAL that exhibits an improvement in any enzyme property as compared to a reference PAL polypeptide, such as a wild- type PAL polypeptide. Improved properties include but are not limited to such
20 properties as increased protein expression, increased thermoactivity, increased thermostability, increased pH activity, increased stability, increased enzymatic activity, increased substrate specificity and/or affinity, increased specific activity, increased resistance to substrate and/or end-product inhibition, increased chemical stability, improved chemoselectivity, improved solvent stability, increased tolerance to acidic pH, increased
25 tolerance to proteolytic activity (i.e., reduced sensitivity to proteolysis), reduced aggregation, increased solubility, reduced immunogenicity, and altered temperature profile. Preferred engineered phenylalanine ammonia lyases or fragments thereof of the present invention are the engineered phenylalanine ammonia lyases described in WO 2018/148633 A1.

30 The term “partially embedded engineered phenylalanine ammonia lyase” as used herein shall mean that the engineered phenylalanine ammonia lyase is not fully covered by the protective layer, thus, the engineered phenylalanine ammonia lyase is not fully embedded in the protective layer. In one embodiment less than 50% of the engineered phenylalanine ammonia

lyase of interest are covered by the protective layer, though typically more at least 70% will be covered, thus improving protection of the engineered phenylalanine ammonia lyase. In a preferred embodiment, at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95% of the engineered phenylalanine ammonia lyase of interest is covered by the protective layer. In another preferred embodiment, around 70% to around 95%, more preferably around 80% to around 95%, even more preferably around 90% to around 95%, most preferably around 90% to around 95, 96, 97, 98 or 99 % of the engineered phenylalanine ammonia lyase of interest are covered by the protective layer. In a particularly preferred embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the engineered phenylalanine ammonia lyase of interest is covered by the protective layer. In a more particularly preferred embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the engineered phenylalanine ammonia lyase of interest is covered by the protective layer, wherein the active site is not covered.

15 The term “fully embedded engineered phenylalanine ammonia lyase” as used herein shall mean that the engineered phenylalanine ammonia lyase of interest according to the invention is fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered. Preferably the engineered phenylalanine ammonia lyase or a fragment thereof according to the invention is fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered.

25 The term “at least partially embedded engineered phenylalanine ammonia lyase” as used herein shall mean that the engineered phenylalanine ammonia lyase is at least partially embedded and may be fully embedded by the protective layer. Thus “at least partially embedded engineered phenylalanine ammonia lyase” means that the protective layer covers from about 30% and 100% of the engineered phenylalanine ammonia lyase or a fragment thereof, preferably from about 50% to about 100%, more preferably from about 80% to about 100%, even more preferably from about 90% to about 100%, most preferably from about 95% to about 100 %, wherein the active site is preferably covered.

The term “functional constituent” as used herein refers to a constituent which after being immobilized to the surface of the protective layer retains its characteristic, functional

property. A functional constituent in the sense of the present invention is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

5 The term “percent (%) sequence identity” as used herein refers to comparisons amount polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by
10 determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical amino acid
15 residue occurs in both sequences or an amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences.

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The term “Reference sequence” is used herein to refer to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 amino acid residues in length, at least 25 residues in length, at least 50
25 residues in length, at least 100 residues in length or the full length of the polypeptide.

The term “Comparison window” as used herein refers to a conceptual segment of at least about 20 contiguous amino acids residues wherein a sequence may be compared to a reference sequence of at least 20 contiguous amino acids and wherein the portion of the sequence in the
30 comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The comparison window can be longer than 20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

The term “polymer comprising repeat units wherein each repeat unit comprises at least one amino group” as used herein refers to a polymer comprising a number of repeat units (monomers), wherein each repeat unit comprises at least one amino group. A preferred
5 polymer comprises a number of repeat units (monomers), wherein each repeat unit contains one amino group, in particular one primary amino group.

The term “polymer comprising repeat units wherein each repeat unit comprises at least one thiol group” as used herein refers to a polymer comprising a number of repeat units
10 (monomers), wherein each repeat unit comprises at least one thiol. A preferred polymer comprises a number of repeat units (monomers), wherein each repeat unit contains one thiol group.

The term “polycarbophil-cysteine conjugates” as used herein refers to conjugates which
15 comprise cysteine covalently attached to polycarbophil. Such conjugates can be produced as referred in e.g. Bernkop-Schnurch and Thaler, 2000, Journal of Pharmaceutical Sciences 89(7):901-9.

The term “polylysine” as used herein refers to α -polylysine and or ϵ -polylysine (ϵ -poly-L-lysine, EPL), preferably ϵ -polylysine. α -polylysine is a synthetic polymer, which can be
20 composed of either L-lysine or D-lysine. ϵ -polylysine (ϵ -poly-L-lysine, EPL) is typically produced as a homopolypeptide of approximately 25–30 L-lysine residues.

The term “polycysteine” as used herein can be composed of either L-cysteine or D-cysteine
25 and is preferably composed of L-cysteine and comprises preferably between 2 and 30 cysteine residues, more preferably between 2 and 5 cysteine residues.

The term “polyglucosamin” as used herein refers to linear amino-polysaccharides composed of D-glucosamine and N-acetyl-D-glucosamine units linked by (1-4) glycosidic bonds.
30 Polyglucosamine contains free amine (-NH₂) groups and may be characterized by the proportion of N-acetyl-D-glucosamine units and D-glucosamine units, which is expressed as the degree of deacetylation (DDA) of the fully acetylated polymer chitin. A preferred polyglucosamin of the present invention is selected from the group consisting of chitin,

chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof. Most preferred is a chitosan or a derivative thereof.

The term “chitosan or a derivative thereof” as used herein refers to a chitosan or chitosan
5 derivative thereof including a salt thereof which has preferably a molecular weight of 2 000
Da or more, preferably in the range 25 000 - 2 000 000 Da and more preferably about 50 000 -
350 000 Da, most preferably about 50 000 – 190 000 Da or 190 000 – 310 000 Da. The term
“derivative” in relation to chitosan includes ester, ether or other derivatives formed by
reaction of acyl or alkyl groups with the OH groups. Examples are O-alkyl ethers of chitosan,
10 O-acyl esters of chitosan. Suitable derivatives are given e.g. in G.A.E. Roberts, Chitin
Chemistry, MacMillan Press Ltd, London, 1992. Suitable salts of chitosan include nitrates,
phosphates, sulphates, xanthates, hydrochlorides, glutamates, lactates, acetates.

In a first aspect the present invention provides a composition comprising a solid carrier, an
15 engineered phenylalanine ammonia lyase or a fragment thereof immobilized on the surface of
the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase or a
fragment thereof by embedding the engineered phenylalanine ammonia lyase or a fragment
thereof, and a functional constituent immobilized on the surface of the protective layer,
wherein the functional constituent immobilized on the surface of the protective layer is a
20 polymer comprising repeat units wherein each repeat unit comprises at least one amino group
and/or at least one thiol group.

The engineered phenylalanine ammonia lyase or a fragment thereof can be immobilized on
the surface of the solid carrier by non-covalent binding or covalent binding. Non-covalent
25 binding includes p-p (aromatic) interactions, van der Waals interactions, H-bonding
interactions, and electrostatic interactions like e.g. ionic interactions. Preferably, the
engineered phenylalanine ammonia lyase or fragment thereof is immobilized on the surface of
the solid carrier by covalent binding or by covalent binding via a linker.

A solution of the engineered phenylalanine ammonia lyase or a fragment thereof usually
30 comprises the protein or a fragment thereof in a buffer solution. Buffers which can be used are
usually phosphate, chloride, citrate, MES, MOPS, HEPES, PIPES, ACES or mixtures thereof.
The solution may additionally contain sugar alcohols or non-ionic surfactants as described
herein.

In one embodiment the solid carrier is selected from the group of organic particles, inorganic particles, organic-inorganic particles, self-assembling organic particles, silica particles, gold particles, titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP). The particle size is usually measured by measuring the diameter of the particles and is usually between 1 nm and 1000 nm, preferably between 10 nm and 100 nm, particularly about 50 nm. In case the solid carrier is a monodisperse particle, the size is usually between 1 nm and 1000 nm, preferably between 10 nm and 100 nm, particularly about 50 nm. In case the solid carrier is a polydisperse particle, the size is usually between 1 nm and 1000 μ m, preferably between 10 nm and 100 μ m, particularly between 50 nm and 50 μ m. In one embodiment the composition comprises a solid carrier wherein the solid carrier comprises at least 15%, preferably at least 20%, in particular between 15% and 25%, more particular between 15% and 20 %, immobilized engineered phenylalanine ammonia lyase or fragment thereof per dry weight of the solid carrier.

