

Method for immobilization of proteins

The field of the invention

The present invention relates to a method of producing a composition, the composition
5 comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the
solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the
protein or a fragment thereof, and optionally 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
10 least one amino group and/or at least one thiol group. The present invention also relates to the
composition obtainable by the method.

Background of the invention

Proteins such as enzymes are frequently needed, e.g. in industrial applications, diagnostics or
15 for therapeutic use. In order to stabilize the proteins and/or to provide resistance to various
types of stresses it has been suggested in the prior art to immobilize the proteins on the surface
of a carrier and to protect them with a layer of protective material. Such an approach has been
described e.g. in WO2015/014888 A1 which discloses a biocatalytical composition comprising
a solid carrier, a functional constituent like an enzyme and a protective layer for protecting the
20 functional constituent by embedding the functional constituent at least partially and a process
to produce such biocatalytical composition. Nevertheless, the method described in
WO2015/014888 A1 is difficult to use in large scale as the load of protein per dry weight of
particle is low. Thus there is a need for providing improved immobilization methods in order to
obtain a high protein load per dry weight of particle, enabling the particles produced to have
25 high enzymatic activity.

Summary of the invention

The present invention provides a method of producing a composition, the composition
comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the
30 solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the
protein or a fragment thereof, and optionally 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:

- 5 (a) providing a solid carrier, wherein the solid carrier is provided in suspension;
- (b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment thereof is added to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;
- 10 (c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the protein or the fragment thereof; and optionally
- (d) immobilizing a functional constituent on the surface of the protective layer, wherein the
- 15 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 provides a composition comprising a solid carrier, a protein or a

20 fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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

25 least one thiol group, wherein the composition is obtainable by the methods described herein.

It has been surprisingly found by the inventors of the present application that the methods as provided by the present invention and described herein can be used in large scale protein immobilization as they provide a high load of immobilized protein per dry weight of particle

30 which significantly increases the activity of particles.

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 PAL surface to the protective layer. (A) PAL quantification performed on reaction supernatants of PAL-based silica nanoparticles NP-1(1), NP-1(2) and NP-1. (B) PAL loading per dry weight of SNP. (C) PAL activities of SNPs 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 PAL resistance to external stresses. PAL-based silica nanoparticles 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 PAL-based silica nanoparticles 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 PAL-based silica nanoparticles 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 PAL-based silica nanoparticles NP-1 (n=5) or inactive nanoparticles 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.

Figure 7) shows the plasmatic concentration of Phe in BTBR-*Pah^{emu2}*/J mice. BTBR-*Pah^{emu2}*/J mice having *ad libitum* access to drinking water containing L-Phe were dosed intraduodenally with PAL-based silica nanoparticles NP-1 (0.581U; 7mg), inactive nanoparticles 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^{emu2}*/J mice. (B) Graph shows the normalized plasmatic concentration of Phe in BTBR-*Pah^{emu2}*/J mice.

Figure 8) shows absorbance of nanoparticles PAL-based silica nanoparticles NP-1, NP-1(1) and NP-1(2) at 460 nm.

Figure 9) shows a schematic representation of the process for the production of the composition of the invention: a) a disaccharidase or fragment thereof (indicated as “Protein”) is immobilized on the solid carrier; b) and c) a protective layer grows around the immobilized disaccharidase or fragment thereof embedding the immobilized disaccharidase or fragment thereof; and d) a functional constituent is immobilized on the surface of the protective layer.

Figure 10) shows the added value of the covalent bonding of the lactase surface to the protective layer. (A) Lactase quantification performed on reaction supernatants of lactase-based silica nanoparticles NP-2(1), NP-2(2) and NP-2. (B) Lactase loading per dry weight of SNP.

Figure 11) shows the disaccharidase activity of the nanoparticles. (A) Lactase activity (in U/g) of lactase-based silica nanoparticles NP-2 after exposure to lactose. (B) Invertase activity (in U/mg) of invertase-based silica nanoparticles NP-3 after exposure to sucrose. (C) Isomaltase activity (in U/g) of isomaltase-based silica nanoparticles NP-4 after exposure to isomaltose. (D) Isomaltase and Invertase activity (in U/g) of invertase/isomaltase-based silica nanoparticles NP-5 after exposure to isomaltose and sucrose, respectively.

Figure 12) shows the *in vitro* biocompatibility of a representative model of nanoparticles on an 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 inactive nanoparticles NP-1 (0.5mg/mL and 1mg/mL) for 16h. TEER data was normalized to the control point consisting in the equilibrium value before the addition of inactive nanoparticles NP-1 (defined as control) and set at 100%. The

graph represents the time course profile evolution of averaged normalized TEER data over 16h. The dash line represents the untreated condition. (B) *In vitro* evaluation of inflammatory effects on intestinal epithelial barrier. Differentiated Caco2-HT29-MTX-E12 and M0-differentiated THP-1 were co-cultured and exposed to inactive nanoparticles NP-1(1mg/mL) at 37°C for 16h. Lipopolysaccharide (LPS) (50 and 100ug/mL) was used as positive control to induce inflammatory response. TEER data was normalized with the equilibrium value before the addition of inactive nanoparticles NP-1 or LPS set at 100%. The graph represents the time course profile evolution of averaged normalized TEER data over 16h in presence of inactive nanoparticles NP-1.

Figure 13) shows the differences in the size of cecum in rats. Wistar rats were daily dosed intraduodenally with lactase-based silica nanoparticles NP-2, inactive silica nanoparticles NP-1, or the vehicle and immediately gavaged with lactose over a period of 15 days. At termination, the size of the cecum was evaluated. (A) Pictures of the gastrointestinal tract in rats. The circles show the cecum. (B) MRI images of the gastrointestinal tract in rats. The arrows show the cecum. (C) Histogram shows the cecum size in cm³ assessed by MRI imaging. *p<0.05, **p<0.01 by one-way ANOVA test.

Figure 14) shows the *in vitro* digestion of sucrose on a model of intestinal barrier. Differentiated Caco-2/HT29-MTX-E12 co-culture were exposed to different amount of invertase-based silica nanoparticles NP-3 (0.5mU or 1mU) in presence of sucrose for 4h at the apical side of the barrier. The hydrolysis of sucrose was evaluated by the quantification of glucose in the basal side of the barrier. The graph shows the time course profile evolution of the accumulation of glucose over 4h.

Figure 15) shows absorbance of nanoparticles lactase-based silica nanoparticles NP-2, NP-2(1) and NP-2(2) at 460 nm.

Figure 16) shows a schematic representation of the process for the production of the composition of the invention: a) to a solid carrier, a lipase with closed lid, a protease, an amylase and an agent (displayed as round circle) which interacts with the lid domain of the lipase is provided and the lipase with an open lid, the protease and the amylase are immobilized on the solid carrier; b) and c) a protective layer grows around the immobilized lipase with an open lid, the protease and the amylase, embedding all three enzymes) and d) a functional constituent is immobilized on the surface of the protective layer.

Figure 17) shows the biodistribution of inactive DOTA-labelled silica nanoparticles functionalized with chitosan ^{111}In -NP-1 (n=5) and inactive DOTA-labelled silica nanoparticles non-functionalized ^{111}In -NP-2 (n=4) (%ID) in minipigs. (A) SPECT/CT images were acquired at 0.25, 3, 8 and 24 hours after intraduodenal dosing with ^{111}In -NP-1 and ^{111}In -NP-2. Graphs represent the relative quantification (in %) of nanoparticles at imaging time points for the small intestine compartment. (B) Histograms show the area under the curves (AUC) of ^{111}In -NP-1 and ^{111}In -NP-2 residence time in the small intestine. (C) Blood sampling were taken at 0.25, 3, 8 and 24 hours after intraduodenal dosing with ^{111}In -NP-1 or free ^{111}In . Histograms represents the relative quantification of radioactivity in the blood compartment.

Figure 18) shows the activities of lipase and/or a fragment thereof, protease and/or a fragment thereof and amylase and/or a fragment thereof comprised by pancreatin as immobilized and protected on the nanoparticles. (A) Lipase activity (in U/g) of pancreatin-based silica nanoparticles NP-3 after exposure to olive oil. (B) Protease activity in (U/mg) of pancreatin-based silica nanoparticles NP-5 after exposure to casein. (C) Amylase activity (in U/g) of pancreatin-based silica nanoparticles NP-3 after exposure to amylase substrate solution.

Figure 19) shows the relative quantification of plasmatic triglycerides (TG) of pancreatic duct ligated (PDL) rats. PDL rats were dosed with pancreatin-based silica nanoparticles NP-3 (n=1) or inactive nanoparticles NP-4 (n=1) intraduodenally and 5 minutes later they were gavaged with triolein. Blood samples were taken before and at 0.25, 0.5, 1, 1.5, 2, 4, 6h after treatment for plasma extraction and analysis by LC-MS. Graphs show the peak area of triolein (TG(54:3)).

Figure 20) shows the comparison of plasmatic triglycerides (TG) concentration between healthy minipigs and pancreatic duct ligated (PDL) minipigs treated with pancreatin-based silica nanoparticles NP-3. Healthy minipigs (n=1) and PDL minipig (n=1) received olive oil, and sequentially, PDL minipig was dosed with pancreatin-based silica nanoparticles NP-3. Blood samples were taken before dosing and at 0.083, 0.25, 0.5, 1, 2, 3,4, and 6h after the dosing for TG analysis. (A). Graphs show the plasmatic concentration of TG in minipigs determined with Konelab analyzer. (B) Histograms show the area under the curves (AUC) of plasmatic concentration of TG in minipigs.

Figure 21) shows the fecal fat content of minipigs under high fat diet. Healthy and PDL minipigs were fed with high fat diet over a period of 25 days. PDL minipigs were dosed with pancreatin-based silica nanoparticles NP-3 twice a day for 10 days. Feces were collected at

days 8, 9 and 10, and fecal homogenates were analyzed by near infrared spectroscopy for fecal fat quantification. (A) The histograms show the absolute fecal fat measurements. (B) The graph shows the normalized fecal fat homogenate fat content relative to healthy and untreated PDL minipigs.

5 **Figure 22)** shows the *in vitro* biocompatibility of pancreatin-based silica nanoparticles NP-5 on an 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 increasing amount of pancreatin-based silica nanoparticles NP-5 or pancreatin (from 32.9 to 263.6 U/m²) for 20h. TEER data was
10 normalized with the equilibrium value before the addition of pancreatin-based silica nanoparticles NP-5 or pancreatin set at 100%. The graph represents the time course profile evolution of averaged normalized TEER data over 20h. The dash line represents the untreated condition. (B) Confocal images of tight junctions on the intestinal barrier model. Differentiated Caco-2/HT29-MTX-E12 co-culture were exposed to pancreatin-based silica nanoparticles NP-
15 5 or pancreatin (263.6 U/m²) for 20h. Cells were stained with zonula occludens 1 (ZO-1) and assessed by confocal microscopy (white signal on images).

Figure 23) shows the added value of the covalent bonding of pancreatin surface to the protective layer. (A) Protein quantification performed on reaction supernatants of pancreatin-based silica nanoparticles NP-3 and NP-3(1). (B) Pancreatin loading per dry weight of SNP.
20 (C) Lipase activities of SNPs expressed in $\mu\text{mol/min}$. (D) Pancreatin-specific activities expressed in U/g of pancreatin.

Figure 24) shows absorbance of nanoparticles pancreatin-based silica nanoparticles NP-3 and NP-3(1) at 460 nm.

Figure 25) shows a schematic representation of the process for the production of the
25 composition of the invention: a) to a solid carrier, a lipase or a fragment thereof with closed lid and an agent (displayed as round circle) which interacts with the lid domain of the lipase or a fragment thereof is provided and the lipase or a fragment thereof with an open lid is immobilized on the solid carrier; b) and c) a protective layer grows around the immobilized lipase or the fragment with an open lid thereof embedding the immobilized lipase or the
30 fragment thereof.

Figure 26) shows the 3D structure of pancreatic lipase in a) its inactive conformation (with closed lid) and b) active conformation (with opened lid). The active site of pancreatic lipase is

covered by a lid that prevents substrates from reaching the enzyme active site (Figure 26a).

The opening of the lipase lid is induced by interactions with an agent which interacts with the lid domain of the lipase e.g. bile salts and/or a protein cofactor called colipase, allowing the stabilization of the active conformation of pancreatic lipase (Figure 26b).

- 5 **Figure 27** shows the kinetics of lipase substrate hydrolysis by recombinant human pancreatic lipase (HRL) with or without colipase (CLPS): a) in its free form; b) immobilized at the surface of silica nanoparticles (SNPs) and protected in an organosilica layer made of APTES, TEOS and Benzyltriethoxysilane (ATB).

- Figure 28** shows the kinetics of lipase substrate hydrolysis by porcine pancreatic lipase (PL) with or without colipase (CLPS) immobilized at the surface of silica nanoparticles (SNPs) and protected in an organosilica layer made of APTES, TEOS and Benzyltriethoxysilane (ATB).

Figure 29 shows the kinetics of lipase substrate hydrolysis by free human recombinant lipase (HRL) using increasing concentrations of sodium taurocholate (NaTc).

- Figure 30** shows the kinetics of lipase substrate hydrolysis by porcine pancreatic lipase (PL) immobilized at the surface of silica nanoparticles (SNPs) and protected in an organosilica layer made of APTES, TEOS and Benzyltriethoxysilane (ATB) with or without sodium taurocholate (NaTc).

Figure 31 shows the 3D structure of pancreatic lipase activated by a colipase-mimicking peptide.

- 20 **Figure 32** shows the added value of the covalent bonding of HRL surface to the protective layer. (A) Protein quantification performed on reaction supernatants of HRL-based silica nanoparticles NP-1, NP-1(1), NP-1(2). (B) HRL loading per dry weight of SNP. (C) Lipase activities of nanoparticles expressed in $U_{\mu M/min/g}$ of SNP. (D) HRL-specific activities expressed in $U_{\mu M/min/g}$ of HRL.

- 25 **Figure 33** shows absorbance of nanoparticles HRL-based silica nanoparticles NP-1, NP-1(1), NP-1(2) at 460 nm.

