

Functionalized biocatalytical compositions comprising pancreatic enzymes**The field of the invention**

The present invention relates to a composition comprising a solid carrier, a lipase or a
5 fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a
fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on
the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface
of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment
thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a
10 fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment
thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a
functional constituent immobilized on the surface of the protective layer, wherein the
functional constituent immobilized on the surface of the protective layer is a polymer
comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
15 least one thiol group. The present invention also relates to methods of producing said
composition and uses thereof.

Background of the invention

Exocrine Pancreatic Insufficiency (EPI) is a condition in which the exocrine functions of the
20 pancreas are impaired and its ability to effectively deliver digestive enzymes to the duodenum
is defective. It is a serious health condition that leads to malabsorption of lipids, fat-soluble
vitamins, proteins, and, to a lesser extent, carbohydrates from the digestive tract.

The standard medical therapy to treat clinical symptoms and malabsorption is oral pancreatic
enzyme replacement therapy (PERT). Current approved therapies consist in pancreatic
25 enzyme product (lipase, amylase, protease) from porcine origin, also called pancreatin. 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 current treatment is not efficient and exhibits several limitations: Inefficiency of
the treatment, persistence of the symptoms, low intraluminal survival time of the lipase that is
30 susceptible to proteases degradation (default of fat digestion), possible intolerance to massive
loads of enzymes, and/or gastrointestinal troubles due to the high amount of proteases
ingested. Thus, there is a need to provide compatible and effective compositions for treatment

of Exocrine Pancreatic Insufficiency (EPI).

Summary of the invention

The present invention provides a composition comprising a solid carrier, a lipase or a
5 fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a
fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on
the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface
of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment
thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a
10 fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment
thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a
functional constituent immobilized on the surface of the protective layer, wherein the
functional constituent immobilized on the surface of the protective layer is a polymer
comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
15 least one thiol group.

The present invention also provides a method of producing a composition, the composition
comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the
solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease
or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment
20 thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid
domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a
fragment thereof, the protease or a fragment thereof, and the amylase or a fragment thereof by
embedding the lipase or a fragment thereof, the protease or a fragment thereof and the
amylase or a fragment thereof, and a functional constituent immobilized on the surface of the
25 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;
- 30 (b) providing a lipase or a fragment thereof, a protease or a fragment thereof and an amylase
or a fragment thereof ;
- (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);

(e) immobilizing the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier;

(f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof
5 immobilized on the solid carrier;

(g) 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.

10 The present invention also provides a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI) and a method of enzyme replacement therapy (ERT).

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

15 biodistribution specificity and high bioactivity in vitro and in vivo, show low cytotoxicity and surprisingly high biocompatibility, do not disrupt the intestinal barrier if localized in the gastrointestinal tract and do restore lipase digestive function in an EPI animal model, thus making them extremely promising for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI).

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Brief description of the figures

Figure 1) 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
25 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 2) shows the biodistribution of ^{111}In -NP-1 (n=5) and ^{111}In -NP-2 (n=4) (%ID) in
30 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 3) 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 NP-3 after exposure to olive oil. (B) Protease activity (in U/mg) of NP-5 after exposure to casein. (C) Amylase activity (in U/g) of NP-3 after exposure to amylase substrate solution.

Figure 4) shows the relative quantification of plasmatic triglycerides (TG) of pancreatic duct ligated (PDL) rats. PDL rats were dosed with NP-3 (n=1) or 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 5) shows the comparison of plasmatic triglycerides (TG) concentration between healthy minipigs and pancreatic duct ligated (PDL) minipigs treated with NP-3. Healthy minipigs (n=1) and PDL minipig (n=1) received olive oil, and sequentially, PDL minipig was dosed with 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 6) 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 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.

Figure 7) shows the *in vitro* biocompatibility of 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 NP-5 or pancreatin (from 32.9 to 263.6 U/m²) for 20h. TEER data was normalized with the equilibrium value before the addition of 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 NP-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 8) shows the added value of the covalent bonding of the enzyme surface to the protective layer. (A) Protein quantification performed on reaction supernatants of NP-3 and NP-3(1). (B) Pancreatin loading per dry weight of SNP. (C) SNP activities expressed in $\mu\text{mol/min}$. (D) Pancreatin-specific activities expressed in U/g of pancreatin.

Figure 9) shows absorbance of nanoparticles NP-3 and NP-3(1) at 460 nm.

Detailed description of the invention

The present invention relates to a composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

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

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

any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The invention is not restricted to the details of any foregoing embodiments.

- 5 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.

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

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

- 15 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 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
20 size of the solid carrier is usually between 1 nm and 1000 μm , preferably between 10 nm and 100 μm , particularly about 50 nm.

- The term “linker” or “cross-linker” which are used synonymously herein refers to any linking reagents containing groups, which are capable of binding to specific functional groups (e.g.
25 primary amines, sulfhydryls, etc.). A linker in the context of the present invention usually connects the surface of the solid carrier with the enzyme e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the 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 enzyme e.g. the lipase or a fragment thereof, the protease or a
30 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 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.