Usually monodisperse particles or polydisperse particles, preferably monodisperse particles are used as solid carrier in the present invention. In a preferred embodiment the monodisperse particles are spherical monodisperse particles. In a further preferred embodiment, the polydisperse particles are non-spherical polydisperse particles.

The solid carrier is usually provided in suspension. Suspension of the solid carrier can be e.g. in water, buffer or non-ionic surfactants or mixtures thereof, preferably in mixtures of water and non-ionic surfactants. Non-ionic surfactants are usually selected from the group consisting of ethoxylated sorbitan esters like PEG-40 sorbitan diisostearate, polysorbate 80 (PS80), polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60); block copolymers like poloxamer 124, poloxamer 188, poloxamer 331, poloxamer 407, fatty acids ethoxylates like PEG-5 oleate, PEG-8 stearate, polyoxyl 40 stearate, polyoxyl 15 hydroxystearate, fatty alcohol ethoxylates like steareth 40; fatty acid esters like ascorbyl palmitate, beeswax, polyglyceryl 3-oleate, propylene glycol monocaprylate, propylene glycol monolaurate; fatty alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). Buffers which can be used in the method of the present invention are phosphate, piperazine-N,N'-bis(2-ethanesulfonic acid), 2-Hydroxy-3-

morpholinopropanesulfonic acid, N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), (3-(N-morpholino)propanesulfonic acid), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 3-(N,N-Bis[2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid, N,N-Bis(2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid, N-[Tris(hydroxymethyl)methyl]glycine, Diglycine, 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid, N,N-Bis(2-hydroxyethyl)glycine, N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid.

10 In one embodiment the surface of the solid carrier is modified to introduce a molecule or functional chemical group as anchoring point i.e. as anchoring point for the engineered phenylalanine ammonia lyase or for the linker connecting the engineered phenylalanine ammonia lyase to the solid carrier. Preferably, said anchoring point is an amine functional chemical group or moiety. As a non-limiting example, an amino-modified surface of the solid carrier e.g. an amino-modified silica surface may be used as modified solid carrier. Such an amino-modified surface of the solid carrier may be obtained by reacting a solid carrier having a silica surface with an amino silane, e.g. with APTES. Thus, in a preferred embodiment, the solid carrier is a solid carrier having a silica surface with an amino-modified surface, more preferably a solid carrier obtained by reacting the solid carrier having a silica surface with an amino silane, e.g. with APTES. Such a modified carrier may form an amide linkage between the engineered phenylalanine ammonia lyase and the amine group at the surface of the carrier material or an amide linkage between the linker and the amine group at the surface of the carrier material. In one embodiment the introduced molecule or functional chemical group as anchoring point is homogeneously distributed on the surface of the solid carrier.

25 In some embodiments the protective layer has a defined thickness of about 1 to about 200 nm, usually 1 to about 100 nm, preferably about 1 to about 50nm, more preferably about 1 to about 25 nm, even more preferably about 1 to about 20 nm, in particular about 1 to about 15 nm. The most preferred defined thickness is about 1 to about 10 nm. In some embodiments the layer has a defined thickness of about 5 to about 100 nm, preferably about 5 to about 50 nm, more preferably about 5 to about 25 nm, even more preferably about 5 to about 20 nm, in particular about 5 to about 15 nm. The most preferred defined thickness is about 5 to about 10 nm. The protective layer is usually porous and the pore size is between 1 and 100 nm,

preferably between 1 and 20 nm.

In one embodiment, the engineered phenylalanine ammonia lyase or fragment thereof is partially embedded by the protective layer. In a preferred embodiment the engineered
5 phenylalanine ammonia lyase or fragment thereof is at least partially embedded by the protective layer. In a more preferred embodiment, the engineered phenylalanine ammonia lyase or fragment thereof is fully embedded by the protective layer.

In one embodiment, the protective layer embeds the solid carrier and embeds the engineered phenylalanine ammonia lyase or fragment thereof thereof immobilized on the surface of the
10 solid carrier. In one embodiment, the functional constituent immobilized on the surface of the protective layer, is not embedded by the protective layer. Preferably, the protective layer fully embeds the solid carrier and fully embeds the engineered phenylalanine ammonia lyase or fragment thereof immobilized on the surface of the solid carrier. More preferably, the protective layer fully embeds the solid carrier and fully embeds the engineered phenylalanine
15 ammonia lyase or fragment thereof immobilized on the surface of the solid carrier and the functional constituent immobilized on the surface of the protective layer is not embedded by the protective layer. If the protective layer fully embeds the solid carrier and fully embeds the engineered phenylalanine ammonia lyase or fragment thereof immobilized on the surface of the solid carrier, the engineered phenylalanine ammonia lyase or fragment thereof is fully, i.e.
20 100% covered by the protective layer, i.e. that also the active site is covered and the solid carrier is fully, i.e. 100% covered by the protective layer.

In a preferred embodiment, the engineered phenylalanine amino lyase or a fragment thereof comprises or consists of an amino acid sequence having at least 90%, at least 95%, at least
25 96%, or at least 97% sequence identity to the sequence of SEQ ID NO: 1. In one embodiment, the engineered phenylalanine ammonia lyase or a fragment thereof is SEQ ID NO: 2, 3, 4 or 5. In a particular preferred embodiment the engineered phenylalanine ammonia lyase or a fragment thereof comprises the polypeptide as shown in SEQ ID NO: 5.

30 The protective layer thickness can be measured, by using a microscope such as scanning electron microscope (SEM), transmission electron microscopy (TEM), scanning probe microscopy (SPM), light scattering methods or by ellipsometry.

The composition of the present invention is usually produced in a reaction vessel like a reactor. The formation of the protective layer is usually carried out by forming the respective protective layer by building blocks, wherein the building blocks build the protective layer in a polycondensation reaction. The polycondensation can be effected in different solvents, preferably in aqueous solution. Polycondensation can be easily controlled and stopped if appropriate, allowing achievement of a defined thickness of the protective layer. The choice of the building blocks, which can be used to build the protective layer, may depend on the known structure of the engineered phenylalanine ammonia lyase in order to adapt the affinity of the protective layer according to optimal and/or desired parameters. As building blocks for the protective layer usually structural building blocks and protective building blocks are used to build the protective layer. Structural building blocks which can be used are e.g. tetraethylorthosilicate (designated herein as "TEOS" or "T"). Protective building blocks which can be used are e.g. 3-Aminopropyltriethoxysilane (designated herein as "APTES" or "A"), Propyltriethoxysilane (designated herein as "PTES" or "P"), Isobutyltriethoxysilane (designated as "IBTES"), Hydroxymethyltriethoxysilane (designated herein as "HTMEOS" or H), Benzyltriethoxysilane (designated herein as "BTES"), Ureidopropyltriethoxysilane (designated as "UPTES"), or Carboxyethyltriethoxysilane (designated herein as "CETES"). Structural building blocks are usually precursors of inorganic silica, capable of forming 4 covalent bonds in the layer formed. Protective building blocks are usually organosilanes, bearing an organic moiety endowed with the ability to interact with the engineered phenylalanine ammonia lyases. Preferred structural building blocks are tetravalent silanes, in particular tetra-alkoxy-silanes. Preferred protective building blocks are trivalent silanes, in particular tri-alkoxy-silanes. More preferred structural building blocks are mixtures of tetravalent silanes and trivalent silanes, in particular mixtures of tetra-alkoxy-silanes and tri-alkoxy-silanes. Even more preferred structural building blocks are selected from the group consisting of tetraethylorthosilicate, tetra-(2-hydroxyethyl)silane, and tetramethylorthosilicate. Even more preferred protective building blocks are selected from the group consisting of carboxyethylsilanetriol, benzylsilanes, propylsilanes, isobutylsilanes, n-octylsilanes, hydroxysilanes, bis(2-hydroxyethyl)-3-aminopropylsilanes, aminopropylsilanes, ureidopropylsilanes, (N-Acetylglycyl)-3-aminopropylsilanes, hydroxy(polyethyleneoxy)propyl]triethoxysilanes, in particular selected from benzyltriethoxysilane, propyltriethoxysilane, isobutyltriethoxysilane, n-octyltriethoxysilane, hydroxymethyltriethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltriethoxysilane, 3-

Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglycyl)-3-aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutylmethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrimethoxysilane, aminopropyltrimethoxysilane, ureidopropyltrimethoxysilane (N-Acetylglycyl)-3-aminopropyltrimethoxysilane or selected from benzyltrihydroxyethoxysilane, propyltrihydroxyethoxysilane, isobutyltrihydroxyethoxysilane, n-octyltrihydroxyethoxysilane, hydroxymethyltrihydroxyethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrihydroxyethoxysilane, aminopropyltrihydroxyethoxysilane, ureidopropyltrihydroxyethoxysilane (N-Acetylglycyl)-3-aminopropyltrihydroxymethoxysilane.