Detailed description of the invention

- The present invention relates to a method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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:

- 5 (a) providing a solid carrier, wherein the solid carrier is provided in suspension;
- (b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment thereof is added to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;
- 10 (c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the protein or the fragment thereof; and optionally
- (d) immobilizing a functional constituent on the surface of the protective layer, wherein the
- 15 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 provides a composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect

20 the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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 described herein.

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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 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.

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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

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
5 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 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.

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

The term “about” refers to a range of values $\pm 10\%$ of a specified value. For example, the
15 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 is a monodisperse particle or a polydisperse particle, more preferably a monodisperse particle. The solid carrier usually comprises organic particles, inorganic particles, organic-inorganic
20 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 1 nm and 1000 μm , preferably between 10 nm and 100 μm , particularly about 50 nm.

25 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 connects the surface of the solid carrier with the protein or a fragment thereof i.e. with the enzyme e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the
30 amylase or a fragment thereof. 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 protein or a fragment thereof i.e. the enzyme e.g. the lipase or a fragment thereof, the protease or a fragment thereof

- and the amylase or a fragment thereof may be bound to an unoccupied binding-site of the linker. Alternatively, the linker may firstly bind to the protein or a fragment thereof i.e. the enzyme e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof and then the linker bound to the enzyme e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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.
- 10 The term “protective layer” as used herein refers to a layer for protecting the functional properties of the protein or fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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
- 15 covalent binding and the immobilized protein or fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof usually by non-covalent binding. The protective layer is formed on the surface of the solid carrier to protect the protein or the fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the solid carrier. The
- 20 protective layers are usually homogeneous layers where at least 50%, preferably at least 70%, more preferably at least 90% of the protein or fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof are embedded in the protective layer.
- 25 The term, "protein or a fragment thereof" includes naturally occurring proteins or a fragment thereof and also includes artificially engineered proteins or a fragment thereof. Artificially engineered proteins or a fragment thereof are e.g. variants or functionally active fragments of the protein. The terms “fragment of a protein”, “fragment thereof” in relation to the protein and “functionally active fragment of a protein” are thus used synonymously herein. By
- 30 “variants or functionally active fragments thereof” in relation to the protein of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the protein. Such variants include

naturally occurring allelic variants and non-naturally occurring variants. 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.

Preferably the functionally active fragment or variant has at least about 80% sequence identity
5 more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the protein. A fragment of a protein as defined herein does usually have the same functional properties as the protein i.e. the full length protein from which it is derived. A fragment of a protein contains usually between 100 and 1000 amino acids, preferably between 150 and 500
10 amino acids, more preferably between 300 and 450 amino acids. A preferred protein or a fragment thereof of the present invention is an enzyme or a fragment thereof, more preferred is an enzyme or fragment thereof selected from the group consisting of hydrolases and lyases, or a fragment thereof. An even more preferred protein or a fragment thereof of the present invention is selected from the group consisting of a lipase or a fragment thereof, a protease or a
15 fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a disaccharidase or a fragment thereof.

The term, "lipase or a fragment thereof" includes naturally occurring lipases or a fragment
20 thereof and also includes artificially engineered lipases or a fragment thereof. Artificially engineered lipases or a fragment thereof are e.g. variants or functionally active fragments of the lipase. The terms "fragment of a lipase", "fragment thereof" in relation to the lipase and "functionally active fragment of a lipase" are thus used synonymously herein. By "variants or functionally active fragments thereof" in relation to the lipase of the present invention is meant
25 that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the lipase. Such variants include naturally occurring allelic variants and non-naturally occurring variants. 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. Preferably
30 the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of

the lipase. A fragment of a lipase as defined herein does usually have the same functional properties as the lipase i.e. the full length enzyme from which it is derived and includes at least the lid domain and the substrate binding region. A fragment of a lipase contains usually between 100 and 450 amino acids, preferably between 150 and 400 amino acids, more preferably between 200 and 350 amino acids. A preferred lipase or a fragment thereof is a lipase or a fragment thereof extracted from pancreas, more preferably extracted from pancreas of porcine origin, even more preferably a lipase or a fragment thereof comprised by pancreatic. In a preferred embodiment the lipase or a fragment thereof is a recombinant human pancreatic lipase (HRL) or a fragment thereof or a porcine pancreatic lipase or a fragment thereof, preferably a recombinant human pancreatic lipase (HRL) or a fragment thereof, more preferably a full length recombinant human pancreatic lipase (HRL).

The term, "protease or a fragment thereof" includes naturally occurring proteases or a fragment thereof and also includes artificially engineered proteases or a fragment thereof. Artificially engineered proteases or a fragment thereof are e.g. variants or functionally active fragments of the protease. The terms "fragment of a protease", "fragment thereof" in relation to the protease and "functionally active fragment of a protease" are thus used synonymously herein. By "variants or functionally active fragments thereof" in relation to the protease of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the protease. Such variants include naturally occurring allelic variants and non-naturally occurring variants. 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. Preferably the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the protease. A fragment of a protease as defined herein does usually have the same functional properties as the protease from which it is derived. A fragment of a protease contains usually between 50 and 200 amino acids, preferably between 75 and 175 amino acids, more preferably between 100 and 150 amino acids. A preferred protease or a fragment thereof is a protease or a fragment thereof extracted from pancreas, more preferably extracted from

pancreas of porcine origin, even more preferably a protease or a fragment thereof comprised by pancreatin.

The term, "amylase or a fragment thereof" includes naturally occurring amylases or a fragment thereof and also includes artificially engineered amylases or a fragment thereof. Artificially engineered amylases or a fragment thereof are e.g. variants or functionally active fragments of the amylase. The terms "fragment of an amylase", "fragment thereof" in relation to the amylase and "functionally active fragment of an amylase" are thus used synonymously herein. By "variants or functionally active fragments thereof" in relation to the amylase of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the amylase. Such variants include naturally occurring allelic variants and non-naturally occurring variants. 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.

Preferably the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the amylase. A fragment of an amylase as defined herein does usually have the same functional properties as the amylase from which it is derived. A fragment of a amylase contains usually between 100 and 550 amino acids, preferably between 200 and 500 amino acids, more preferably between 300 and 450 amino acids. A preferred amylase or a fragment thereof is an amylase or a fragment thereof extracted from pancreas, more preferably extracted from pancreas of porcine origin, even more preferably an amylase or a fragment thereof comprised by pancreatin.

The term "pancreatin" also known as and used interchangeably herein with "pancreatic enzymes" as used herein refers to pancreatic enzyme preparation derived from porcine pancreatic glands and comprises a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof. The term "pancreatin" as used herein also comprises formulated pancreatic enzymes like capsules comprising pancreatic enzymes e.g. Zenpep ®.

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 Alal67-Serl68-Glyl69 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", "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 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 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 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 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. In a preferred embodiment, the engineered phenylalanine ammonia lyase or a fragment thereof comprises or consists of an amino acid sequence having at least 90%, at least 95%, at least 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.

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 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%,
5 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.

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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
15 fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered.

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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
20 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.

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The term, "disaccharidase or a fragment thereof" includes naturally occurring disaccharidases or a fragment thereof and also includes artificially engineered disaccharidases or a fragment thereof. Disaccharidases are glycoside hydrolases, enzymes that break down certain types of sugars called disaccharides into simpler sugars called monosaccharides. In the human body,
30 disaccharidases are made mostly in an area of the small intestine's wall called the brush border. Disaccharidases includes e.g. lactase, maltase, isomaltase, trehalase and sucrase (which is also named invertase). Artificially engineered disaccharidases or a fragment thereof are e.g. variants

or functionally active fragments of the disaccharidase. The terms “fragment of a disaccharidase”, “fragment thereof” in relation to a disaccharidase and “functionally active fragment of a disaccharidase” are thus used synonymously herein. By “variants or functionally active fragments thereof” in relation to the disaccharidase of the present invention is meant that

5 the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the disaccharidase. Such variants include naturally occurring allelic variants and non-naturally occurring variants. 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. Preferably

10 the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the disaccharidase. A fragment of an disaccharidase as defined herein does usually have the same functional properties as the disaccharidase from which it is derived. A fragment of a

15 disaccharidase contains usually between 100 and 1000 amino acids, preferably between 300 and 800 amino acids, more preferably between 500 and 700 amino acids.

The term “partially embedded disaccharidase” as used herein shall mean that the disaccharidase is not fully covered by the protective layer, thus, the disaccharidase is not fully embedded in the

20 protective layer. In one embodiment less than 50% of the disaccharidase of interest are covered by the protective layer, though typically at least 70% will be covered, thus improving protection of the disaccharidase. 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 disaccharidase of interest is covered by the protective layer. In another preferred embodiment,

25 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 disaccharidase 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 disaccharidase of interest is covered by the protective

30 layer. In a more particularly preferred embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the disaccharidase of interest

is covered by the protective layer, wherein the active site is not covered.

The term “fully embedded disaccharidase” as used herein shall mean that the disaccharidase of interest according to the invention is fully, i.e. 100% covered by the protective layer, i.e. that
5 also the active site is covered.

The term “at least partially embedded disaccharidase” as used herein shall mean that the disaccharidase is at least partially embedded and may be fully embedded by the protective layer. Thus “at least partially embedded disaccharidase” means that the protective layer covers from
10 about 30% and 100% of the disaccharidase 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.

15 The term “partially embedded protein” as used herein shall mean that the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is not fully covered by the protective layer, thus, the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is not fully embedded in the protective layer. In one embodiment less than 50% of the protein e.g. the
20 lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof are covered by the protective layer, though typically at least 70% will be covered, thus improving protection of the protein. 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 protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a
25 fragment thereof 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 protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof are covered by the protective layer. In a particularly preferred
30 embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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 protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is covered by the protective layer, wherein the active site is not covered.

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The term “fully embedded protein” as used herein shall mean that the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase 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. Preferably the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof according to the invention are fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered.

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The term “at least partially embedded protein” as used herein shall mean that the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is at least partially embedded and may be fully embedded by the protective layer. Thus “at least partially embedded protein” means that the protective layer covers from about 30% and 100% of the protein or a fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase 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.

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The term “agent which interacts with the lid domain of the lipase or a fragment thereof” as used herein refers to an agent which normally binds to the lid domain of the lipase or a fragment thereof and/or to the region of the lipase or a fragment thereof surrounding the lid domain, thereby causing the lid domain to shift the lipase or a fragment thereof to the open conformation and/or to maintain the open conformation of the lipase or the fragment thereof. The lid domain of lipases is normally an amphipathic structure; in the closed conformation, their hydrophilic side faces the solvent, while the hydrophobic side is directed toward the catalytic pocket (Brocca S., Secundo F., Ossola M., Alberghina L., Carrea G., Lotti M. (2003). Sequence of the lid affects activity and specificity of *Candida rugosa* lipase isoenzymes. Protein Sci. 12, 2312–2319. 10.1110/ps.0304003). As the lipase shifts to the open

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conformation, the hydrophobic face becomes exposed and contributes to the substrate-binding region. Preferably the agent which interacts with the lid domain of the lipase or a fragment thereof, causes the lipase or a fragment to be locked in its active conformation. When locked in its active conformation the lipase is normally fully activated. The agent which interacts with the lid domain of the lipase or a fragment thereof so that the the lipase or a fragment thereof is in the open conformation include a colipase or a fragment thereof, a colipase-mimicking peptide, and an amphipathic molecule.

The term “amphipathic molecule” as used herein refers to a molecule like a chemical compound containing both polar (water-soluble) and nonpolar (not water-soluble) portions in its structure. It may also relate to a molecule like a chemical compound having both hydrophobic and hydrophilic regions. Amphipathic molecules include bile salts, phospholipids, and nonionic detergents.

The terms “open conformation” or “open conformation of a lipase or a fragment thereof” which are used interchangeably herein refer to the conformation of the lipase or the fragment thereof where substrates can enter the lipases’ active sites and be converted. In the closed conformation entrance of substrates to the active site of the lipase or a fragment thereof and its conversion is limited or not possible. The conformation of the lipase or fragment thereof i.e. whether the lipase is in open or closed confirmation can be determined by X-ray crystallography, enzymatic activity study, site-directed spin labeling (SDSL) methods and electron paramagnetic resonance (EPR).

The term “colipase or a fragment thereof” as used herein includes naturally occurring colipases or a fragment thereof and also includes artificially engineered colipases or a fragment thereof. Artificially engineered colipases or a fragment thereof are e.g. variants or functionally active fragments of the lipase. By “variants or functionally active fragments thereof” in relation to the colipase of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the colipase. Such variants include naturally occurring allelic variants and non-naturally occurring variants. 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. Preferably the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the lipase. A fragment of a colipase as defined herein does have
5 the same functional properties as the colipase from which it is derived. A preferred colipase is the colipase with Uniprot number: P02703.

The term “colipase mimicking peptide” as used herein refers to a peptide consisting of between 10 and 40 amino acids, allowing specific amino acid residues to be geometrically located in the
10 right position to interact with amino acids of the pancreatic lipase structure, inducing stretching of the lipase conformation and opening of the lid, and thereby having the same functional properties as the colipase. A colipase mimicking peptide which can be used in the present invention is preferably the peptide as shown in SEQ ID NO: 6.

15 The term “bile salt” as used herein refers to bile acids conjugated with taurine or glycine and include sodium taurocholate, sodium glycocholate, sodium glycodeoxycholate, sodium taurodeoxycholate, sodium glycochenodeoxycholate, and sodium taurochenodeoxycholate.

The term “nonionic detergent” as used herein refers to a surfactant and include tetra ethylene
20 glycol mono-octyl ether, octyl- β -D-glucopyranoside, N,N-dimethyldodecylamine-N-oxide, and β -octylglucoside.

The term “phospholipids” as used herein refers to a class of lipids whose molecule has a hydrophilic "head" containing a phosphate group and two hydrophobic "tails" derived from
25 fatty acids, joined by an alcohol residue (usually a glycerol molecule). Phospholipids include lecithin and lysolecithin.

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.
30 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.

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 group. 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 composed of
20 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 and
25 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 ($-NH_2$) 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.

- 5 The term “chitosan or a derivative thereof” as used herein refers to a chitosan or chitosan 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 chitosan derivatives includes ester, ether or other derivatives formed by reaction of acyl or
- 10 alkyl groups with the OH groups. Examples are O-alkyl ethers of chitosan, 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.
- 15 In a first aspect the present invention provides a method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent
- 20 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;
- (b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is
- 25 added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment thereof is added to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;
- (c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected
- 30 the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the protein or the fragment thereof; and optionally

(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 In one embodiment, the method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the
10 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, comprises the following steps:

(a) providing a solid carrier, wherein the solid carrier is provided in suspension;
(b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment
15 thereof is added to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;
(c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently
20 binds the protective layer to the protein or the fragment thereof.

In a further aspect the present invention provides a method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally a functional constituent
25 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;
30 (b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment

thereof is added to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;

(c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected
5 the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the protein or the fragment thereof; and optionally

(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
10 least one thiol group, with the proviso that the protein or a fragment thereof is not a lipase or a fragment thereof, a protease or a fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a disaccharidase or a fragment thereof, preferably with the proviso that the protein or a fragment thereof is not a lipase or a
15 fragment thereof, a protease or a fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a disaccharidase or a fragment thereof as described herein.

In one embodiment, the method of producing a composition, the composition comprising a
20 solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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
25 least one amino group and/or at least one thiol group, comprises the following steps:

(a) providing a solid carrier, wherein the solid carrier is provided in suspension;
(b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment thereof is added to the suspension comprising the solid carrier and the linker, wherein the
30 linker connects the solid carrier with the protein or a fragment thereof;
(c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected

the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the protein or the fragment thereof, with the proviso that the protein or a fragment thereof is not a lipase or a fragment thereof, a protease or a fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof
5 comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a disaccharidase or a fragment thereof, preferably with the proviso that the protein or a fragment thereof is not a lipase or a fragment thereof, a protease or a fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a
10 disaccharidase or a fragment thereof as described herein.

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

20 A solution of a protein or a fragment thereof usually 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. A solution of the protein or
25 a fragment thereof can be prepared by e.g. dissolving the protein or fragment thereof in water to reconstitute the stock buffer of the protein or a fragment thereof.

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
30 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 μm , preferably between 10 nm and 100 μm , particularly between 50 nm and 50 μm . In one
5 embodiment the composition comprises a solid carrier wherein the solid carrier comprises at least 4%, preferably at least 10%, more preferably at least 20%, even more preferably between 4% and 50%, in particular between 10% and 40%, more particular between 25% and 35 %, even more particular between 15% and 25% immobilized protein or a fragment thereof per dry weight of the solid carrier.

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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.

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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),
20 polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60); block 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
25 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-
30 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.

5

In one embodiment, the agent which interacts with the lid domain of the lipase or a fragment thereof is added to a suspension of the solid carrier, preferably the agent which interacts with the lid domain of the lipase or a fragment thereof is added together with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof to the suspension of the solid carrier prior to immobilization of the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier. The lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof can be added in form of pancreatin the suspension of the solid carrier prior to immobilization of the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier

The immobilization of the protein or a fragment thereof, e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier is usually carried out by adding a solution comprising a protein or a fragment thereof, e.g. a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof, or pancreatin or a solution thereof, to the suspension of the solid carrier. In a preferred embodiment the immobilization of the protein or a fragment thereof, e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier is carried out by providing a suspension of the solid carrier and providing a solution comprising protein or a fragment thereof, e.g. a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof, or pancreatin or a solution thereof, wherein the suspension of the solid carrier is incubated with the solution comprising protein or a fragment thereof, e.g. a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof, or pancreatin or a solution thereof, to allow the protein or a fragment thereof, e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof to bind on the surface of the solid carrier. In a more preferred embodiment the immobilization of a protein or a fragment thereof, e.g. a lipase

on the solid carrier, a protease or a fragment thereof and an amylase or a fragment thereof, or pancreatin or a solution thereof, is carried out by providing a suspension of the solid carrier, providing a solution comprising a protein or a fragment thereof, e.g. a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof, or pancreatin or a solution thereof, and providing a solution of the agent which interacts with the lid domain of the lipase or a fragment thereof, wherein the suspension of the solid carrier is incubated with the solution comprising protein or a fragment thereof, e.g. a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof, or pancreatin or a solution thereof, and with the solution of the agent which interacts with the lid domain of the lipase or a fragment thereof to allow the protein or a fragment thereof, e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof to bind on the surface of the solid carrier.

In a further preferred embodiment the protein or a fragment thereof is immobilized on the solid carrier by a linker, preferably a bi-functional cross-linker, binding to the protein or a fragment thereof or to a fragment thereof and to the surface of the solid carrier, preferably a linker, preferably a bi-functional cross-linker, binding to the protein or to a fragment thereof and to the surface of the solid carrier by covalent binding.

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 protein or a fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof or for the linker connecting the protein or a fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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 protein or a

fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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
5 homogeneously distributed on the surface of the solid carrier.

In one embodiment the agent which interacts with the lid domain of the lipase or a fragment thereof is selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide, and an amphipathic molecule. Preferably, the agent which interacts with the
10 lid domain of the lipase or a fragment thereof is selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide, and a bile salt, more preferably selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide, and sodium taurocholate, even more preferably selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide as shown in SEQ ID NO:6, and
15 sodium taurocholate. Most preferably the agent which interacts with the lid domain of the lipase or a fragment thereof is a bile salt, in particular sodium taurocholate.

In one embodiment the agent which interacts with the lid domain of the lipase or a fragment thereof interacts specifically with the lid domain of the lipase or a fragment thereof so that the
20 lipase or a fragment thereof shifts to and/or maintains the open conformation.

In one embodiment from about 50% to 100%, preferably from about 80% to 100%, more preferably from about 90% to 100%, even more preferably about 100% of the lipase or a fragment thereof immobilized on the surface of the solid carrier is in the open conformation.
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
30 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 protein or fragment thereof, e.g. the lipase or a fragment thereof, the
5 protease or a fragment thereof and the amylase or a fragment thereof is partially embedded by
the protective layer. In a preferred embodiment the protein or a fragment thereof, e.g. the
lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment
thereof is at least partially embedded by the protective layer. In a more preferred embodiment
the protein or a fragment thereof, e.g. the lipase or a fragment thereof, the protease or a
10 fragment thereof and the amylase or a fragment thereof is fully embedded by the protective
layer.

In one embodiment, the protective layer embeds the solid carrier and embeds the protein or a
fragment thereof, e.g. the lipase or a fragment thereof, the protease or a fragment thereof and
the amylase or a fragment thereof immobilized on the surface of the solid carrier. In one
15 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 protein or a fragment thereof, e.g. the lipase or a fragment thereof,
the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the
surface of the solid carrier. More preferably, the protective layer fully embeds the solid carrier
20 and fully embeds the protein or a fragment thereof, e.g. the lipase or a fragment thereof, the
protease or a fragment thereof and the amylase or a 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 protein or a fragment thereof, e.g. the lipase or a fragment
25 thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized
on the surface of the solid carrier, the protein or a fragment thereof, e.g. the lipase or a
fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is
fully, i.e. 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.

30

In a preferred embodiment the lipase or a fragment thereof, the protease or a fragment thereof
and the amylase or a fragment thereof used in the present invention are comprised by

pancreatin. In a more preferred embodiment pancreatin is used to immobilize the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the surface of the solid carrier.

- 5 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.

- 10 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 performed 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
- 15 the building blocks, which can be used to build the protective layer, may depend on the known structure of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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
- 20 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
- 25 (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 proteins e.g. the lipase or a fragment thereof, the protease or a
- 30 fragment thereof and the amylase or a fragment thereof. 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-Acetylglucyl)-3-aminopropylsilanes, hydroxy(polyethyleneoxy)propyltriethoxysilanes, in particular selected from benzyltriethoxysilane (BTES), propyltriethoxysilane, isobutyltriethoxysilane, n-octyltriethoxysilane, hydroxymethyltriethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltriethoxysilane, 3-Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglucyl)-3-aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutyltrimethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrimethoxysilane, 3-Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglucyl)-3-aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutyltrimethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrimethoxysilane, aminopropyltrimethoxysilane, ureidopropyltrimethoxysilane (N-Acetylglucyl)-3-aminopropyltrimethoxysilane or selected from benzyltrihydroxyethoxysilane, propyltrihydroxyethoxysilane, isobutyltrihydroxyethoxysilane, n-octyltrihydroxyethoxysilane, hydroxymethyltrihydroxyethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrihydroxyethoxysilane, aminopropyltrihydroxyethoxysilane, Ureidopropyltrihydroxyethoxysilane (N-Acetylglucyl)-3-aminopropyltrihydroxymethoxysilane.

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

The reaction time of the building blocks with the solid carrier carrying the immobilized enzymes can depend on the length of the linker, if a linker is used, and the size of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a

fragment thereof. 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
5 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 further more preferred embodiment the protein or a fragment thereof e.g. the lipase or a
10 fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof 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 protein or a fragment thereof e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase
or a fragment thereof and by using a linker, preferably a cross-linker binding to the anchoring
15 point and the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof.

In one embodiment the introduced molecule as anchoring point and/or the linker are
homogeneously distributed on the surface of the solid carrier.

20

Step (a) of the method is usually carried out by providing the solid carrier in suspension in water, buffer or non-ionic surfactants 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 buffer is present in the suspension, even more preferably in suspension
25 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 protein or a fragment thereof on the solid carrier in step b) of the present method is usually carried out by adding a solution of the protein or a fragment thereof to the suspension of the solid carrier. Preferably a linker to connect the solid carrier with the protein or a fragment
30 thereof is added to the suspension of the solid carrier prior to adding the solution of the protein or a fragment thereof to the suspension of the solid carrier. In a preferred embodiment the immobilization of the protein or a fragment thereof on the solid carrier is carried out by

providing a suspension of the solid carrier and adding a solution of the protein or a fragment thereof, wherein the suspension with the added solution of the protein or a fragment thereof is incubated to allow the protein to bind on the surface of the solid carrier. In a more preferred embodiment the immobilization of the protein or a fragment thereof on the solid carrier in step

5 b) is carried out by i) adding a linker to the suspension comprising the solid carrier provided in step (a), and ii) adding adding a solution of the protein or a fragment thereof, to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof, preferably wherein the suspension with the added solution of the protein or a fragment thereof is incubated to allow the protein to bind on the surface of the

10 solid carrier. 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 suspension comprising the solid carrier and the linker, prior to adding the solution of the protein or a fragment thereof. In a preferred embodiment the surface of the solid carrier is at least partly modified to improve

15 immobilization of the protein or a fragment thereof on the solid carrier. In particular, the surface of the solid carrier is at least partly modified before the protein or a fragment thereof is immobilized. The surface of the solid carrier can be at least partly modified by adding a molecule as anchoring point for the protein or a fragment thereof to the surface of the solid carrier as described supra.

20

The suspension comprising the solid carrier is usually incubated after each addition step described above to allow a reaction between e.g. the solid carrier and/or the molecule as anchoring point, the solid carrier and the linker and, the solid carrier comprising the linker and the protein or a fragment thereof, respectively, so that the protein or a fragment thereof

25 connects to the solid carrier, preferably the surface of the solid carrier, via the linker, preferably by covalent binding, thereby immobilizing the protein or a fragment thereof on the solid carrier.

In one embodiment in step (b) the protein or a fragment thereof is immobilized on the solid carrier by connecting the solid carrier with the protein or a fragment thereof via a linker,

30 wherein the solid carrier is connected with the protein or a fragment thereof by covalent binding between the linker and the solid carrier and between the linker and the protein or a fragment thereof. The linker connects the surface of the solid carrier with the protein or a

fragment thereof by preferably covalent binding. More preferably the linker is added to the suspension of the solid carrier in i) of step (b), in a molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b), preferably the linker is added to the suspension of the solid carrier in step (b), in a 1 fold to 5 1000 fold molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b), more preferably the linker is added to the suspension of the solid carrier in step (b), in a 2 fold to 300 fold molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b), even more preferably the linker is added to the suspension of the solid 10 carrier in step (b), in a 4 fold to 250 fold molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b).

In a preferred embodiment the linker which has not connected the solid carrier with the protein 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 further more preferred embodiment the linker which 15 has not connected the solid carrier with the protein 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 protein 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 protein or a fragment thereof in step (b), or a part 20 thereof, covalently binds the protective layer to the protein or the fragment thereof in step (c).

The amount of the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b) after addition of the protein in ii), 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 25 adding the linker to the the suspension of the solid carrier provided in step (a) in (i) of step (b) and adding the solution of the protein or a fragment thereof to the suspension comprising the solid carrier and the linker in ii) of step (b). 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 suspension of the solid carrier provided in step (a) in (i) of step (b) and adding 30 the protein or a fragment thereof to the suspension comprising the solid carrier and the linker in ii) of step (b) and there is no washing step between any of steps (a) to (c).

In a preferred embodiment the cross-linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated

5 sulfhydryls, sulfhydryl-reactive 2-pyridyldithiol, BSOE (Bis[2-(succinimidocarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3'-dithiobispropionimide·2 HCl), DST (Disuccinimidyl tartarate), Sulfo-LC-SMPT (4-Sulfosuccinimidyl-6-methyl-α-(2-pyridyldithio)toluamido]hexanoate), SPDP (N-Succinimidyl

10 3-(2-pyridyldithio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate), SMPT (4-Succinimidylcarbonyl-methyl-α-[2-pyridyldithio]toluene), DPDPB (1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane), DTME (Dithio-bismaleimidoethane), BMDB (1,4 bismaleimide-2,3-dihydroxybutane). More preferably said cross-linker is selected from glutaraldehyde, disuccinimidyl tartrate,

15 disuccinimidyl suberate, bis[sulfosuccinimidyl] suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls (e.g. sulfhydryl-reactive 2-pyridyldithio) and a colipase-mimicking peptide, wherein the colipase-mimicking peptide can be functionalized with a chemical group that enable

20 covalent binding to the solid carrier surface. In a more preferred embodiment the cross-linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOE (Bis[2-(succinimidocarbonyloxy)ethyl]sulfone),

25 DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3'-dithiobispropionimide·2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimide-2,3-dihydroxybutane). More preferably said cross-linker is selected from glutaraldehyde, disuccinimidyl tartrate, disuccinimidyl suberate, bis[sulfosuccinimidyl] suberate, ethylene

30 glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls (e.g. sulfhydryl-reactive 2-pyridyldithio). Most preferred is glutaraldehyde.