Particular preferred building blocks are TEOS as structural building block and APTES, PTES, and/or HTMEOS, preferably APTES as protective building block. In particular TEOS as structural building block and APTES as protective building block are used to build the protective layer.

The reaction time of the building blocks with the solid carrier depends on the length of the linker, if a linker is used, and the size of the engineered phenylalanine ammonia lyase. The reaction is usually carried out for a time period of between 0.5 to 10 hours, preferably between 1 and 5 hours, more preferably between 1 and 4 hours, even more preferably between 2 and 4 hours, preferably in aqueous solution and preferably at room temperature of about 5 to about 25 °C or at about 20 °C. The formation of the protective layer can be stopped by actively stopping the polycondensation reaction e.g. by removing the non-reacted building blocks e.g. by a washing step or by self-stopping of the polycondensation reaction caused by a limited amount of building blocks.

In a furthermore preferred embodiment the engineered phenylalanine ammonia lyase is immobilized on the solid carrier by at least partly modifying the surface of the solid carrier by introducing a molecule as anchoring point as described supra for the engineered phenylalanine ammonia lyase and by using a linker, preferably a cross-linker binding to the anchoring point and the engineered phenylalanine ammonia lyase.

After the protective layer has been formed, the solid carrier comprising the engineered phenylalanine ammonia lyase and the protective layer can be stored. Storing is usually accomplished e.g. by washing the composition formed e.g. with a buffer and storing it
5 suspended or solved in that buffer for a desired time period. In a preferred embodiment the solid carrier comprising the engineered phenylalanine ammonia lyase and the protective layer is stored at a constant temperature between 2 to 25 °C. In a further preferred embodiment, the solid carrier comprising the engineered phenylalanine ammonia lyase and the protective layer is stored 5 to 48 hours, preferably 10 to 30 hours. More preferably the solid carrier comprising
10 the engineered phenylalanine ammonia lyase and the protective layer is stored at a constant temperature between 2 to 25 °C, preferably at room temperature for 10 to 30 hours.

In one embodiment, the functional constituent binds to mucus.

In one embodiment a polymer comprising repeat units wherein each repeat unit comprises at
15 least one amino group and/or at least one thiol group is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group.

In one embodiment a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is a polymer comprising repeat units wherein each repeat unit comprises at least one thiol group.

20 In one embodiment a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin, a polymerized silane-PEG-NH₂ and a polymerized silane comprising an amino group. In a preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, is selected from
25 the group consisting of a polyglucosamin, a polymerized silane-PEG-NH₂ and polymerized APTES.

In a more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin selected from the group consisting of chitin, chitosan,
30 polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof; a polymerized silane-PEG-NH₂; and a polymerized silane comprising an amino group, preferably a polymerized APTES. In an even more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at

least one thiol group, is a polyglucosamin, preferably a polyglucosamin selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof, more preferably a chitosan or a derivative thereof.

A preferred polyglucosamin of the present invention is selected from the group consisting of
5 chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof. Most preferred is a chitosan or a derivative thereof. A preferred silane-PEG-NH₂ of the polymerized silane-PEG-NH₂ is selected from the group consisting of silane-PEG₄-NH₂, silane-PEG₂₀₀₀-NH₂, and silane-PEG₅₀₀₀-NH₂. A preferred polymerized silane comprising an amino group is selected from the group consisting of
10 APTES, amino-butyl-TES, amino-pentyl-TES, amino-hexyl-TES, amino-heptyl-TES, and amino-octyl-TES, and is in particular APTES.

In a further embodiment a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin, a polymerized silane-PEG-NH₂, a polymerized silane
15 comprising an amino group, a polymerized silane comprising a thiol group, a polycarbophil-cysteine conjugate, a polymerized silane-PEG-thiol and a polycysteine. In a further more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin selected from the group consisting of chitin, chitosan,
20 polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof; a polymerized silane-PEG-NH₂; a polymerized silane comprising a thiol group, preferably a polymerized MPTS; a polycarbophil-cysteine conjugate; a polymerized silane-PEG-thiol; and a polycysteine. In an even more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, is
25 a polyglucosamin or a polymerized silane comprising a thiol group, preferably a polyglucosamin selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof, more preferably a chitosan or a derivative thereof or a polymerized silane comprising a thiol group, a polycarbophil-cysteine conjugate, and a polymerized silane-PEG-thiol, preferably a
30 polymerized silane comprising a thiol group.

In a particular embodiment, the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, is selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratin,

dermatan or a derivative thereof in particular chitosan or a derivative thereof, a polymerized silane-PEG-NH₂ selected from the group consisting of polymerized silane-PEG₄-NH₂, polymerized silane-PEG₂₀₀₀-NH₂, polymerized silane-PEG₅₀₀₀-NH₂, a polymerized silane comprising an amino group which is preferably polymerized APTES and a polymerized silane comprising a thiol group, which is preferably polymerized MPTS.

In one embodiment a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin, a polymerized silane-PEG-NH₂, a polymerized silane comprising an amino group and a polymerized silane comprising a thiol group. In a preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, is selected from the group consisting of a polyglucosamin, a polymerized silane-PEG-NH₂, polymerized APTES and polymerized MPTS.

In a more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof; a polymerized silane-PEG-NH₂; a polymerized silane comprising an amino group, preferably a polymerized APTES; and a polymerized silane comprising a thiol group, preferably polymerized MPTS. In a particular embodiment, the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, is selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratin, dermatan or a derivative thereof, most particular chitosan or a derivative thereof.

In one embodiment a polymer comprising repeat units wherein each repeat unit comprises at least one thiol group is selected from the group consisting of a polymerized silane comprising a thiol group, a polycarbophil-cysteine conjugate, a polymerized silane-PEG-thiol and a polycysteine, and is preferably selected from the group consisting of a polymerized silane comprising a thiol group, a polycarbophil-cysteine conjugate, and a polymerized silane-PEG-thiol, and is more preferably a polymerized silane comprising a thiol group, and is most preferably polymerized MPTS. In one embodiment a polymerized silane comprising a thiol group is preferably polymerized MPTS.

In one embodiment 5% to 100%, preferably 10% to 100%, more preferably 50% to 100%, of the surface of the protective layer is covered with a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

- 5 In one embodiment the functional constituent is immobilized on the surface of the protective layer by binding, preferably covalent binding. In a preferred embodiment the functional constituent is immobilized on the surface of the protective layer by non-covalent binding, preferably by electrostatic interactions. In a more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
10 least one thiol group is immobilized on the surface of the protective layer by covalent binding.

- In one embodiment the functional constituent is immobilized on the surface of the protective layer using a spacer binding to the surface of the protective layer and the functional constituent. Thus in one embodiment the present invention comprises a composition
15 comprising a solid carrier, an engineered phenylalanine ammonia lyase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase or a fragment thereof by embedding the engineered phenylalanine ammonia lyase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the
20 surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, wherein the functional constituent is immobilized on the surface of the protective layer by a spacer. Examples of such a spacer include a polyethylene such as PEG4, PEG2000, PEG5000. A functional constituent immobilized on the surface of the protective layer, by a spacer is usually produced
25 by firstly reacting the spacer with the functional constituent, so that the spacer binds to the functional constituent and then the functional constituent bound to the spacer is reacted with the the surface of the protective layer.

- The immobilization of the functional constituent to the surface of the protective layer is
30 usually carried out in a reaction vessel like a reactor by suspending the solid carrier carrying the engineered phenylalanine ammonia lyase embedded in a protective layer as described supra in e.g. in water, buffer or non-ionic surfactants or mixtures thereof, preferably in mixtures of water and non-ionic surfactants. Non-ionic surfactants are usually selected from

the group consisting of ethoxylated sorbitan esters like PEG-40 sorbitan diisostearate, polysorbate 80 (PS80), polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60); bock co-polymers like poloxamer 124, poloxamer 188, poloxamer 331, poloxamer 407, fatty acids ethoxylates like PEG-5 oleate, PEG-8 stearate, polyoxyl 40 stearate, polyoxyl 15 hydroxystearate, fatty alcohol ethoxylates like steareth 40; fatty acid esters like ascorbyl palmitate, beeswax, polyglyceryl 3-oleate, propylene glycol monocaprylate, propylene glycol monolaurate; fatty alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). The functional component is then added to the suspension to react usually under stirring with the surface of the protective layer to immobilize the functional constituent on the surface of the protective layer. Ususally such obtained composition is washed and resuspended into water, buffer or non-ionic surfactants or mixtures thereof. Immobilization takes place by non-covalent binding e.g. electrostatic binding or by covalent binding of the functional constituent. The functional constituent may be immobilized by chemically modifying the surface of the protective layer and the functional constituent using e.g. "click chemistry" such as copper-catalyzed click chemistry (Copper-catalysed azide-alkyne cycloaddition, see e.g. Kolb et al. (2001) Angew. Chem. 40(11)2004-2021) or by copper free click chemistry (Wittig G, A Chem Ber, 1961, 94, 3260) ., e.g. the solid carrier carrying the engineered phenylalanine ammonia lyase embedded in a protective layer as described supra is first reacted with a reactive compound like an ethynyl compound and the functional constituent is modified by adding a reactive compound e.g. an azide residue and then both components are reacted to immobilize the functional constituent on the surface of the protective layer.

25 In a further aspect the present invention provides the composition as described supra for use as a medicament.

In a further aspect the present invention provides the composition for use in a method for the prevention, delay of progression or treatment of phenylketonuria (PKU). Also provided is the use of the composition as described herein for the manufacture of a medicament for the prevention, delay of progression or treatment of phenylketonuria (PKU) in a subject. Also provided is the use of the composition as described herein for the prevention, delay of progression or treatment of phenylketonuria (PKU) in a subject. Also provided is a method for

the prevention, delay of progression or treatment of phenylketonuria (PKU) in a subject, comprising administering to said subject a therapeutically effective amount of the composition as described herein. Preferably, the composition when administered to a subject in the method of the invention degrades phenylalanine in the intestine of the subject.

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A composition according to the invention is preferably a pharmaceutical composition and comprises a therapeutically effective amount of the composition as described herein and one or more suitable pharmaceutically acceptable carrier. A pharmaceutical composition according to the invention is suitable for oral administration to a subject. If not indicated otherwise, a pharmaceutical composition according to the invention is prepared in a manner known per se.

10

The composition, e.g. the pharmaceutical composition of the invention may be administered accordingly for a continuous period of one week or a part thereof, for two weeks, for three weeks for four weeks, for five weeks or for six weeks and then stopped for a period of one week, or a part thereof, for two weeks, for three weeks, for four weeks, for five weeks, or for six weeks.

15

The composition, e.g. the pharmaceutical composition of the present invention may conveniently be administered in unit dosage forms. Units ("U") of enzyme activity can be described in terms of weight or mass of substrate hydrolyzed per unit time. Units ("U") can be described in terms of nmol substrate converted per hour (or nmol/hr). In an exemplary treatment regime between 60 U to 1,000 U of engineered PAL comprised by the composition.

20

The expression "effective amount" or "therapeutically effective amount" as used herein refers to an amount capable of invoking one or more of the desired effects in a subject receiving the composition of the present invention. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

25

The terms "treatment"/"treating" as used herein includes: (1) delaying the appearance of clinical symptoms of the state, disorder or condition developing in an animal, particularly a mammal and especially a human, that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; (2) inhibiting the state, disorder or condition (e.g. arresting,

30

reducing or delaying the development of the disease, or a relapse thereof in case of maintenance treatment, of at least one clinical or subclinical symptom thereof); and/or (3) relieving the condition (i.e. causing regression of the state, disorder or condition or at least one of its clinical or subclinical symptoms). The benefit to a patient to be treated is either
5 statistically significant or at least perceptible to the patient or to the physician. However, it will be appreciated that when a medicament is administered to a patient to treat a disease, the outcome may not always be effective treatment.

As used herein, "delay of progression" means increasing the time to appearance of a symptom
10 or slowing the increase in severity of a symptom. Further, "delay of progression" as used herein includes reversing or inhibition of disease progression. "Inhibition" of disease progression or disease complication in a subject means preventing or reducing the disease progression and/or disease complication in the subject.

15 Preventive treatments comprise prophylactic treatments. In preventive applications, the pharmaceutical combination of the invention is administered to a subject suspected of having, or at risk for developing the above mentioned diseases or disorders. In therapeutic applications, the pharmaceutical combination is administered to a subject such as a patient already suffering from the above mentioned diseases or disorders, in an amount sufficient to
20 cure or at least partially arrest the symptoms of the disease. Amounts effective for this use will depend on the severity and course of the disease, previous therapy, the subject's health status and response to the drugs, and the judgment of the treating physician.

In the case wherein the subject's condition does not improve, the pharmaceutical combination of the invention may be administered chronically, which is, for an extended period of time,
25 including throughout the duration of the subject's life in order to ameliorate or otherwise control or limit the symptoms of the subject's disease or condition.

In the case wherein the subject's status does improve, the pharmaceutical combination may be administered continuously; alternatively, the dose of drugs being administered may be temporarily reduced or temporarily suspended for a certain length of time (i.e., a "drug
30 holiday"). Once improvement of the patient's condition has occurred, a maintenance dose of the pharmaceutical combination of the invention is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, is optionally reduced, as a function of the symptoms, to a level at which the improved disease is retained.

In a further aspect the present invention provides a method of producing a composition as described supra, e.g. a composition comprising a solid carrier, an engineered phenylalanine ammonia lyase or a fragment thereof immobilized on the surface of the solid carrier, a
5 protective layer to protect the engineered phenylalanine ammonia lyase or a fragment thereof by embedding the engineered phenylalanine ammonia lyase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
10 least one thiol group; the method comprising the following steps:
(a) providing a solid carrier;
(b) immobilizing an engineered phenylalanine ammonia lyase or a fragment thereof on the solid carrier;
15 (c) forming a protective layer on the surface of the solid carrier to protect the engineered phenylalanine ammonia lyase or the fragment thereof immobilized on the solid carrier;
(d) immobilizing a functional constituent on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
20 least one thiol group.

Step (a) is usually carried out by providing the solid carrier in suspension in water, non-ionic surfactants or a buffer or mixtures thereof, preferably in suspension in water and/or non-ionic surfactants, more preferably in suspension in water and/or non-ionic surfactants wherein no
25 buffer is present in the suspension, even more preferably in suspension in mixtures of water and non-ionic surfactants in particular in suspension in mixtures of water and non-ionic surfactants wherein no buffer is present in the suspension. The immobilization of the engineered phenylalanine ammonia lyase on the solid carrier in step b) of the present method is usually carried out by adding a solution of the engineered phenylalanine ammonia lyase to
30 the suspension of the solid carrier. Preferably a linker to connect the solid carrier with the engineered phenylalanine ammonia lyase is added to the suspension of the solid carrier prior to adding the solution of the engineered phenylalanine ammonia lyase to the suspension of the solid carrier. In a preferred embodiment the immobilization of the engineered phenylalanine

ammonia lyase on the solid carrier is carried out by providing a suspension of the solid carrier and adding a solution of the engineered phenylalanine ammonia lyase, wherein the suspension with the added solution of the engineered phenylalanine ammonia lyase is incubated to allow the enzyme to bind on the surface of the solid carrier. In a more preferred embodiment the

5 immobilization of the engineered phenylalanine ammonia lyase or a fragment thereof on the solid carrier in step b) is carried out by i) adding a linker to the solid carrier provided in step (a), preferably adding a linker to a suspension of the solid carrier provided in step a), and ii) adding the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, preferably adding a solution of the engineered phenylalanine ammonia lyase or a fragment thereof, to the

10 solid carrier and the linker or to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof. In one embodiment, a building block of the protective layer, preferably a monomer of a building block of the protective layer, more preferably an organosilane, even more preferably a triethoxysilane, in particular APTES, is added to the

15 solid carrier and the linker or to the suspension comprising the solid carrier and the linker, prior to adding the solution of the engineered phenylalanine ammonia lyase or a fragment thereof. In a preferred embodiment the surface of the solid carrier is at least partly modified to improve immobilization of the engineered phenylalanine ammonia lyase on the solid carrier. In particular, the surface of the solid carrier is at least partly modified before the engineered

20 phenylalanine ammonia lyase is immobilized. The surface of the solid carrier can be at least partly modified by adding a molecule as anchoring point for the engineered phenylalanine ammonia lyase to the surface of the solid carrier as described supra.