In one embodiment, after the protective layer has been formed, the solid carrier comprising the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof and the protective layer can be stored. Storing is usually accomplished
5 e.g. by washing the composition formed e.g. with a buffer and storing it suspended or solved in that buffer for a desired time period. In a preferred embodiment the solid carrier comprising the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof and the protective layer is stored at a constant temperature between 2 to 25 °C. In a further preferred embodiment, the solid carrier comprising protein e.g. the lipase or
10 a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof and the protective layer is stored 5 to 48 hours, preferably 10 to 30 hours. More preferably the solid carrier comprising the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof and the protective layer is stored at a constant temperature between 2 to 25 °C, preferably at room temperature for 10 to
15 30 hours.

A preferred method of the present invention is a method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or the fragment thereof by
20 embedding the protein or the fragment thereof, and optionally 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, 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 a protein or a fragment thereof on the solid carrier, wherein preferably the
30 surface of the solid carrier is at least partly modified before the protein or a fragment thereof is immobilized on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier or ii) 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) a solution of the protein or a fragment thereof is added to the the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;

(c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to protein or the fragment thereof; and optionally

(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.

A further preferred method of the present invention is a method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or the fragment thereof by embedding the protein or the fragment thereof, and optionally 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, 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 a protein or a fragment thereof on the solid carrier, wherein preferably the surface of the solid carrier is at least partly modified before the protein 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) a solution of the protein or a fragment thereof is added to the the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;

(c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected

the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to protein or the fragment thereof.

In one embodiment, the optional functional constituent binds to mucus.

5 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 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

10 wherein each repeat unit comprises at least one thiol 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 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 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, 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 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 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
5 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, 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
10 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 a thiol group, preferably a polymerized MPTS; a polycarbophil-cysteine conjugate; a polymerized silane-PEG-thiol; and a
15 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 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,
20 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 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
25 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
30 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
- 5 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
- 10 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,
- 15 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 APTES and a polymerized silane comprising an thiol group which is MPTS.
- 20
- 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
- 25 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.
- 30
- 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.

- In one embodiment the optional functional constituent is immobilized on the surface of the protective layer by binding, preferably covalent binding. In a preferred embodiment the optional 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 least one thiol group is immobilized on the surface of the protective layer by covalent binding.
- 10 In one embodiment the optional 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 comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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 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 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.
- 25 The immobilization of the optional functional constituent to the surface of the protective layer is usually carried out in a reaction vessel like a reactor by suspending the solid carrier carrying the protein e.g. the enzyme 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); block 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
5 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. Usually such obtained composition is washed and resuspended
10 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. t. 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.
15 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 protein e.g. the enzyme 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
20 functional constituent on the surface of the protective layer.

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

25 In a further aspect the present invention provides the composition as described herein for use in a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy, or for use in a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI). In a preferred embodiment the present invention provides the composition for use in a method for the prevention, delay of progression
30 or treatment of exocrine pancreatic insufficiency (EPI). In a further preferred embodiment the present invention provides the composition for use in a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy.

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 exocrine pancreatic insufficiency (EPI) in a subject. Also provided is the use of the composition as described herein for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI) in a subject. Also provided is a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI) in a subject, comprising administering to said subject a therapeutically effective amount of the composition as described herein. Also provided herein is the use of the composition as described herein for the manufacture of a medicament for a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy. Also provided is the use of the composition as described herein in a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy in a subject. Also provided is a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy, in a subject, comprising administering to said subject a therapeutically effective amount of the composition as described herein.

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In a further aspect the present invention provides the composition as described herein 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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In a further aspect the present invention provides the composition as described herein for use in a method for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances. In one embodiment the present invention provides the composition for use in a method for the prevention, delay of progression or treatment of lactase deficiency or sucrase-isomaltase deficiency. Lactase deficiency includes primary (hereditary) lactase deficiency, secondary (acquired) lactase deficiency and congenital

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lactase deficiency and is preferably secondary lactase deficiency. Sucrase-isomaltase deficiency includes Congenital Sucrase-Isomaltase Deficiency (CSID) which is preferred. 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 lactase deficiency, sucrase-isomaltase
5 deficiency, and/or disaccharidoses intolerances in a subject. Also provided is the use of the composition as described herein for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances in a subject. Also provided is a method for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances in a subject,
10 comprising administering to said subject a therapeutically effective amount of the composition as described herein.

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
15 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. The composition, e.g. the pharmaceutical composition of the invention may be administered according for a continuous period of one week or a part thereof, for two weeks, for three
20 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. 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.

25 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
30 provided herein.

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, 5 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 statistically significant or at least perceptible to the patient or to the physician. However, it will 10 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 of or slowing the increase in severity of a symptom. Further, "delay of progression" as used 15 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.

Preventive treatments comprise prophylactic treatments. In preventive applications, the 20 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 cure or at least partially arrest the symptoms of the disease. Amounts effective for this use will depend on the 25 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, including throughout the duration of the subject's life in order to ameliorate or otherwise 30 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 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 composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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 described supra.

In a further aspect the present invention provides a composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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 described supra; with the proviso that the protein or a fragment thereof is not a lipase or a fragment thereof, a protease or a fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a disaccharidase or a fragment thereof, preferably with the proviso that the protein or a fragment thereof is not a lipase or a fragment thereof, a protease or a fragment thereof, an amylase or a fragment thereof, pancreatin or a protein or a fragment thereof comprised by pancreatin, an engineered phenylalanine ammonia lyase (PAL) or a fragment thereof, and a disaccharidase or a fragment thereof as described herein.

Solid carrier, protein or a fragment thereof, protective layer to protect the protein or a

fragment thereof and the optional functional constituent of the composition are as described supra.

Examples

Phenylalanine Ammonia Lyase

Material and Methods:

Reagents:

- 5 - 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.
- 10 - Chitosan 95/500 P was purchased from Heppe Medical Chitosan GmbH.
- 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
- 15 cancer cell line) was purchased from the European Collection of Authenticated Cell Cultures (ECACC).
- 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
- 20 Streptomycin), L-Glutamine 200mM (100x), Dulbecco's Phosphate Buffered Saline DPBS (1X), 0.25% Trypsin-EDTA (1X), DMEM, white DMEM, were purchased from Gibco.
- Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free was purchased from corning.
- Animal diet Altromin 1324 was purchased at Altromin international.
- 25 - Animal diet phenylalanine free 5LF2 was purchased at LabDiet.
- The catheters were purchased at Instech Laboratories.

Synthesis of silica nanoparticles (SNPs):

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

5 ***Production of PAL-based silica nanoparticles 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 10 20°C, 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.

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Production of inactive nanoparticles 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 25 was 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 30 rpm. The particles 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.

5 ***Production of PAL-based silica nanoparticles NP-1 variants:***

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, PAL-based silica 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).
10 The reaction 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
15 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, 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
20 (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 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,
25 400 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 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 PAL-based silica nanoparticles (NP-1(2)) in buffer. Nanoparticles were washed after each chemical step
30 resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 59 nm) in phosphate buffer (25 mM, pH 7.5), 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. 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
5 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
10 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
15 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, PAL-based silica nanoparticles (NP-1) were produced in H₂O/PS80 (8
20 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
25 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 PAL-based silica nanoparticles NP-1(1), NP-1(2), and NP-1 at 460 nm was measured after the
30 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” of “Example 1: Phenylalanine Ammonia

Lyase Immobilization and Protection “ 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 PAL-based silica nanoparticles 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 PAL-based silica nanoparticles 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 PAL-based silica nanoparticles 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 PAL-based silica nanoparticles 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 PAL-based silica nanoparticles NP-1 efficacy in rats*

- 5 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
10 urine was sampled in Eppendorf tubes and stored at -80°C until shipped for analysis.

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

- 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,
15 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 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
20 (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).

- 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
25 collision energy 10, 20 and 30. Data analysis was performed in Thermo quan Browser software.

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

- The study was performed in male and female BTBR-Pah^{enu2}/J mice (8 weeks of age) of the
30 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 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 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 PAL-based silica nanoparticles 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
5 (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: Q₁ resolution: 0.7; Q₃ resolution: 0.7;
10 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:

15 **Example 1: Enhancing PAL Stability through Covalent Attachment to the protective layer**

To demonstrate that covalent bonding of the protective layer to PAL surface via glutaraldehyde cross-linking further improves enzyme stability compared with immobilized proteins protected by an organosilica layer with which it interacts only electrostatically, a series of three experiments was carried out.

20 In a first experiment, PAL-based silica nanoparticles NP-1(1) were produced in non-buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth). In a second experiment, PAL-based silica 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, PAL-based silica nanoparticles NP-1 were
25 produced in non-buffered conditions without any intermediate washing steps (*i.e.* unreacted glutaraldehyde still present in the reaction 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
30 maintained (NP-1) increases the enzyme immobilization yield by a factor of 2 (Fig. 2A), 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)).

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 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 higher activity in the presence of a buffer (Fig. 2 D). In summary, covalent attachment of the protective layer to PAL surface unexpectedly enhances its load and stability compared to PAL protected with an organosilica layer via electrostatic interactions only.

Example 2: *Phenylalanine ammonia lyase (PAL) activity of PAL-based silica nanoparticles 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. 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*

PAL-based silica nanoparticle 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.

Example 4: *In vitro* biocompatibility and efficacy of PAL-based silica nanoparticles 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 PAL-based silica nanoparticles NP-1 in rats

- 5 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
10 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.

15 **Example 6: *In vivo* therapeutic efficacy of PAL-based silica nanoparticles 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
20 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
25 (acidity and exposure to proteases). Our approach to control the Phe level in patients is to 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.

- 30 Mice having access to L-Phe supplemented drinking water were dosed twice a day with NP-1, 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.

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.

Disaccharidase

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, lactase (USP reference standard), invertase, bovine serum albumin (BSA), invertase activity assay kit, lactose, lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. BSA, lactase and invertase were dissolved in water to reconstitute the stock buffer.

- Chitosan 95/500 P was purchased from Heppe Medical Chitosan GmbH

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).

- THP-1 (human acute monocytic leukemia cell line) was purchased from LGC.

- 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), MEM Non-Essential Amino Acids (100x), L-Glutamine 200mM (100x), Dulbecco's Phosphate Buffered Saline DPBS (1X), 0.25% Trypsin-EDTA (1X), RPMI 1640 Medium, DMEM, HEPES, Sodium pyruvate, D-glucose, β-mercaptoethanol were purchased from Gibco.

- Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free was purchased from corning.

- Animal diet Altromin 1319 and AIN 93G modified 200g polysaccharides were purchased at Altromin international
- The catheters were purchased at Instech Laboratories
- Glucose meter TB100 Holtex kit was bought from MediSafe
- 5 - Oligo- α -1,6-Glucosidase 13A from Bifidobacterium adolescentis, Recombinant (Isomaltase) was purchased from Creative Enzymes at a concentration of 1mg/mL in 35 mM NaHepes buffer, pH 7.5, 750 mM NaCl, 200 mM imidazol, 3.5 mM CaCl₂, 0.02% sodium azide and 25% (v/v) glycerol.

10 ***Synthesis of silica nanoparticles:***

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
15 20000 g for 20 min and washed successively with ethanol and water. Particle size measurement was carried out on SEM micrographs acquired at a magnification of 150000x using the image analysis software Olympus stream motion.

Enzyme shielding and surface functionalization

20 - *Production of lactase-based silica nanoparticles NP-2:*

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
25 20°C, 400 rpm. Lactase (7 mg/mL, 0.1 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 lactase using APTES (8.4 mM) and TEOS (125.9 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 resuspended in
30 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 μ g/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.

- *Lactase-based silica nanoparticles NP-2 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, lactase-based silica nanoparticles (NP-2(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, 69 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.3 mM).

10 The reaction 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 (3.3 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 (3.3 mM) and stirring the
15 reaction mixture 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). Lactase (5.2 mg/mL, 0.1 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 lactase using APTES (6.5 mM) and TEOS (93 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The
20 particles 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). NP-2(1) were cured overnight in a 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 lactase-based silica
25 nanoparticles (NP-2(2)) in buffer. Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) was added APTES (3.3 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
30 mg/L). Then, glutaraldehyde (3.3 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 (3.3 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). Lactase (5.2 mg/mL, 0.1 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 lactase using APTES (6.5 mM) and TEOS (93 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). NP-2(2) were cured overnight in a water bath at 20°C.

In a third experiment, lactase-based silica nanoparticles (NP-2) were produced in H₂O/PS80 (8 mg/L) according to the section headed “Production of NP-2” above. To keep the excess amount of glutaraldehyde which has not linked the solid carrier to the lactase 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 bonding of the protective layer to the lactase. The covalent binding of the protective layer to lactase can be observed by the appearance of a yellow/orange colour that has an absorbance maximum at 460 nm. This colour 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 lactase and the organosilica layer. The absorbance of lactase-based silica nanoparticles NP-2(1), NP-2(2), and NP-2 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-2” of “Example 2: Disaccharidase immobilization and protection” above, showing an higher absorbance at 460 nm for NP-2 than for NP-2(1) and NP-2(2) (see Figure 15). NP-2(1) and NP-2(2) still show absorbance to some degree at this wavelength, as imine bonds are also formed during enzyme immobilization. However, the absorbance of NP-2 is significantly higher indicating an additional formation of imine bonds caused by covalent binding of the protective layer to lactase.

- *Production of invertase-based silica nanoparticles NP-3:*

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 performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Invertase (1.726 mg/mL, 0.03 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 invertase using APTES (5.4 mM) and TEOS (81.3 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 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 115 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-3 was cured overnight in a water bath at 20°C.

- *Production of isomaltase-based silica nanoparticles NP-4:*

To SNPs (10 mg/mL, 59 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.6 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.6 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.6 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Isomaltase (3.55 mg/mL, 0.05 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 invertase using APTES (5.8 mM) and TEOS (88 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 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 82 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-4 was cured overnight in a water bath at 20°C.

- *Production of invertase/isomaltase-based silica nanoparticles NP-5:*

To SNPs (10 mg/mL, 59 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.6 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.6

mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.6 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Isomaltase (1.77 mg/mL, 0.025 mM) and invertase (4.05 mg/mL, 0.07 mM) were 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 enzymes using APTES (5.8 mM) and TEOS (88 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 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 82 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-5 was cured overnight in a water bath at 20°C.