The suspension comprising the solid carrier is usually incubated after each addition step

25 described above to allow a reaction between e.g. the solid carrier and the molecule as anchoring point, the solid carrier and the linker and, the solid carrier comprising the linker and the engineered phenylalanine ammonia lyase or a fragment thereof, respectively, so that the engineered phenylalanine ammonia lyase or a fragment thereof connects the solid carrier, preferably the surface of the solid carrier, with the engineered phenylalanine ammonia lyase

30 (PAL) or a fragment thereof via the linker, preferably by covalent binding, thereby immobilizing the engineered phenylalanine ammonia lyase or a fragment thereof on the solid carrier.

In one embodiment in step (b) the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof is immobilized on the solid carrier by connecting the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof via a linker, preferably by connecting the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof via a linker, wherein the solid carrier is connected with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof by covalent binding between the linker and the solid carrier and between the linker and the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof. Preferably in step b), i) a linker is added to the solid carrier provided in step (a), and ii) the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof. The linker used is as described supra and connects the surface of the solid carrier with the engineered phenylalanine ammonia lyase by preferably covalent binding. More preferably the linker is added to the solid carrier in step (b), in a molar excess to the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, preferably the linker is added to the solid carrier in step (b), in a 1 fold to 1000 fold molar excess to the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, more preferably the linker is added to the solid carrier in step (b), in a 2 fold to 300 fold molar excess to the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, even more preferably the linker is added to the solid carrier in step (b), in a 4 fold to 250 fold molar excess to the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, in particular the linker is added to the solid carrier in step (b), in a four fold molar excess to the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof.

In a preferred embodiment the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b), is present during formation of a protective layer on the surface of the solid carrier in step (c). In a more preferred embodiment the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the phenylalanine ammonia lyase (PAL) or the fragment thereof in step (c). In a furthermore preferred embodiment the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b) is not removed in step (b) or step (c) or in between step (b) and (c). In a particular embodiment the linker which has not connected the solid carrier with the

engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b) is not removed in step (b) or step (c) or in between step (b) and (c) and the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the phenylalanine ammonia lyase (PAL) or the fragment thereof in step (c). The amount of the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b) is usually between 30% and 70%, preferably between 40% and 60 %, more preferably around 50% of the amount of linker added to the solid carrier in step (b). In one embodiment there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof to the solid carrier and the linker in ii). In one embodiment there is no washing step between any of steps (a) to (c). In one embodiment there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof to the solid carrier and the linker in ii) and there is no washing step between any of steps (a) to (c).

In one embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls, sulfhydryl-reactive 2-pyridyldithiol, BSO COES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate)), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate)), DTBP (Dimethyl 3,3'-dithiobispropionimidate·2 HCl), DST (Disuccinimidyl tartarate), Sulfo-LC-SMPT (4-Sulfosuccinimidyl-6-methyl-a-(2-pyridyldithio)toluamido]hexanoate)), SPDP (N-Succinimidyl 3-(2-pyridyldithio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate), SMPT (4-Succinimidylloxycarbonyl-methyl-a-[2-pyridyldithio]toluene), DPDPB (1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane), DTME (Dithio-bismaleimidoethane), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane) and is preferably glutaraldehyde.

In a preferred embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene

glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOCOES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3 '-Dithiobis[sulfosuccinimidyl]propionate),
5 DTBP (Dimethyl 3,3 '-dithiobispropionimide·2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane) and is preferably glutaraldehyde.

The formation of the protective layer according to step (c) of the present method is usually carried out by forming the respective protective layer with building blocks, wherein the
10 building blocks build the protective layer in a polycondensation reaction as described supra. The immobilization of a functional constituent on the surface of the protective layer according to step (d) of the present method is usually carried out as described supra.

In one embodiment the protective layer is formed by building blocks, wherein as building blocks structural building blocks and protective building blocks are used to form the
15 protective layer, wherein the structural building blocks are precursors of inorganic silica, capable of forming 4 covalent bonds in the layer formed and the protective building blocks are organosilanes as described supra.

In one embodiment the protective layer embeds from about 30% to about 100% of the
20 engineered phenylalanine ammonia lyase.

In one embodiment the solid carrier is selected from the group of organic particles, inorganic particles, organic-inorganic particles, self-assembled organic particles, silica particles, gold particles, magnetic particles and titanium particles and is preferably a silica particle, more
25 preferably a silica nanoparticle (SNP).

A preferred method of the present invention is a method of producing a composition, the composition comprising a solid carrier, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect
30 the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof by embedding the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat

units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:

- (a) providing a solid carrier, wherein the solid carrier is provided in suspension, preferably wherein the solid carrier is provided in suspension in water and/or non-ionic surfactants, more preferably wherein the solid carrier is provided in suspension in mixtures of water and non-ionic surfactants;
- (b) immobilizing an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof on the solid carrier, wherein preferably the surface of the solid carrier is at least partly modified before the engineered phenylalanine ammonia lyase or a fragment thereof is immobilized on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier or i) a linker is added to the suspension of the solid carrier after the at least partly modification of the surface of the solid carrier and ii) the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, preferably a solution of the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof is added to the the suspension of the solid carrier and the linker, wherein the linker connects the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof;
- (c) forming a protective layer on the surface of the solid carrier to protect the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the phenylalanine ammonia lyase (PAL) or the fragment thereof;
- (d) immobilizing a functional constituent on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

Also provided is a composition comprising a solid carrier, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof by embedding the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a

polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group, wherein the composition is obtainable by the methods, in particular by the preferred method of the invention as described supra.

Examples

Material and Methods:

Reagents:

- Tetraethyl orthosilicate 99% (TEOS), (3-aminopropyl)-triethoxysilane (APTES), ammonium hydroxide (ACS grade, 28–30%), ethanol (ACS grade, anhydrous), glutaraldehyde (grade I, 25% in water), polysorbate 80, acetic acid, bovine serum albumin (BSA), Tris buffer, L-Phenylalanine, pancreatin, pronase were purchased from Sigma-Aldrich. BSA, pancreatin, and pronase were dissolved in water to reconstitute the stock buffer.
- Chitosan 95/500 P was purchased from Heppe Medical Chitosan GmbH
- 10 - Engineered PAL (SEQ ID NO: 5) was provided by Nestlé Health Science at a concentration of 90 mg/mL in 25 mM Sodium Phosphate, 250 mM Sodium Chloride, 5% D-Mannitol, 0.2% Poloxamer 188, pH 7.5.
- Caco-2 (human colorectal adenocarcinoma cell line) and HT29-MTX-E12 (human colon cancer cell line) was purchased from the European Collection of Authenticated Cell Cultures (ECACC).
- 15 - ThinCert™ cell culture insert plates (1.0µm membrane) were purchased from Greiner bio-one.
- Fetal Bovine Serum, Penicillin/Streptomycin (10'000 U/ml Penicillin/10'000 µg/ml Streptomycin), L-Glutamine 200mM (100x), Dulbecco's Phosphate Buffered Saline DPBS (1X), 0.25% Trypsin-EDTA (1X), DMEM, white DMEM, were purchased from Gibco.
- 20 - Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free was purchased from corning.
- Animal diet Altromin 1324 was purchased at Altromin international.
- Animal diet phenylalanine free 5LF2 was purchased at LabDiet.
- 25 - The catheters were purchased at Instech Laboratories

Synthesis of silica nanoparticles (SNPs):

- Silica nanoparticles (50 nm) have been synthesized following the original Stöber process as described in WO2015/014888 A1. Briefly, ethanol, distilled water (6 M) and ammonium hydroxide (0.13 M) were mixed and stirred at 400 rpm for 1 h. TEOS (0.28 M) was added, and the solution was stirred at 400rpm at 20°C for 22h. The solution was then centrifuged at 20000 g for 20 min and washed successively with ethanol and water. Particle size measurement was
- 30

carried out on SEM micrographs acquired at a magnification of 150000x using the image analysis software Olympus stream motion.

5 ***Production of NP-1:***

To SNPs (10 mg/mL, 55 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.9 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.9 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.9 mM) and stirring the reaction mixture for 10 min at 20°C,
10 400 rpm. Engineered PAL (11.9 mg/mL, 1 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized engineered PAL using APTES (7.7 mM) and TEOS (80.8 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and
15 resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 121 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in NaCl (0.9%)/PS80 (8 mg/L). NP-1 was cured overnight in a water bath at 20°C.

20

Production of NP-2:

To SNPs (10 mg/mL, 56 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.8 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.8 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was
25 performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. A BSA solution was added to achieve a final BSA concentration of 1.42 mg/mL, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized BSA using APTES (7.5 mM) and TEOS (75.4 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles
30 were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 121 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min

at 20000 rcf and washed 3 times in NaCl (0.9%)/PS80 (8 mg/L). NP-2 was cured overnight in a water bath at 20°C.

Production of NP-1 variants:

5 The following experiments explored the impact of covalently linking an enzyme to a protective layer on enzyme stability and enzyme activity, respectively.

In a first experiment, nanoparticles (NP-1(1)) were produced in H₂O/PS80 (8 mg/L). Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 59 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.9 mM). The reaction
10 mixture was allowed to react for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). Then, glutaraldehyde (3.9 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A priming was performed by adding APTES (3.9 mM) and stirring the reaction mixture for 10 min at 20°C,
15 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). Engineered PAL (11.9 mg/mL, 1 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized engineered PAL using APTES (7.7 mM) and TEOS (80.8 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were
20 washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 121 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O/PS80 (8 mg/L). NP-1(1) were cured overnight in a
25 water bath at 20°C.

In a second, comparative experiment, enzyme immobilisation and formation of the protective layer were carried out according to WO2015/014888 A1 to produce nanoparticles (NP-1(2)) in buffer. Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 59 nm) in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L)
30 was added APTES (3.9 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Then, glutaraldehyde (3.9 mM) was added, and the reaction mixture was stirred for 10 min at 20°C,

400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). A priming was performed by adding APTES (3.9 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Engineered PAL (11.9 mg/mL, 1 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized engineered PAL using APTES (7.7 mM) and TEOS (80.8 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 121 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). NP-1(2) were cured overnight in a water bath at 20°C.

In a third experiment, nanoparticles (NP-1) were produced in H₂O/PS80 (8 mg/L) according to the section headed “Production of NP-1” above. To keep the excess amount of glutaraldehyde which has not linked the solid carrier to the engineered PAL in the reaction mixture, the nanoparticles were not washed between each chemical step. Therefore, glutaraldehyde was still present during layer growth and caused a covalent binding of the protective layer to the engineered PAL. The covalent binding of the protective layer to the engineered PAL can be observed by the appearance of a yellow/orange color that has an absorbance maximum at 460 nm. This color is due to the formation of an imine bond by reaction between the aldehyde functions of the glutaraldehyde linker and the primary amines of the amino acids of the engineered PAL and the organosilica layer. The absorbance of nanoparticles NP-1(1), NP-1(2), and NP-1 at 460 nm was measured after the organosilica layer formation and final particles washing i.e. after the organosilica layer was formed and the particles were washed 3 times in H₂O/PS80 and resuspended in H₂O/PS80 as described the section headed “Production of NP-1” above, showing a much higher absorbance at 460 nm for NP-1 than for NP-1(1) and NP-1(2) (see Figure 8). NP-1(1) and NP-1(2) still show absorbance to some degree at this wavelength, as imine bonds are also formed during enzyme immobilization. However, the absorbance of NP-1 is significantly higher indicating an additional formation of imine bonds caused by covalent binding of the protective layer to the engineered PAL.

Activity assay of NP-1:

In a 96-well plate, NP-1 (20 μ L, 1.75 mg/mL) in Tris buffer (0.1 M, pH 7.5) was mixed with L-Phe solution (180 μ L, 50 mM). PAL kinetics was monitored for 30 minutes at 37°C in a spectrophotometer at $\lambda = 290$ nm.

Resistance to external stresses:***- Resistance to acidic conditions***

Engineered PAL or NP-1 were incubated at pH4 over a period of 24. The enzymatic activity was assessed at 0, 1, 3, 6 and 24h as described in “Activity assay of NP-1”.

- Resistance to proteases

Engineered PAL or NP-1 were treated with pancreatin (30mU) or pronase (0.8U) and incubated at 37°C under shaking at 300rpm over a period of 4h. The enzymatic activity was assessed at 0, 0.1, 0.25, 0.5, 1, 2 and 4h as described in “Activity assay of NP-1”-

Cell culture:

For all experiments, cells were cultured at 37°C and 5% CO₂.

Caco2 (human colorectal adenocarcinoma cell line) and HT29-MTX-E12 (human colon cancer cell line) cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 1% non-essential amino-acid and 100 U/mL penicillin/streptomycin. For the development of the intestinal barrier model, cells were seeded at a density of 2.6×10^5 cells/cm² in transwell PET inserts (1 μ m pore size). All cell models were used for experiments on day 21. For the co-culture, Caco-2 and HT-29-MTX-E12 cells were used at a ratio 75%-25%.

Transepithelial electrical resistance

The integrity of the cell barrier was assessed by the measurement of the transepithelial electrical resistance (TEER) using the CellZscope system (NanoAnalytics). After cell culture medium refreshment and treatment with nanoparticles, automated measurements of the TEER for up to 24h every 15 minutes with a range from 1Hz to 100'000Hz.

In vitro metabolism of Phe

Differentiated Caco-2/HT29-MTX-E12 co-culture in white DMEM supplemented with 1% heat-inactivated fetal calf serum, 2mM L-glutamine, 1% non-essential amino-acid and 100 U/mL penicillin/streptomycin (here after referred as “cell medium”).

The intestinal barrier was exposed to NP-1 (9.7mU) or engineered PAL (9.7mU) in presence of pancreatin (30mU) for 6h at the apical side of the barrier. At each timepoint, aliquots of 150uL were withdrawn from the basolateral sides of the intestinal barrier and replaced with the same volume of pre-warmed cell medium. The barrier was further incubated at 37°C. The absorbance of the withdrawn aliquot samples was measured at 290nm to quantify the level of trans-cinnamic acid (TCA).

Animals:

All animal experimentations were carried out under a license approved by the National Animal Experiments Inspectorate under the Ministry of Food, Agriculture and Fisheries of Denmark.

- Wistar rats:

The study was performed in male Wistar rats (8 weeks of age) of the stock from Janvier, France.

○ Diet and drinking water:

The rats were fed with a pelleted complete diet “Altromin 1324” available *ad libitum*. They had access *ad libitum* to drinking water.

○ Duodenum catheterization

Animals were anesthetized with isoflurane (2-4%) in an induction chamber before being moved to a nose cone with isoflurane for the surgery. A catheter (C30PU-RDD1444, Instech Laboratories) was placed in the duodenum on the antimesenteric side close to the opening of the biliopancreatic duct. The catheter was ligated to intestinal wall and subcutaneously tunneled to the neck of the animals where it is exteriorized. The abdomen and the incision in the neck were thereafter closed with sutures. The animals were kept on heating during the entire procedure and closely monitored until fully recovered from anesthesia.

○ Evaluation of NP-1 efficacy in rats

Before dosing and d5-L-Phe administration, animals were starved for 4h. Then, rats were dosed intraduodenally with NP-1 (0.85U) or NP-2 (8.5mg) and immediately gavaged with 3,6mg of d5-L-Phe. Rats were hosted in metabolic cage for 24h.