- *Production of inactive nanoparticles NP-1:*

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 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 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-1 was cured overnight in a water bath at 20°C. SNPs-BSA-AT were cured overnight at 20°C.

30

Disaccharidases activity assays:

- *Lactase activity assay*

To a suspension of lactase-based silica nanoparticles NP-2 (30 μ L, 2.3 mg/mL) in phosphate buffer (100 mM, pH 6.5)/MgCl₂ (5 mM) was added a lactose solution (100 μ L, 50 mg/mL). The reaction mixture was incubated in a thermomixer for 20 minutes at 37°C, 750 rpm. Samples were collected every 2.5 minutes and the glucose formation was monitored using a
5 blood glucose meter.

- *Invertase activity assay*

Invertase-based silica nanoparticles NP-3 activity was assessed using the Sigma invertase assay kit. A 1X reaction buffer (94 μ L) was added to NP-3 (94 μ L, 147 μ g/L). Next, 1X sucrose
10 solution (11.76 μ L) was added. The reaction mixture was incubated for 20 minutes at 37°C, 300 rpm in a thermomixer. The sample was centrifuged at 20000 rcf for 5 min. The supernatant was collected and 85 μ L was transferred to a 96-well plate. 90 μ L of a master reaction mix (prepared by mixing an enzyme mix, a dye reagent, and the assay buffer) was added to each well. The reaction mixture was incubated for 20 minutes in the dark at room
15 temperature. The absorbance was measured at $\lambda = 570$ nm.

- *Isomaltase activity assay*

Isomaltase-based silica nanoparticles NP-4 activity was assessed using a glucose meter. An isomaltose solution (25 μ L, 100 mM) was equilibrated for 5 minutes at 37°C, 700 rpm. Then,
20 to the isomaltose solution was added NP-4 (25 μ L, 67 μ g) in phosphate buffer 50 mM, pH 6.8. The reaction mixture was incubated for 10 min at 37°C, 700 rpm. Samples were collected after 2 min, 5 min and 10 min and glucose concentrations were determined using a glucose meter.

- *Co-immobilized isomaltase-invertase activity assay*

25 Invertase/isomaltase-based silica nanoparticles NP-5 isomaltase activity was assessed using a glucose meter. An isomaltose solution (25 μ L, 100 mM) was equilibrated for 5 minutes at 37°C, 700 rpm. Then, to the isomaltose solution was added NP-5 (25 μ L, 67 μ g) in phosphate buffer 50 mM, pH 6.8. The reaction mixture was incubated for 10 min at 37°C, 700 rpm. Samples were collected after 2 min, 5 min and 10 min and glucose concentrations were
30 determined using a glucose meter.

Invertase/isomaltase-based silica nanoparticles NP-5 invertase activity was assessed using a glucose meter. A sucrose solution (25 μ L, 100 mM) was equilibrated for 5 minutes at 37°C,

700 rpm. Then, to the sucrose solution was added NP-5 (25 μ L, 67 μ g) in phosphate buffer 50 mM, pH 6.8. The reaction mixture was incubated for 10 min at 37°C, 700 rpm. Samples were collected after 2 min, 5 min and 10 min and glucose concentrations were determined using a glucose meter.

5

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.

THP-1 (Human monocytic leukaemia cell line) cells were maintained in culture in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, and 100 U/mL penicillin/streptomycin.

For THP-1 differentiation into macrophages, THP-1 cells were cultured in differentiation medium: RPMI 1640 with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 100 U/mL penicillin/streptomycin, 10mM HEPES, 1mM sodium pyruvate, 2,5g/L glucose and 50pM β -mercaptoethanol. THP-1 were differentiated into M0-macrophages by 24h incubation with 150nM phorbol 12-myristate 13-acetate (PMA) followed by 24h incubation in differentiation medium.

20

- Intestinal barrier model

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%.

25

- Immunocompetent intestinal barrier model

For the development of the immunocompetent intestinal barrier model, M0 differentiated THP-1 were added to the intestinal barrier model at day 21. Immune cells were attached to the posterior side of the Transwell membrane contained the previously differentiated co-culture with a 25% Matrigel solution *via* the drop method.

30

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
5 for up to 24h every 15 minutes with a range from 1Hz to 100'000Hz.

In vitro digestion of sucrose

Differentiated Caco-2/HT29-MTX-E12 co-culture in PBS were exposed to NP-3(0.5mU and 1mU) in presence of sucrose for 4h at the apical side of the barrier. At each timepoint, aliquots
10 of 150uL were withdrawn from the basolateral sides of the intestinal barrier and replaced with the same volume of pre-warmed PBS. The barrier was further incubated at 37°C. The absorbance of the withdrawn aliquot samples was measured at 570nm to quantify the level of glucose.

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

20

- Diet and drinking water:

For maintenance diet, the rats were fed with a pelleted complete diet "Altromin 1319" available *ad libitum*. They had access *ad libitum* to drinking water.

One week before the treatment and over the experimentation period, the rats were fed with low
25 sugar diet (AIN 93G modified 200g polysaccharides) available *ad libitum*.

- 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
30 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.

- *Dosing and lactose administration*

- 5 Before dosing and lactose administration, animals were starved for 4h. Then, rats were dosed intraduodenally with lactase-based silica nanoparticles NP-2 (97U), inactive nanoparticles NP-1(54mg) or vehicle (1,5mL of NaCl0,9%-polysorbate 80 8mg/mL) and immediately gavaged with 3g of lactose. Rats were daily dosed and gavaged over a period of 15 days.

10 - *Cecum analysis*

At the end of the experimentation, all animals were scanned under full anesthesia (scan time approx. 5min) on a Bruker Pharmascan 7 Tesla with a rat volume coil to assess the cecum size. Regions of interest were drawn on the cecum in all acquired slices. MRI images were used to determine the volume of the cecum.

- 15 At the necropsy, pictures of the gastrointestinal tract were taken to visualize the dilatation of the cecum.

Results:

20 **Example 1: *Enhancing Lactase Loading through Covalent Attachment to the protective layer***

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

- 30 Lactase quantification was performed on the reaction supernatants to determine lactase immobilization yield at the surface of lactase-based silica nanoparticles NP-2(1), NP-2(2) and NP-2. The results show that surprisingly enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-2) increases the enzyme immobilization yield by

a factor of twenty-six (Fig. 10A), resulting in a 24-fold increase of enzyme loading per dry weight of SNP (Fig. 10B) compared to buffered conditions where glutaraldehyde is removed by washing steps (NP-2(2)). Similarly, enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-2) results in a 2 times higher enzyme loading per dry weight of SNP (Fig. 10B) compared to unbuffered conditions where glutaraldehyde is removed by washing steps (NP-2(1)).

In summary, covalent attachment of the protective layer to the lactase surface unexpectedly enhances its load compared to lactase protected with an organosilica layer via electrostatic interactions only.

Example 2: Disaccharidases activity of lactase-based silica nanoparticles NP-2, invertase-based silica nanoparticles NP-3, isomaltase-based silica nanoparticles NP-4 and invertase/isomaltase-based silica nanoparticles NP-5

The biocatalytic activity of four different immobilized and protected disaccharidases was assessed. The results as displayed in Figure 11 report the enzymatic activity of each nanoparticle: lactase activity on lactase-based silica nanoparticles NP-2 (Fig. 11A), invertase activity on invertase-based silica nanoparticles NP-3 (Fig. 11B), isomaltase activity on isomaltase-based silica nanoparticles NP-4 (Fig. 11C) and the dual enzyme activities (isomaltase and invertase) on invertase/isomaltase-based silica nanoparticles NP-5 (Fig. 11D).

These data show that the disaccharides reach the catalytic site of the enzymes and are cleaved with high enzymatic activity. The validation of the biocatalytic activity on NP-2, NP-3, NP-4 and NP-5 confirms the possibility to apply the strategy of immobilization and protection to a wide range of disaccharidases that could be used for therapeutic purposes.

Example 3: Biocompatibility of the nanoparticles for gastrointestinal applications

The intestinal mucosa consists in a single layer of epithelial cells closely attached by intercellular tight junctions next to a subepithelial region that contains the *lamina propria*. It acts as a barrier between the environment and the internal milieu. Its integrity is a key parameter to ensure the protection of the body against undesirable contaminants such as microorganisms. The *lamina propria* includes a diffuse lymphoid tissue constituted by immune cells that maintain homeostasis or respond to a breakdown of epithelial protection.

To evaluate the biocompatibility of the nanoparticles, we developed a representative model for lactase-based silica nanoparticles NP-2 and invertase-based silica nanoparticles NP-3. It consists in inactive nanoparticles NP-1, a nanoparticle with the same functionalized outer surface as NP-2 and NP-3 but without enzymatic activity.

- 5 To evaluate the safety of the nanoparticles, we first focused on the maintenance of the intestinal barrier integrity in presence of inactive nanoparticles NP-1 (Fig. 12A). The transepithelial electrical resistance (TEER) measurements across Caco2-HT29-MTX-E12 cell monolayers shows that the integrity of the intestinal epithelial barrier remains intact when in contact with NP-1 for 24h. This result demonstrates the *in vitro* biocompatibility of NP-1.
- 10 To further characterize the impact of the nanoparticles on the intestinal barrier, we evaluated the ability of NP-1 to trigger an inflammatory response. To this purpose, we developed an immunocompetent intestinal barrier model and monitored its integrity. Figure 12B shows a decrease in integrity of the barrier in a dose dependant manner when treated with LPS, a proinflammatory component. This loss of integrity reveals the recruitments of macrophages
- 15 from the basal to the apical side of the barrier.
- Most importantly, the TEER measurements showed that the integrity of the epithelial barrier remained intact when in contact with NP-1 (Fig. 12B). NP-1 does not stimulate the recruitment of macrophages at the apical side of the intestinal barrier, and it can be concluded that NP-1 does not induce inflammation.
- 20 Altogether, these results demonstrate the *in vitro* safety of the nanoparticles for gastrointestinal applications.

Example 4: *In vivo* efficacy of lactase-based silica nanoparticles NP-2

- Lactose malabsorption is attributable to an imbalance between the amount of ingested lactose
- 25 and the capacity for lactase to hydrolyse the disaccharide. Digestion and absorption of lactose takes place in the small intestine. In case of lactose malabsorption, undigested lactose reaches the large intestine and comes into contact with the intestinal microbiota. The bacterial lactose fermentation leads to the production of short chain fatty acids and gasses resulting notably in an enlargement of the cecum.
- 30 In rats daily gavaged with high dose of lactose for 15 days, greater substrate availability for fermentation in the large intestine led to an enlargement of the cecum compared to rats fed with a normal diet without additional intake of lactose (Conditions “Vehicle” vs “No lactose”

respectively) (Fig. 13). Importantly, a significant cecum size reduction is reported in rats dosed with lactase-based silica nanoparticles NP-2, while the administration of inactive nanoparticles (NP-1) has no impact on cecum size reduction. These results demonstrate the *in vivo* biocatalytic activity of lactase-based silica nanoparticles NP-2 for the digestion of lactose.

5 Enzyme-replacement therapies with microbial exogenous lactase are available but the results about the exact rate of efficacy are discordant (Montalto *et al.*, World J Gastroenterol 2006, Jan 14;12(2):187-91). Rules used to calculate the amount of lactase are 7500 units for 16 grams of lactose. Besides this close relationship between the amount of lactose to be hydrolysed and the enzyme units required, the stomach pH and bile salt concentrations
10 influence the efficacy of exogenous lactase as well as the lack of specific localization of the enzyme in the intestine.

In Figure 13, the *in vivo* efficacy of lactase-based silica nanoparticles NP-2 is demonstrated with a dose of 97 units for 3g of lactose. When compared to the dosage of currently available lactase preparation, this dose surprisingly corresponds to a 14.5 lower dose. So, this set of data
15 demonstrate the high value of NP-2 for lactose digestion and highlights the therapeutic potential of lactase-based silica nanoparticles NP-2 for patients with defective disaccharide digestion like patients with lactose malabsorption.

Example 5: *In vitro* efficacy of invertase-based silica nanoparticles NP-3

20 Congenital Sucrase-Isomaltase Deficiency (CSID) is characterized by complete, or almost complete lack of sucrose activity and varying degrees of reduction in isomaltase activity. The efficacy of invertase-based silica nanoparticles NP-3 to digest sucrose was assessed on a model of intestinal barrier. Differentiated Caco2-HT29-MTX-E12 cell monolayers were exposed at their apical side to NP-3 in presence of its substrate for 4h. The quantification of
25 the product of sucrose hydrolysis in the basal compartment of the barrier is shown on Figure 14. The graph reports a dose dependant accumulation of glucose in the basolateral side of the barrier in presence of increasing amount of invertase-based silica nanoparticles NP-3 while no glucose is detected in the untreated intestinal barrier. This result demonstrates the *in vitro* efficacy of invertase-based silica nanoparticles NP-3 and underlines the use of NP-3 for
30 therapeutic applications.

Lipase, Amylase and Protease

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, 5 25% in water), Chelex® 100 sodium form, polysorbate 80, pancreatin (4xUSP specifications), bovine serum albumin (BSA), potassium phosphate monobasic, potassium phosphate dibasic, sodium taurocholate hydrate, acetic acid, olive oil, gum arabic from acacia tree, sodium chloride, sodium hydroxide, Trizma base, hydrogen chloride, ammonium acetate, sodium acetate, pancreatin, bile salts, butanol, methanol, isopropanol, acetonitrile, NH₄ acetate, 10 amylase activity assay kit, 4% buffered formaline, Triton X-100 were purchased from Sigma-Aldrich. BSA and pancreatin were dissolved in water to reconstitute the stock buffer.
- p-SCN-Bn-DOTA was purchased from Macrocyclics.
- Benzyltriethoxysilane (B, 96%) was purchased from abcr GmbH.
- Chitosan 95/500 P was purchased from Heppe Medical Chitosan GmbH
- 15 - Indium chloride (¹¹¹In) was purchased from Curium
- Animal diet Altromin 1324 and Altromin 9033 were purchased at Altromin international
- The catheters were purchased at Instech Laboratories
- 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 20 (ECACC).
- 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), MEM Non-Essential Amino Acids (100x), L-Glutamine 200mM (100x), 25 Dulbecco's Phosphate Buffered Saline DPBS (1X), 0.25% Trypsin-EDTA (1X), RPMI 1640 Medium, DMEM were purchased from Gibco.
- Antizoonula occludens 1 (ZO-1) antibody, goat anti-rabbit IgG conjugated to Alexa Fluor 488 and mounting medium containing DAPI were purchased at ThermoFischer Scientific.