○ *Urine sampling, metabolic cages*

Urine was collected in metabolic cages for 24h. The total urine output was obtained, and the urine was sampled in Eppendorf tubes and stored at -80°C until shipped for analysis.

○ *Measurement d5-hippuric acid in urine of rats:*

- 5 Quantification of analytes of interest was performed using the LC system: Thermo Vanquish Horizon Binary Pump and the mass spectrometer: Thermo Q Exactive. Urine samples were prepared as follow: 50uL of urine sample were spiked on 5uL of ISTD (100µM 13C6-HIP, final conc. 2µM in each sample), and 200uL of 100% methanol was added before vortexing. After 20min on ice, samples were centrifugated for 10 min at 16000g at 4°C. The supernatant was
10 transferred into total recovery MS glass vials for analysis.

The injection volume used was 2.5uL and the run time was 4.8 min at a flow rate of 1mL/min. Mobile phase A was H₂O, formic acid (0.1%) and mobile phase B was Methanol, formic acid (0.1%). Chromatographic separation was carried out using Waters Premier BEH C18 column (50mmx2.1mm) (with the following gradient: from 10% B to 100% B).

- 15 The MS was performed by using the mass spectrometer Thermo Q Exactive with the acquisition mode DDA top5. The MS parameters were the following: MS1 resolution: 70'000 and MS2 resolution: 17'500. The HCD fragmentation was performed with normalized stepped collision energy 10, 20 and 30. Data analysis was performed in Thermo quan Browser software.

20 - *BTBR-Pah^{enu2}/J:*

The study was performed in male and female BTBR-Pah^{enu2}/J mice (8 weeks of age) of the stock from The Jackson Laboratory, USA.

○ *Diet and drinking water:*

- The mice were fed with a phenylalanine free diet (5LF2, LabDiet) available *ad libitum*. They
25 had access *ad libitum* to drinking water.

○ *Duodenum catheterization*

- Animals were anesthetized with isoflurane (2-4%). An incision was made to open the abdominal cavity through the linea alba, and a catheter (C19PB-MGI1923, Instech Laboratories) was placed in the duodenum through the antimesenteric side of the duodenum. The tip of the catheter
30 was advanced and positioned close to the opening of the biliopancreatic duct. The catheter was ligated to the intestinal wall and tunneled subcutaneously to the neck of the animals where it was exteriorized and closed. The abdominal wall and the incision in the neck were thereafter

closed with sutures. The animals were kept on a warm bed during the entire procedure and will be closely monitored until fully recovered from anesthesia.

○ *Evaluation of NP-1 efficacy in mice*

Before being used in the studies, mice were maintained under phenylalanine free diet for at least 3 days, then the drinking water was supplemented with L-Phe at low concentration (0.03g/L) for 3 days, after which the concentration of L-Phe was increased in the drinking water to 0.5g/L. Mice had access to L-Phe supplemented drinking water *ad libitum* during the night phase. Mice were dosed intraduodenally with NP-1 (0.581U, 7mg), NP-2 (7mg) or engineered PAL (0.581U) twice per day over a period of 12 days. Blood samples were taken in EDTA at days 0, 4, 6, 8, 10 and 12. Blood samples were then centrifuged (10 min, 4°C, 2000 x g), and a minimum of 20 uL plasma was transferred into Eppendorf tubes and stored at -80°C until analysis for Phe content.

○ *Measurement of plasmatic Phe of mice:*

Quantification of analytes of interest was performed using the LC system: Thermo Vanquish Horizon Binary Pump and the mass spectrometer: Thermo TSQ Quantiva.

Plasma samples were prepared as follows: 20uL of plasma sample were centrifuged at 13.2krpm for 10 min at 4°C. Ten microliters of supernatant were added to 10uL of ISTD (1000µM D5-Phe) and 80µL 100% MeOH. The samples were then vortex and centrifuged at 13.2krpm for 10 min at 4°C, after which 50uL of supernatant were dried under a gentle stream of nitrogen at 30°C. Five hundred microliters of 0.1% (v/v) formic acid in water were added and the samples were shaken at 900rpm for 10min at 15°C before centrifugation (13.2krpm for 10 min at 4°C). Finally, 350uL of supernatant were transferred into total recovery glass vials for analysis.

The injection volume used was 2uL and the run time was 5 min at a flow rate of 1mL/min. Mobile phase A was H₂O, formic acid (0.1%) and mobile phase B was Methanol, formic acid (0.1%). Chromatographic separation was carried out using Waters Premier BEH C18 column (50mmx2.1mm) (with the following gradient: from 100% A to 100% B).

The MS is performed by using the mass spectrometer Thermo TSQ Quantiva with the acquisition mode: Selected reaction monitoring.

The MS parameters were the following: Q&1 resolution: 0.7; Q3 resolution: 0.7; fragmentation: CID fragmentation with argon (1.5mTorr). The analyte concentration was calculated from the peak area ratio of Phe to the internal standard d5-Phe. Data analysis was performed in Thermo quan Browser software.

Results:**Example 1: *Enhancing Enzyme Stability and Loading through Covalent Attachment to the protective layer***

In a first experiment, nanoparticles NP-1(1) were produced in non-buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth).
5 In a second experiment, nanoparticles NP-1(2) were produced in buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth).
In a third experiment, nanoparticles NP-1 were produced in non-buffered conditions without any intermediate washing steps (*i.e.* unreacted glutaraldehyde still present in the reaction
10 mixture during layer growth).

Protein quantification was performed on the reaction supernatants to determine PAL immobilization yield at the surface of NP-1(1), NP-1(2) and NP-1. The results show that surprisingly enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-1) increases the enzyme immobilization yield by a factor of 2 (Fig. 2A),
15 resulting in a doubling of enzyme loading per dry weight of SNP (Fig. 2B) compared to buffered conditions where glutaraldehyde is removed by washing steps (NP-1(2)). Similarly, enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-1) results in a 1.5 times higher enzyme loading per dry weight of SNP (Fig. 2B) compared to unbuffered conditions where glutaraldehyde is removed by washing steps (NP-1(1)).

20 The biocatalytic activity of PAL immobilized and protected on NP-1(1), NP-1(2), and NP-1 was evaluated. Even more surprisingly than the increase in load by the enzyme immobilization where the presence of glutaraldehyde is maintained, was the threefold increase in nanoparticle specific activity compared to buffered conditions where glutaraldehyde is removed by washing steps and the two times increase compared to unbuffered conditions where glutaraldehyde is
25 removed by washing steps (Fig. 2C). This extremely surprising threefold increase in nanoparticle specific activity goes hand in hand with the specific activity (Units / g PAL) of the enzyme immobilized in unbuffered conditions where the presence of glutaraldehyde is maintained, which is comparable to the specific activity of the enzyme immobilized in buffered conditions. This result is completely unexpected as the enzyme is supposed to have a much
30 higher activity in the presence of a buffer (Fig. 2 D). In summary, covalent attachment of the protective layer to the enzyme surface unexpectedly enhances its load and stability compared to enzymes protected with an organosilica layer via electrostatic interactions only.

Example 2: Phenylalanine ammonia lyase (PAL) activity of NP-1

The biocatalytic activity of immobilized and protected engineered PAL was assessed. The result as displayed in figure 3 reports a PAL activity on NP-1. This shows the ability of the shielded functionalized SNP to access and to convert L-Phe despite the shield and the functionalization.

- 5 The validation of the biocatalytic activity on NP-1 confirms the possibility of using the nanoparticles for therapeutic purposes.

Example 3: Resistance to external stresses

- NP-1 is a nanoparticle that has been developed for gastrointestinal applications. Due to the physiological properties of the gastrointestinal tract, NP-1 will be submitted to various stresses. To ensure a sustained activity of NP-1 in the gastrointestinal tract, the protection of immobilized engineered PAL was assessed. First, NP-1 or engineered PAL were submitted to acidic conditions (pH4). The monitoring of the PAL activity over a period of 24h reveals a sustained enzymatic activity on NP-1 (Fig.4B) while the free form of engineered PAL loses its activity over the time (Fig.4A). These data demonstrated the protection of the immobilized and protected engineered PAL on NP-1 in an acidic environment.