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

Enzyme shielding and protein shielding, and surface functionalization

- Production of inactive DOTA-labelled silica nanoparticles functionalized with chitosan NP-1:

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, DOTA (3.8 mM) was added and reacted for 1 hour at 50°C. DOTA-labeled particles were washed 3 times by centrifugation (20 min at 20000 rcf) in H₂O/PS80 (8 mg/L, Chelex), resuspended in H₂O/PS80 (8 mg/L, Chelex) and tip sonicated. To DOTA-labeled particles 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 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 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-1 was cured overnight in a water bath at 20°C. SNPs-BSA-AT were cured overnight at 20°C.

- Production of inactive DOTA-labelled silica nanoparticles non-functionalized NP-2:

Immobilization of the enzyme and shielding of the enzyme was conducted similar as described in WO2015/014888:A1. 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, DOTA (3.8 mM) was added and reacted for 1 hour at 50°C. DOTA-labeled particles
5 were washed 3 times by centrifugation (20 min at 20000 rcf) in H₂O / PS80 (8 mg/L, Chelex), resuspended in H₂O/PS80 (8 mg/L, Chelex) and tip sonicated. To DOTA-labeled particles 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 performed by adding APTES (3.8 mM) and stirring the
10 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 were centrifuged 5 min at 20000 rcf and washed 3
15 times in NaCl (0.9%)/PS80 (8 mg/L). NP-2 was cured overnight in a water bath at 20°C.

- Production of pancreatin-based silica nanoparticles NP-3:

To SNPs (10 mg/mL, 55 nm) in phosphate buffer (20 mM, pH 8), 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.
20 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, 400 rpm. Sodium taurocholate (2 mM) and pancreatin (20 g/L) were successively 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 pancreatin using
25 APTES (7.7 mM), TEOS (40.4 mM) and benzyltriethoxysilane (35 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were centrifuged (5 min at 1000 rcf) and washed 3 times (by centrifugation during 5 min at 1000 rcf) in phosphate buffer (20 mM, pH 8), PS80 (8 mg/L) and resuspended in phosphate buffer (20 mM, pH 8), PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the
30 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 1000 rcf) and washed 3 times in phosphate buffer (0.120 M, pH 6), PS80 (8 mg/L) and resuspended in phosphate buffer (0.120 M, pH 6), PS80 (8 mg/L) to yield NP-3. NP-3 was cured overnight in a water bath at 20°C.

5 - ***Production of inactive silica nanoparticles NP-4:***

To SNPs (10 mg/mL, 55 nm) in phosphate buffer (20 mM, pH 8), 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
10 mixture for 10 min at 20°C, 400 rpm. BSA (11.1 g/L) 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 BSA using APTES (7.7 mM), TEOS (40.4 mM) and benzyltriethoxysilane (35 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were centrifuged (5 min at 1000 rcf) and washed 3 times (by centrifugation during 5
15 min at 1000 rcf) in phosphate buffer (20 mM, pH 8), PS80 (8 mg/L) and resuspended in phosphate buffer (20 mM, pH 8), 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 1000 rcf) and washed 3 times in phosphate buffer
20 (0.120 M, pH 6), PS80 (8 mg/L) and resuspended in phosphate buffer (0.120 M, pH 6), PS80 (8 mg/L) to yield NP-4. NP-4 was cured overnight in a water bath at 20°C.

- ***Production of pancreatin-based silica nanoparticles NP-5:***

To SNPs (10 mg/mL, 58 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.7 mM). The
25 reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.7 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.7 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Pancreatin (23.5 mg/mL) comprising lipase and/or a fragment thereof, protease and/or a fragment thereof and amylase and/or a fragment thereof was added, and the reaction
30 mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lactase using APTES (7.2 mM) and TEOS (75.6 mM). The resulting suspension was allowed to react for 2 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 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 103 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-5 was cured overnight in a water bath at 20°C.

Production of pancreatin-based silica nanoparticles NP-3 variants:

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, comparative experiment, enzyme immobilisation and formation of the protective layer were carried out according to WO2015/014888 A1 to produce pancreatin-based silica nanoparticles (NP-3(1)) in buffer. Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) was added APTES (3.1 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 (3.1 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 (3.1 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). Sodium taurocholate (2 mM) and pancreatin (15 g/L) were successively 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 pancreatin using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 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). NP-3(1) were cured overnight in a water bath at 20°C.

In a second experiment, pancreatin-based silica nanoparticles (NP-3) were produced in phosphate buffer (20 mM, pH 8), PS80 (8 mg/L) according to the section headed "Production

of NP-3” of “Example 3: Lipase, Amylase and Protease Immobilization and Protection” above. To keep the excess amount of glutaraldehyde which has not linked the solid carrier to pancreatin 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 pancreatin. The covalent binding of the protective layer to pancreatin can be observed by the appearance of a yellow/orange colour that has an absorbance maximum at 460 nm. This colour 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 pancreatin and the organosilica layer. The absorbance of pancreatin-based silica nanoparticles NP-3(1) and NP-3 at 460 nm was measured after the organosilica layer formation and final particles washing (see Figure 24), showing a much higher absorbance at 460 nm for NP-3 than for NP-3(1). NP-3(1) still shows absorbance to some degree at this wavelength, as imine bonds are also formed during enzyme immobilization. However, the absorbance of NP-3 is significantly higher indicating an additional formation of imine bonds caused by covalent binding of the protective layer to pancreatin.

Radioactive labeling with Indium 111 (¹¹¹In):

For the labeling of nanoparticles, additional steps have been added to the process of nanoparticles described in the section “Enzyme shielding and protein shielding, and surface functionalization”, “Production of inactive DOTA-labelled silica nanoparticles non-functionalized NP-2” and “Production of inactive DOTA-labelled silica nanoparticles functionalized with chitosan NP-1”.

The nanoparticles were labeled with a specific activity of 500-400 MBq/g nanoparticles according to the protocol stated below.

All used buffers were pretreated with Chelex®.

Nanoparticles were incubated with ¹¹¹Lu (0.02M HCl) and ammonium acetate (1M, pH 5.4) for 12h at 45°C under continuous stirring. Nanoparticles were centrifuged at 5000g for 5min and resuspended in sodium acetate (20mM, pH 5) with polysorbate 80 (8mg/L). Then, the nanoparticles were resuspended in DTPA (1mM, pH 5) and incubated overnight at room temperature (RT) for quenching. The nanoparticles were then washed and resuspended in 0.9% sodium chloride with polysorbate 80 (8mg/L).

Pancreatic lipase activity assay:

The olive oil solution was prepared by mixing olive oil / gum arabic / water (1 / 8.25 / 0.75).

The buffer solution was prepared by dissolving Trizma base (0.6 g/L) and sodium chloride (2.34 g/L) in nanopure water. The bile salts solution was prepared by dissolving sodium

5 taurocholate (80 g/L) in water.

The activity assay was performed by mixing the olive oil solution (13.8 mL), the buffer solution (11 mL), the bile salts solution (2.8 mL) and water (12.4 mL) in a bioreactor at 37°C. The pH was adjusted to 9.2 by adding a sodium hydroxide solution (0.1 M). NP-3 (1.4 mL, 10 mg/mL) was washed twice in water and added to the bioreactor. Lipase kinetics was monitored by

10 measuring the amount of sodium hydroxide added to the reaction mixture to maintain the pH at 9 for 10 minutes.

Pancreatic protease activity assay:

To the enzyme sample (50 µL, 0.2 mg/mL) in phosphate buffer (50 mM, pH 7.4) was added a
15 casein solution (250 µL, 0.65% w/v). The reaction mixture was incubated for 30 minutes at 37 °C, 750 rpm. The sample was centrifuged for 5 minutes at 20000 rcf and the supernatant was collected. To the supernatant (200 µL) was added TCA (166.7 µL). The sample was incubated for 30 minutes at 37°C, 750 rpm. The sample was centrifuged for 5 minutes at 20000 rcf and the supernatant was collected. To the supernatant (200 µL) was added Na₂CO₃ (500 µL, 500
20 mM) and Folin reagent (100 µL, 0.5 M). The sample was incubated for 30 minutes at 37°C, 750 rpm. The absorbance was measured at 660 nm on 200 µL of the resulting solution.

Pancreatic amylase activity assay:

Pancreatin-based silica nanoparticles NP-3 amylase activity was assessed using the Sigma
25 amylase assay kit. In a 96-well plate, the enzyme sample (2 µL, 18.2 mg/mL) was mixed with the activity buffer (48 µL). The amylase substrate solution (100 µL) was added to the well and the sample kinetics was monitored at 37°C for 30 minutes in the spectrophotometer at $\lambda = 405$ nm.

30 ***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.

- **Rats:**

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

○ *Diet and drinking water:*

5

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

○ *Pancreatic duct ligation (PDL) and duodenum catheterization:*

10 Animals were anesthetized with isoflurane (2-4%) in an induction chamber before being moved to a nose cone with isoflurane for the surgery. With the rat in a supine position on a heated table the abdomen was opened in the midline. The pancreas was located and gently moved to locate the biliopancreatic duct. The pancreatic tissue around the biliopancreatic duct was bluntly dissected to visualize the pancreatic ducts. These were ligated close to the
15 biliopancreatic duct to stop the flow of pancreatic enzymes. After ligation of the pancreatic duct, 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 was exteriorized. The abdomen and the incision in the neck were thereafter closed with sutures.

20

○ *Evaluation of pancreatin-based silica nanoparticles NP-3 efficacy:*

PDL rats were dosed through the duodenum with either inactive nanoparticles NP-4 (7mg) or pancreatin-based silica nanoparticles NP-3 (7mg; 7.5U) and 5 minutes later with triolein (10mg) by oral gavage. Following dosing of triolein, approximately 150 uL of blood was
25 sampled in EDTA at 0.25, 0.5, 1, 1.5, 2, 4, and 6h.

Blood samples were centrifuged (10 min, 4°C, 2000 x g), and a minimum of 50 uL plasma was transferred into Eppendorf tubes and stored at –80°C until analysis for triglycerides content.

○ *Measurement of plasmatic triglycerides of rats:*

30 Quantification of analytes of interest was performed using the LC system: Thermo Vanquish Horizon Binary Pump and the mass spectrometer: Thermo Q Exactive. Plasma samples were prepared according to the BUME method. Ten microliters of plasma sample were mixed with

300 µL of 1-butanol/methanol (3:1, v/v). Samples were incubated for 1h at 20°C under stirring (900rpm). After centrifugation (16 000g, 10min, 20°C), 50uL was transferred into glass vial and used for LC-MS.

The injection volume used was 2.5uL and the run time was 7.5 min at a flow rate of 1mL/min.

- 5 Mobile phase A was 60% acetonitrile, 40% H₂O, 5 mM NH₄ acetate and mobile phase B was 90% isopropanol, 10% acetonitrile, 5 mM NH₄ acetate. Chromatographic separation was carried out using Waters Premier BEH C18 column (50mmx2.1mm) column (with the following gradient: from 15% B to 99% B).

- 10 The MS is 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. The targeted extraction of EIC was TG 54:3 for triolein and the most abundant triglycerides. Data analysis was performed in Thermo quan Browser software.

15 - **Minipigs:**

The study was performed on female Göttingen minipigs of the stock from Ellegaard Göttingen Minipigs A/S, Denmark.

○ *Diet and drinking water:*

- 20 On regular basis, the minipigs were fed with a pelleted complete diet “Altromin 9033” offered with a daily ration of approximatively 250g.

For the evaluation of NP-3 efficacy (chronic dosing), the diet was changed to high fat diet where the daily ration of Altromin 9033 was completed with olive oil (1:10 – olive oil: altromin) and 100g of apple sauce. The high fat diet was initiated 15 days before the start of

25 the dosing and maintained during the chronic administration of the treatment.

The animal had access *ad libitum* to domestic quality drinking water.

○ *SPECT/CT imaging*

- SPECT/CT scanning (Clinical D670 SPECT/CT, GE) were initiated 15 min, 3, 8, 24, 48 and
- 30 72 hours post dosing (+/- ½ h) of the In-111-labeled nanoparticles. For the control animal no SPECT/CT scanning were initiated and 15 min, 3, 8, and 24 hours post dosing of the free In-111. The SPECT acquisition time was decided based on the count rate at the day of scanning.

SPECT imaging included two field of views (FOV) to cover the area ranging from the stomach to the rectum. The two FOVs were scanned with the FOV including the stomach and the duodenum as the first acquisition. Prior to the CT scan procedure, an intravenous infusion of an iodine-containing contrast medium (Ultravist®, 370 mg/mL, 1 mL/kg, flow rate 2 mL/s) was performed to improve the visibility of the organs for image analysis. FOV for CT imaging included the entire animal.

The animals were imaged on site and were transported from the holding room to the scanner under anesthesia.

For quantification of the uptake of In-111-labeled compounds, region of interest was drawn over relevant parts of the gastrointestinal organs (3 compartments- small intestines, colon, and rectum) identified from the CT image data. The uptake is expressed as % ID (percentage injected dose) and SUV (standardized uptake value). All data analysis was performed by single viewer to avoid intra-observed deviations.

15 ○ *Pancreatic duct ligation (PDL) and duodenum catheterization:*

Animals were pre-medicated with Zoletil mixture (6.25 mL of Rompun Vet® (Xylazine, 20 mg/mL) + 1.25 mL Ketaminol Vet® (Ketamine, 100 mg/mL) + 2.5 mL Torbugesic Vet® (Butorphanol, 10 mg/mL) in one vial of Zoletil Vet 50® (125 mg Tiletamin + 125 mg Zolazepam)) injected IM/IV in the stable. Once sedated the pig were intubated, and anaesthesia maintained on inhalation of isoflurane 2-4%. An ear vein catheter was placed unilaterally. The animal was positioned in left lateral recumbency and the right flank prepared for surgery lege artis.

An incision was made in the right flank and the duodenum and pancreas were gently located. Two ligatures were placed around the pancreatic duct 2-3 mm apart and the duct was incised between the ligatures to stop the flow of pancreatic enzymes.

After ligation of the pancreatic duct, a catheter (Dog duodenal catheter 7F, SAI Infusion Technologies) 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 back of the animals where it is exteriorized. The flank incision was thereafter sutured, and a protective bandage is applied around the abdomen.

30 ○ *Pre- and post-operative analgesia and antibiotics*

The animals were treated with preemptive analgesia in the form of intramuscular NSAID prior to surgery. Post-operative analgesia consisted of oral NSAID once daily for 4 days, and additionally low-dose percutaneous opioid patches for 72 hours as need.

Antibiotics (amoxicillin) were administered intramuscularly prior to surgery and completed by
5 oral administrations once daily for 4 days after surgery.

○ *Evaluation of pancreatin-based silica nanoparticles NP-3 efficacy:*

▪ *Single dosing:*

PDL minipigs were dosed through the intra duodenal catheter with either inactive nanoparticles
10 NP-4 (1g) or pancreatin-based silica nanoparticles NP-3 (1g; 1365U) and sequentially with
olive oil (14g) in the intra duodenal catheter. Following dosing of olive oil, 5mL of blood was
sampled in EDTA at 0, 0.0833, 0.25, 0.5, 1, 2, 3, 4 and 6 hours.

Blood samples were centrifuged (10 min, 4°C, 2000 × g), and a minimum of 500 uL plasma
was transferred into Eppendorf tubes and stored at –80°C until analysis for triglycerides
15 content.

▪ *Chronic dosing:*

PDL minipigs were dosed through the intra duodenal catheter with either inactive nanoparticles
NP-4 (1g) or pancreatin-based silica nanoparticles NP-3 (1g; 1365U) on a daily basis twice a
20 day before receiving their meal over a period of 10 days.

The feces of individual animals were collected for a period of 72 hours before the treatment
(Days -3, -2 and -1) and at the end of the treatment (Days 8, 9 and 10). Feces samples were
kept at –20°C until analysis.

25 ○ *Measurement of plasmatic triglycerides of minipigs:*

Quantification of triglycerides in plasma of minipigs was assessed using the Konelab systems
following the specifications of the supplier.

○ *Measurement of fecal fat content of minipigs*

30 The quantification of fecal fat content was assessed by Near Infra-Red spectroscopy (NIR)
using the Impana FT 9700 (Perkin Elmer). Stools were weighed, homogenized and placed in
open glass Petri dish for analysis by NIR (700-2500nm). For each sample, measurements were

read at three different spots. Results were expressed as the mean fat concentration of the three spot specimens (grams of fat per 100 g of stool wet weight).

Cell culture:

- 5 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 x
10 10⁵cells/cm² in transwell PET inserts (1µm 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

- 15 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.

Confocal microscopy

- 20 Cells on the insert were washed with phosphate-buffered saline and fixed with 4% buffered formaline. Fixed cells were permeabilized in a solution of PBS containing 1% Triton-X 100 (Reference). Aspecific bindings were blocked using a solution of PBS containing 3% bovine serum album (BSA). Cells were incubated for 2h at RT with the primary antibody against
25 zonula occludens 1 (ZO-1; 1:100). After washing, cells were incubated for 2h at RT in the dark with the corresponding fluorescent labelled secondary antibody: goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1000). For image acquisition, membranes were cut from the inserts, placed between two coverslips with a drop of mounting medium containing DAPI and imaged using a confocal laser scanning microscope (FluoView, FV3000, Olympus, Tokyo,
30 Japan).

Results:**Example 1: Biodistribution of chitosan-functionalized nanoparticles in minipigs**

To evaluate the benefits of chitosan-functionalized nanoparticles to specifically remain in the gastrointestinal tract of minipigs *via* mucus binding, inactive DOTA-labelled silica nanoparticles non-functionalized (NP-2) and inactive DOTA-labelled silica nanoparticles functionalized with chitosan (NP-1) were administrated into the duodenum of minipigs by endoscopy. At different time points (15min to 24h), SPECT/CT images were acquired and the biodistribution of the nanoparticles in the gastrointestinal tract was analysed using a 3-compartment analysis including small intestines, colon, and the rectal part of the colon. The quantification of the NP-2 and NP-1 in the small intestine is shown on Figure 17A. When comparing SPECT/CT derived biodistribution data from animals dosed with NP-1 and NP-2, it shows that NP-1 retained in the small intestine 2,7 times longer (AUC: 170.7 vs 460,9; NP-2 vs NP-1 respectively) than the non-functionalized nanoparticles (Fig. 17B). These results demonstrate the specific retention in the gastrointestinal tract of the NP-1 *in vivo*. Altogether, these *in vivo* data reveals that the surface functionalization of the shielded nanoparticles with chitosan allows a specific targeting of the intestinal mucus, suggesting the possibility to temporary engraft the nanoparticle onto the intestinal wall.

Example 2: Biocatalytic activities of pancreatin-based silica nanoparticles NP-3 and NP-5

Exocrine Pancreatic Insufficiency is a condition in which the exocrine functions of the pancreas are impaired and its ability to effectively deliver digestive enzymes to the duodenum is defective. The standard medical therapy to treat clinical symptoms and malabsorption is oral pancreatic enzyme replacement therapy (PERT). Current approved therapies consist in pancreatic enzyme product (lipase, amylase, protease) from porcine origin.

Pancreatin-based silica nanoparticles NP-3 and NP-5 have been assessed with regard to their enzymatic activities. Enzymatic activity of lipase and amylase of NP-3 is displayed in Figure 18A and Figure 18C. Enzymatic activity of protease of NP-5 is displayed in Figure 18B.

These data show the ability of immobilizing multiples enzymes on the nanoparticles with the perfect maintenance of each enzymatic activity. The validation of these biocatalytic activities on the nanoparticles demonstrates the possibility to apply the strategy of immobilization and protection of enzymes to restore digestive functions that could be used for therapeutic purposes.

Example 3: *In vivo* activity of pancreatin-based silica nanoparticles NP-3 in PDL-rats

Given the importance of fat digestion for patients with exocrine pancreatitis insufficiency (EPI), the validation of pancreatin-based silica nanoparticles NP-3 was assessed by focusing on its ability to digest lipids *in vivo* using PDL-rats as first animal model. PDL-rats were dosed intraduodenally with pancreatin-based silica nanoparticles NP-3 or inactive nanoparticles NP-4 prior being gavaged with triolein (called hereafter dosing sequence). After a single dosing sequence, an increase of plasmatic TG was observed in PDL-rat that received NP-3 (active nanoparticles) compared to PDL-rat that received the inactive nanoparticles (NP-4) (Fig. 19). This result demonstrates the ability of pancreatin-based silica nanoparticles NP-3 to restore lipase digestive function in an EPI animal model.

Example 4: *In vivo* activity of pancreatin-based silica nanoparticles NP-3 in PDL-minipigs

From anatomical and physiological perspectives, the pig model is widely recognised to share high similarities with human gastrointestinal tract. So, PDL-minipigs have been generated to assess the efficacy of pancreatin-based silica nanoparticles NP-3. As previously described in PDL-rats model, the first validation of NP-3 consisted in a single dosing of the nanoparticles followed by the administration of olive oil in PDL-minipig. When compared to a healthy minipigs, the increase of the plasmatic level of TG in PDL-minipig dosed with pancreatin-based silica nanoparticles NP-3 showed a similar kinetic with a maximal level at 3 hours after feeding (Fig. 20A). The calculation of the area under the curve (AUC) demonstrates an extremely surprising digestion rate of 62% with pancreatin-based silica nanoparticles NP-3 compared with a healthy minipig (Fig. 20B). These results confirm the ability of pancreatin-based silica nanoparticles NP-3 to restore lipase digestive function in an EPI animal model.

Example 5: *In vivo* therapeutic efficacy of pancreatin-based silica nanoparticles NP-3 in PDL-minipigs

To further point-out the ability of pancreatin-based silica nanoparticles NP-3 to restore lipase digestive functions, PDL-minipigs receiving a diet enriched with fat were daily dosed with pancreatin-based silica nanoparticles NP-3 over 10 days. As the primary end point of current treatments for EPI patient is the quantification of fat absorption, the benefits of pancreatin-based silica nanoparticles NP-3 were evaluated by measuring the unabsorbed fat excreted in

feces. The comparison of the fecal fat content between healthy- and untreated-PDL-minipigs shows an accumulation of fat in the feces in the EPI animal model that reflects the default of lipase activity (3.42 vs 5.55 g/100g feces, healthy minipigs and untreated PDL-minipigs respectively) (Fig. 21A). Importantly, the level of fecal fat content decreases in PDL-minipigs treated with NP-3 compared to the untreated PDL-minipigs (Fig. 21A). In fact, PDL-minipigs that received daily doses of pancreatin-based silica nanoparticles NP-3 were able to digest 53.4% of the fat intake (Fig. 21B). These results clearly demonstrate the therapeutic efficacy of pancreatin-based silica nanoparticles NP-3 for pancreatic enzyme replacement therapies.

Enzyme-replacement therapies to treat clinical symptoms and malabsorption are available. A minimum dose of 40 000 – 50 000 Units of PERT-lipase is recommended at each main meals and half that dose with snacks. Such doses lead to a heavy pill burden for the patients. However, the treatment is not efficient and exhibits several limitations (persistence of the symptoms, low intraluminal survival time of the lipase, possible intolerance due to massive load of enzymes and gastrointestinal troubles due to massive amount of proteases).

Pancreatin-based silica nanoparticles NP-3 efficacy was compared to free pancreatin. The amount of lipase activity administered with NP-3 was calculated based on the amount of fat ingestion (1 300U per dose for 14g of olive oil), while the amount of lipase activity administered with pancreatin followed the standard of care (40 000U per dose). Surprisingly, pancreatin-based silica nanoparticles NP-3 administered at a dose of 1300 U twice daily significantly improves digestive efficiency by 25% compared to pancreatin, the standard treatment, which was given at a much higher dose of 40000 U twice daily. Indeed, pancreatin at 80000 U per day facilitates 43.6% fat digestion, while NP-3 at 2600 U per day achieves 53.4% fat digestion (Fig. 21B). Pancreatin-based silica nanoparticle NP-3 consists in a nanoparticle functionalized with mucoadhesive component allowing the interaction of the nanoparticles with the intestinal mucus (PCT/EP2023/051194). This results in a temporary engraftment of NP-3 and in a sustained lipase activity on the wall of the intestine. Additionally, the immobilization and protection of lipase on the nanoparticles protect the enzyme from external stresses (WO 2022/223699 A1) and stabilize its activity. So, this set of data demonstrate the added value of pancreatin-based silica nanoparticles NP-3 for lipase digestion and highlight the therapeutic potential of NP-3 for EPI patients.

Example 6: *In vitro* biocompatibility of pancreatin-based silica nanoparticles NP-5

Currently patients with exocrine pancreatic insufficiency take enormous amounts of pancreatin (10-20 pills/day), leading to a daily intake of high amount of proteases. Despite their digestive role, gastrointestinal proteases contribute to the intestinal homeostasis. Any imbalance in the level of proteases can lead to gastrointestinal pathologies (Vergnolle N *et al.*, Gut
5 2016;65:1215–1224).

To evaluate the safety of pancreatin-based silica nanoparticles NP-5, we focused on the maintenance of the intestinal barrier integrity in presence of the nanoparticles and compared it to pancreatin (Fig. 22A). The transepithelial electrical resistance (TEER) measurements across Caco2-HT29-MTX-E12 cell monolayers shows that the integrity of the intestinal epithelial
10 barrier remains intact when in contact with NP-5 for 20h while the treatment with pancreatin leads to a dose-dependent loss of the barrier integrity. To correlate TEER measurements with morphological characteristics of the cell monolayer, tight junction protein Zonula-Occludens-1 (ZO-1) was evaluated by confocal microscopy (Fig. 22B). When cultured in presence of NP-5, ZO-1 staining shows a continuous ring appearance at the cell borders while in presence of
15 pancreatin, the staining of ZO-1 reveals a diffused punctuated protein distribution around intestinal cells. These morphological data confirmed the safety of pancreatin-based silica nanoparticles NP-5 for gastrointestinal application and highlights the added value of our technology for pancreatic enzyme replacement therapies.

20 **Example 7: Enhancing Pancreatin Stability through Covalent Attachment to the protective layer**

In a first experiment, pancreatin-based silica nanoparticles NP-3(1) were produced in buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth). In a second experiment, pancreatin-based silica nanoparticles NP-3 were
25 produced in non-buffered conditions without any intermediate washing steps (*i.e.* unreacted glutaraldehyde still present in the reaction mixture during layer growth).

Protein quantification was performed on the reaction supernatants to determine pancreatin immobilization yield at the surface of NP-3(1), and NP-3. The results show that surprisingly enzyme immobilization under conditions where the presence of glutaraldehyde is maintained
30 (NP-3) increases the enzyme immobilization yield by a factor of 2 (Fig. 23A), resulting in a doubling of enzyme loading per dry weight of SNP (Fig. 23B) compared to buffered conditions where glutaraldehyde is removed by washing steps (NP-3(1)).

The lipase biocatalytic activity of pancreatin immobilized and protected on NP-3(1) and NP-3 was evaluated. Even more surprising than the increase in enzyme immobilization load where the presence of glutaraldehyde is maintained, the specific activity of nanoparticles was increased eleven-fold compared to buffered conditions where glutaraldehyde is removed by washing steps (Fig. 23C). This surprising eleven-fold increase in nanoparticle specific activity is accompanied by an extremely surprising seven-fold increase in pancreatin specific activity (Units / g pancreatin) of the enzyme protected in the presence of glutaraldehyde compared with buffered conditions where glutaraldehyde is removed by washing steps (Fig. 23D). In summary, covalent attachment of the protective layer to pancreatin surface unexpectedly enhances its load, stability and enzyme specific activity compared to pancreatin protected with an organosilica layer via electrostatic interactions only.