- During the digestion, pancreatic enzymes are released, and notably proteases that could affect therapeutic enzymes in the gastrointestinal tract. Thus, NP-1 and engineered PAL were exposed to various proteases at 37°C. First, to mimic the physiological digestive conditions, the PAL activity was assessed after coincubation of NP-1 or engineered PAL with pancreatin (30mU), a mixture of pancreatic enzymes extracted from porcine pancreas. After 4h, both NP-1 and engineered PAL show a sustained PAL activity (Fig.4C). Then, to further evaluate the benefits of the protective shield in harsh conditions, NP-1 or engineered PAL were exposed to pronase (0.88U), a cocktail of purified proteases. The results as displayed in figure 4C show a maintenance of 80% of the PAL activity on NP-1 after 4h of exposure while the enzymatic activity of engineered PAL is lost after 4h. Altogether, these data demonstrate the added value of the immobilization and protection of engineered PAL on the nanoparticles and emphasize the use of NP-1 for gastrointestinal therapeutic applications.

30 **Example 4: In vitro biocompatibility and efficacy of NP-1**

The maintenance of the integrity of the intestinal barrier is fundamental to prevent undesirable luminal contents such as pathogens or food allergens from entering in the body. To evaluate the biocompatibility of nanoparticles, Caco2-HT29-MTX-E12 cell monolayers were exposed to

NP-1 in presence or not of pancreatin to mimic digestive conditions and the transepithelial electrical resistance (TEER) was measured. Data shown on figure 5A report a maintenance of the integrity of the intestinal epithelial barrier when in contact with NP-1 with or without pancreatin for 6h. This result demonstrates the *in vitro* biocompatibility of NP-1 for gastrointestinal applications.

NP-1 has been developed to metabolize Phe in the lumen of the intestine. To evaluate the *in vitro* efficacy of NP-1, Caco2-HT29-MTX-E12 cell monolayers cultivated in cell medium containing 0.4mM of L-Phe were exposed at their apical side to NP-1 (9.7mU) or engineered PAL (9.7mU) in presence or not of pancreatin (30mU) for 6h. The quantification of the product of Phe metabolism in the basal side of the barrier is shown on figure 5B. The graph reports an accumulation of TCA in the basolateral side of the barrier in all conditions. This result demonstrates the *in vitro* efficacy of NP-1 in a digestive environment and suggests the use of NP-1 for therapeutic applications.

Example 5: *In vivo* activity of NP-1 in rats

In order to evaluate the translation from *in vitro* to *in vivo* application, the efficacy of NP-1 was assessed in rats. Rats were dosed intraduodenally with NP-1 (nanoparticles comprising engineered PAL) or NP-2 (nanoparticles comprising bovine serum albumin (BSA) for which Phe is not a substrate) prior being gavaged with d5-Phe. *In vivo*, the product of Phe metabolism, TCA, is rapidly metabolized into hippuric acid. The *in vivo* activity of NP-1 was then evaluated by the quantification of d5-hippuric acid in rat urine collected for 24h post dosing. The significant increase of d5-hippuric acid in rats dosed with NP-1 compared to NP-2 shown in figure 6 demonstrates the ability of NP-1 to digest Phe in the luminal compartment of the intestine, and suggest the use of NP-1 for therapeutic applications.

Example 6: *In vivo* therapeutic efficacy of NP-1 in mice

Phenylketonuria (PKU) is characterized by a deficiency in the intracellular liver enzyme phenylalanine hydroxylase (PAH). PAH catalyzes the conversion of the essential amino acid phenylalanine to tyrosine. PAH deficiency results in an abnormally elevated concentrations of phenylalanine, which is toxic to the brain. The cornerstone of PKU treatment is a low phenylalanine diet in combination with phenylalanine-free L-amino acid. Currently, an enzyme substitution therapy with recombinant phenylalanine ammonia lyase is available for

subcutaneous injections, but this treatment induces hypersensitivity reactions and immune-mediated acute hypersensitivity reactions.

NP-1 has been developed to exhibit a sustained PAL activity in gastrointestinal environment (acidity and exposure to proteases). Our approach to control the Phe level in patients is to
5 degrade the Phe in the intestine (from food intake) to avoid its absorption and its accumulation in the blood.

This therapeutic strategy was assessed using a representative animal model of the disease, BTBR-*Pah*^{enu2}/J mice in which the gene encoding for PAH is mutated.

Mice having access to L-Phe supplemented drinking water were dosed twice a day with NP-1,
10 NP-2 or engineered PAL over 12 days. Impressively, a steady decrease of Phe plasmatic level is reported in mice dosed with NP-1 over the period of the study, while the plasmatic concentration of mice dosed with engineered PAL is unstable (Fig. 7). The normalization of the plasmatic concentration of Phe as presented on Fig. 7B highlights a decrease of 30% of plasmatic Phe concentration in mice at the end of the treatment with NP-1.

15 The interaction of the nanoparticles with the intestinal mucus results in a temporary engraftment of NP-1 and in a sustained PAL activity on the wall of the intestine, while the free form of engineered PAL is flushed down in the intestine. These results validate and anchor the strategy consisting of the degradation of Phe in the intestine and support the surprising therapeutic efficacy of NP-1 for PKU.

Claims

1. A composition comprising a solid carrier, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof immobilized on the surface of the solid carrier, a
5 protective layer to protect the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof by embedding the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit
10 comprises at least one amino group and/or at least one thiol group.
2. The composition according to claim 1, wherein the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is a polyglucosamin selected from the group consisting of chitin, chitosan,
15 polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof.
3. The composition according to claim 1, wherein the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol
20 group is chitosan or a derivative thereof.
4. The composition according to any one of claims 1-3, wherein the functional constituent is immobilized on the surface of the protective layer by non-covalent binding or by covalent binding.
25
5. The composition of any one of claims 1-4, wherein the protective layer embeds the solid carrier and embeds the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof immobilized on the surface of the solid carrier.
- 30 6. The composition of any one of claims 1-5, wherein the functional constituent immobilized on the surface of the protective layer is not embedded by the protective layer.

7. The composition according to any one of claims 1-6, wherein the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof
(a) comprises or consist of an amino acid sequence having at least 95%, at least 96%, or at least 97% sequence identity to the sequence of SEQ ID NO: 1; or
5 (b) is SEQ ID NO: 2, 3, 4 or 5.
8. The composition according to anyone of claims 1-6, wherein the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof comprises the polypeptide as shown in SEQ ID NO: 5.
10
9. The composition of any one of claims 1-8, for use as a medicament.
10. The composition of any one of claims 1-8, for use in a method for the prevention, delay of progression or treatment of phenylketonuria (PKU).
15
11. A method of producing a composition, the composition comprising a solid carrier, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof by embedding the
20 engineered phenylalanine ammonia lyase (PAL) or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:
25 (a) providing a solid carrier;
(b) immobilizing an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof on the solid carrier;
(c) forming a protective layer on the surface of the solid carrier to protect the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof
30 immobilized on the solid carrier;
(d) immobilizing a functional constituent on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at

least one amino group and/or at least one thiol group.

- 5 12. The method of claim 11, wherein in step (b), i) a linker is added to the solid carrier provided in step (a), and ii) the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof.
- 10 13. The method of claim 12, wherein the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b), is present during formation of a protective layer on the surface of the solid carrier in step (c).
- 15 14. The method of claim 12, wherein there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof to the solid carrier and the linker in ii).
- 20 15. The method of any one of claims 12-14, wherein there is no washing step between any of steps (a) to (c).
- 25 16. The method of any one of claims 12-15, wherein the linker which has not connected the solid carrier with the engineered phenylalanine ammonia lyase (PAL) or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the phenylalanine ammonia lyase (PAL) or the fragment thereof in step (c).
- 30 17. The method of any one of claims 12-16, wherein the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOE (Bis[2-(succinimidocarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3 '-

Dithiobis[sulfosuccinimidyl]propionate]), DTBP (Dimethyl 3,3'-dithiobispropionimidate·2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4-bismaleimidyl-2,3-dihydroxybutane).

5 18. The method of any one of claims 12-16, wherein the linker is glutaraldehyde.

19. A composition comprising a solid carrier, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof by embedding the engineered phenylalanine ammonia lyase (PAL) or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group,
10
15 wherein the composition is obtainable by the method of any one of claims 13-18.