Lipase

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, recombinant human pancreatic lipase (HRL, certified reference material), porcine pancreatic lipase (4xUSP specifications), 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester), Tris base, colipase, sodium taurocholate hydrate were purchased from Sigma-Aldrich. HRL, porcine pancreatic lipase and colipase were dissolved in water to reconstitute the stock buffer.
- Benzyltriethoxysilane (B, 96%), was purchased from abcr GmbH.
- The trifluoroacetate salt of peptide Glu-Leu-Gly-Gly-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Arg-Glu-Gly-Gly-Gly-Glu-Arg-Gly-Gly-Gly-Gly-Asn-Gly-Gly-Gly-Gly (SEQ ID NO: 6) having E-azido-Nle-OH at the carboxy end was purchased from Bachem.

Synthesis of silica nanoparticles:

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 400 rpm at 20°C for 22 h. The solution was then centrifuged at

20000 g for 20 min and washed successively with ethanol and water. Particle size measurement was carried out on SEM micrographs acquired at a magnification of 150000x using the image analysis software Olympus stream motion.

5 ***Free recombinant human pancreatic lipase activity assay:***

To a solution of recombinant human pancreatic lipase (3.43 μ L, 1 mg/mL) in Tris buffer (0.1 M, pH 8.4, 56.6 μ L) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (60 μ L, 100 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

10

Free recombinant human pancreatic lipase activity assay in presence of colipase:

To a solution of recombinant human pancreatic lipase (3.43 μ L, 1 mg/mL) and colipase (0.72 μ L, 3 μ g/mL) in Tris buffer (0.1 M, pH 8.4, 55.8 μ L) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (60 μ L, 100 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

15

Immobilized and protected recombinant human pancreatic lipase activity assay:

To a solution of immobilized and protected recombinant human pancreatic lipase (20 μ L, 10 mg/mL) in Tris buffer (0.1 M, pH 8.4, 40 μ L) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (60 μ L, 100 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

20

25 ***Immobilized and protected porcine pancreatic lipase activity assay:***

To a solution of immobilized and protected porcine pancreatic lipase (15 μ g/mL of enzyme) in Tris buffer (0.1 M, pH 8.4) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (50 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

30

Example 1

A) *Recombinant human pancreatic lipase (HRL) immobilization and shielding*

To SNPs (10 mg/mL) 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 performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A recombinant human pancreatic lipase solution (525 µg/mL, 11 µM) 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 lipase using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-HRL-ATB were cured overnight in a water bath at 20 °C.

B) Recombinant human pancreatic lipase (HRL) and colipase (CLPS) co-immobilization and shielding:

To SNPs (10 mg/mL) 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 performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A solution containing recombinant human pancreatic lipase solution (525 µg/mL, 11 µM) and colipase (110 µg/mL) 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 proteins using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-HRL-CLPS-ATB were cured overnight in a water bath at 20 °C.

25 Activation of recombinant human pancreatic lipase (HRL) with colipase

Activation of recombinant human pancreatic lipase with colipase was studied using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 27a). The faster kinetics of lipase substrate hydrolysis by lipase in the presence of colipase than that of lipase alone shows the proper activation of the lipase enzyme by colipase.

To generate activated lipase nanoparticles, recombinant human pancreatic lipase was co-immobilized with colipase, and protected in a hydrophobic organosilica shield on the surface of

silica nanoparticles as described under B) above. Activation of lipase by colipase was assessed using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 27b). The faster kinetics of lipase substrate hydrolysis by lipase co-immobilized with colipase as described under B) above than that of lipase immobilized without the colipase as described under A) above shows an activation of the lipase enzyme by colipase in the shielded nanoparticle being surprisingly more than three times higher than the lipase enzyme without colipase. These results validate the strategy of co-immobilizing lipase and colipase on the surface of silica nanoparticles to produce nanoparticles bearing immobilized and protected lipase maintained in its active conformation.

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Example 2

A) Porcine pancreatic lipase (PL) immobilization and shielding

To SNPs (10 mg/mL) 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 performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A porcine pancreatic lipase solution (1.9 mg/mL, 39 µM) 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 lipase using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-PL-ATB were cured overnight in a water bath at 20 °C.

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B) Porcine pancreatic lipase (PL) and colipase (CLPS) co-immobilization and shielding according to the present invention:

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To SNPs (10 mg/mL) 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 performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A solution containing porcine pancreatic lipase (1.9 mg/mL, 39 µM) and colipase (150 µg/mL) 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 proteins using

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APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-PL-CLPS-ATB were cured overnight in a water bath at 20 °C.

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Activation of porcine pancreatic lipase (PL) with colipase

To generate activated lipase nanoparticles, porcine pancreatic lipase was co-immobilized with colipase, and protected in a hydrophobic organosilica shield on the surface of silica nanoparticles as described under B) above. Activation of lipase by colipase was assessed using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 28). The faster kinetics of lipase substrate hydrolysis by lipase co-immobilized with colipase as described under B) above than that of lipase immobilized without colipase as described under A) above shows the activation of the lipase enzyme by colipase in the shielded nanoparticle being surprisingly three times higher than the lipase enzyme without colipase.

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These results validate the strategy of co-immobilizing lipase and colipase on the surface of silica nanoparticles to produce nanoparticles bearing immobilized and protected lipase maintained in its active conformation. This also demonstrates the versatility of the method with respect to the origin of the enzyme used.

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Example 3

Porcine pancreatic lipase (PL) immobilization and shielding in presence of sodium taurocholate (NaTc):

To SNPs (10 mg/mL) in a mixture of NaTc (2 mM), H₂O and 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.

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Then, glutaraldehyde (3.8 mM) was added and the reaction mixture was stirred for 10 min at 20 °C, 400 rpm. A priming was performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A porcine pancreatic lipase (1.9 mg/mL, 39 μM) was added and the reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm.

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An organosilica layer was grown at the surface of the immobilized lipase using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000

rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-PL-NaTc-ATB were cured overnight in a water bath at 20 °C.

Activation of recombinant human pancreatic lipase (HRL) with sodium taurocholate

5 ***(NaTc)***

Activation of recombinant human pancreatic lipase by sodium taurocholate was studied using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 29). The faster kinetics of lipase substrate hydrolysis by lipase in the presence of increasing concentration of sodium taurocholate than that of lipase alone shows the proper

10 activation of the lipase enzyme by the bile salt.

Activation of porcine pancreatic lipase (PL) with sodium taurocholate (NaTc)

To generate activated lipase nanoparticles, porcine pancreatic lipase was immobilized in presence of sodium taurocholate and protected in a hydrophobic organosilica shield on the surface of silica nanoparticles. Activation of lipase by sodium taurocholate was assessed using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 30). The faster kinetics of lipase substrate hydrolysis by lipase immobilized in presence of sodium taurocholate than that of lipase immobilized without the bile salt shows the activation of the lipase enzyme by sodium taurocholate in the shielded nanoparticle being

20 surprisingly almost three times higher than the lipase enzyme without sodium taurocholate.

These results validate the strategy of immobilizing lipase in presence of sodium taurocholate on the surface of silica nanoparticles to produce nanoparticles bearing immobilized and protected lipase maintained in its active conformation.

25 ***Example 4: Activation of pancreatic lipase (PL) with a colipase-mimicking peptide***

The analysis of the lipase-colipase complex has enabled to identify aminoacid residues responsible for lipase-colipase interactions. Based on these findings, we designed a peptide as shown in SEQ ID NO: 6 (Glu-Leu-Gly-Gly-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Arg-Glu-Gly-Gly-Gly-Glu-Arg-Gly-Gly-Gly-Gly-Asn-Gly-Gly-Gly-Gly-Gly) to mimic colipase interactions with lipase structure, induce conformational changes around the lid and lipase lid opening (Figure 31). The peptide was chemically modified at its carboxyl end by adding a adding

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and-E-azido-Nle-OH group at its carboxy-end to enable cross-linking of the lipase enzyme at the surface of silica nanoparticles by click-chemistry.

Example 5:

5 *Production of HRL-based silica nanoparticles NP-1 variants:*

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, HRL-based silica 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, 69 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.1 mM).
10 The reaction 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 (3.1 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
15 H₂O/PS80 (8 mg/L). A priming was performed by adding APTES (3.1 mM) and stirring the reaction mixture 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). Sodium taurocholate (2 mM) and human recombinant lipase (0.732 g/L, 15.2 µM) were successively added and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the
20 surface of the immobilized HRL using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). NP-1(1) were cured overnight in a water bath at 20°C.

In a second, comparative experiment, enzyme immobilisation and formation of the protective
25 layer were carried out according to WO2015/014888 A1 to produce HRL-based silica nanoparticles (NP-1(2)) in buffer. Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) was added APTES (3.1 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
30 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Then, glutaraldehyde (3.1 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 (3.1 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). Sodium taurocholate (2 mM) and human recombinant lipase (0.732 g/L, 15.2 µM) were successively 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 HRL using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 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). NP-1(2) were cured overnight in a water bath at 20°C.

In a third experiment, HRL-based silica nanoparticles (NP-1) were produced in H₂O/PS80 (8 mg/L). To keep the excess amount of glutaraldehyde which has not linked the solid carrier to the engineered HRL 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 human recombinant lipase. To SNPs (10 mg/mL, 69 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.1 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.1 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.1 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Sodium taurocholate (2 mM) and human recombinant lipase (0.732 g/L, 15.2 µM) were successively 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 HRL using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). NP-1 were cured overnight in a water bath at 20°C.

The covalent binding of the protective layer to HRL 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 HRL and the organosilica layer. The absorbance of HRL-based silica nanoparticles NP-1(1), NP-1(2), and NP-1 at 460 nm was

measured after the organosilica layer formation and final particles washing (see Figure 33A). The results showed that NP-1 absorbed much more light than NP-1(2) at this wavelength. Interestingly, NP-1 and NP-1(1) had similar absorbance values, even though NP-1 appeared darker visually. This suggests that the instrument could not distinguish the formation of imine bonds (due to the low enzyme amount used) because of the strong interference from the particles themselves. To overcome this limitation, we used an inverted microscope to capture images of the NPs (Figure 33B). At the same concentration, NP-1 appeared significantly darker than NP-1(1) under the microscope. This visual confirmation indicates that NP-1 has more imine bonds, likely due to the covalent attachment of the protective layer to the human recombinant lipase.

Enhancing Human Recombinant Lipase Stability and Specific Activity through Covalent Attachment to the protective layer

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

Protein quantification was performed on the reaction supernatants to determine HRL immobilization yield at the surface of HRL-based silica nanoparticles 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 4 (Fig. 32A), resulting in a quadrupling of enzyme loading per dry weight of SNP (Fig. 32B) compared to buffered conditions where glutaraldehyde is removed by washing steps (NP-1(2)).

The lipase biocatalytic activity of HRL immobilized and protected on NP-1(1), NP-1(2) and NP-1 was evaluated. Even more surprising than the increase in enzyme immobilization load when glutaraldehyde is maintained, the specific activity of nanoparticles increased thirty-seven-fold compared with buffered conditions where glutaraldehyde is removed by washing steps,

and two-fold compared with unbuffered conditions where glutaraldehyde is removed by washing steps (Fig. 32C). These surprising 37- and 2-fold increases in nanoparticle specific activity are accompanied by an extremely surprising 10- and 2-fold increase, respectively, in HRL specific activity ($U_{\mu M/min/g}$ of HRL) of the enzyme protected in the presence of
5 glutaraldehyde compared with buffered and unbuffered conditions where glutaraldehyde is removed by washing steps (Fig. 32D). This result is completely unexpected, as the enzyme is expected to have a much higher activity in the presence of a buffer.

In summary, covalent attachment of the protective layer to HRL surface unexpectedly enhances its load, stability and enzyme specific activity compared to enzymes protected with an
10 organosilica layer via electrostatic interactions only.

Claims

1. A method of producing a composition, the composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally 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;
- (b) immobilizing a protein or a fragment thereof on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier and ii) a solution of the protein or of a fragment thereof is added to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein or a fragment thereof;
- (c) forming a protective layer on the surface of the solid carrier to protect the protein or the fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b), or a part thereof, covalently binds the protective layer to the protein or the fragment thereof; and optionally
- (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.
2. The method of claim 1, wherein there is no washing step between adding the linker to the suspension of the solid carrier in (i) of step (b) and adding the protein or a fragment thereof to the suspension comprising the solid carrier and the linker in ii) of step (b).
3. The method of claim 1 or 2, wherein there is no washing step between any of steps (a) to (c).

4. The method of any one of claims 1-3, wherein the solid carrier is provided in suspension in water, buffer or non-ionic surfactants or mixtures thereof.
5. The method of any one of claims 1-3, wherein the solid carrier is provided in suspension in mixtures of water and non-ionic surfactants.
6. The method of any one of claims 1-5, wherein the linker is added to the suspension of the solid carrier in i) of step (b) in a molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b).
7. The method of any one of claims 1-5, wherein the linker is added to the suspension of the solid carrier in i) of step (b) in a 1 fold to 1000 fold molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b).
8. The method of any one of claims 1-5, wherein the linker is added to the suspension of the solid carrier in i) of step (b) in a 4 fold to 250 fold molar excess to the protein or a fragment thereof added to the suspension comprising the solid carrier and the linker in ii) of step (b).
9. The method of any one of claims 1-8, wherein the linker which has not connected the solid carrier with the protein 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).
10. The method of any one of claims 1-9, wherein the amount of the linker which has not connected the solid carrier with the protein or a fragment thereof in step (b) after addition of the protein or a fragment thereof in ii), is between 30% and 70% of the amount of linker added to the suspension of the solid carrier in i) of step (b).
11. The method of any one of claims 1-10, wherein the protein or a fragment thereof is immobilized on the solid carrier by covalent binding via the linker in step (b).

12. The method of any one of claims 1-11, wherein the surface of the solid carrier is at least partly modified before the protein or a fragment thereof is immobilized on the solid carrier in step (b).
- 5 13. The method of any one of claims 1-12, wherein the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOE (Bis[2-(succinimidocarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3 '-
- 10 Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3 '-dithiobispropionimide-2 HCl), DST (Disuccinimidyl tartrate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane).
- 15 14. The method of any one of claims 1-12, wherein the linker is glutaraldehyde.
15. A composition comprising a solid carrier, a protein or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the protein or a fragment thereof by embedding the protein or a fragment thereof, and optionally a
- 20 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 method of any one of claims 1-14.
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