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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 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.

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The 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.

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The term, "lipase or a fragment thereof" includes naturally occurring lipases or a fragment 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 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 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

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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
5 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 pancreatin.

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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
15 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,
20 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
25 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,
30 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 "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 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 more at least 70% will be covered, thus improving protection of the protein. In a preferred embodiment, at least 70%,
5 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 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,
10 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 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
15 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.

20 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%
25 covered by the protective layer, i.e. that also the active site is covered.

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
30 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.

The term “agent which interacts with the lid domain of the lipase or a fragment thereof” as
5 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,
10 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 conformation, the hydrophobic face becomes exposed and contributes to the substrate-
15 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
20 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
25 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
30 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).

- 5 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
- 10 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
- 15 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 the same functional properties as the colipase from which it is derived. A preferred colipase is the colipase with Uniprot
- 20 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 right position to interact with amino acids of the pancreatic lipase structure,
- 25 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: 1.

- The term “bile salt” as used herein refers to bile acids conjugated with taurine or glycine and
- 30 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

glycol mono-octyl ether, octyl- β -D-glucopyranoside, N,N-dimethyldodecylamine-N-oxide, and β -octylglucoside.

5 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 fatty acids, joined by an alcohol residue (usually a glycerol molecule). Phospholipids include lecithin and lysolecithin.

10 The term “functional constituent” as used herein refers to a constituent which after being immobilized to the surface of the protective layer retains its characteristic, functional property. A functional constituent in the sense of the present invention is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

15 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 polymer comprises a number of repeat units (monomers), wherein each repeat unit contains one amino group, in particular one primary amino group.

20 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 (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
25 one thiol group.

The term “polycarbophil-cysteine conjugates” as used herein refers to conjugates which 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
30 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 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.

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

The term “polyglucosamin” as used herein refers to linear amino-polysaccharides composed of D-glucosamine and N-acetyl-D-glucosamine units linked by (1-4) glycosidic bonds.

10 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

15 thereof. Most preferred is a chitosan or a derivative thereof.

The term “chitosan or a derivative thereof” as used herein refers to a chitosan or chitosan 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 -

20 350 000 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 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,

25 xanthates, hydrochlorides, glutamates, lactates, acetates.

In a first aspect the present invention provides a composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof

30 immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a

fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof ,
and a functional constituent immobilized on the surface of the protective layer, wherein the
functional constituent immobilized on the surface of the protective layer is a polymer
comprising repeat units wherein each repeat unit comprises at least one amino group and/or at
5 least one thiol group.

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
10 p-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.

15 A solution of pancreatin, a lipase or a fragment thereof, a protease or a fragment and/or an
amylase or a fragment thereof usually comprises the protein or a fragment thereof or the
proteins 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
20 solution may additionally contain sugar alcohols or non-ionic surfactants as described herein.
A solution of pancreatin, a lipase or a fragment thereof, a protease or a fragment and/or an
amylase or a fragment thereof can be prepared by e.g. dissolving the protein or fragment or
the proteins or a fragment thereof thereof in water to reconstitute the stock buffer of the
protein or a fragment thereof or the proteins or a fragment thereof.

25 In one embodiment the solid carrier is selected from the group of organic particles, inorganic
particles, organic-inorganic particles, self-assembling organic particles, silica particles, gold
particles, titanium particles and is preferably a silica particle, more preferably a silica
nanoparticle (SNP). The particle size is usually measured by measuring the diameter of the
30 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 embodiment the composition comprises a solid carrier wherein the solid carrier comprises at least 15%, preferably at least 25%, in particular between 15% and 40%, more particular between 25% and 35 % immobilized lipase, protease and/or amylase, or a fragment thereof,
5 per dry weight of the solid carrier.

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

The solid carrier is usually provided in suspension. Suspension of the solid carrier can be e.g. in water, buffer or non-ionic surfactants or mixtures thereof, preferably in mixtures of water and non-ionic surfactants. Non-ionic surfactants are usually selected from the group
15 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
20 palmitate, beeswax, polyglyceryl 3-oleate, propylene glycol monocaprylate, propylene glycol monolaurate; fatty alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). Buffers which can be used in the method of the present invention are phosphate, piperazine-N,N'-bis(2-ethanesulfonic acid), 2-Hydroxy-3-
25 morpholinopropanesulfonic acid, N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), (3-(N-morpholino)propanesulfonic acid), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 3-(N,N-Bis[2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid, N,N-Bis(2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid, N-[Tris(hydroxymethyl)methyl]glycine, Diglycine, 4-
30 (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.

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 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 lipase or a fragment thereof, a protease or a fragment thereof and a 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 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 a lipase or a fragment thereof, a protease or a fragment thereof and a amylase or a fragment thereof, or pancreatin or a solution thereof,, wherein the suspension of the solid carrier is incubated with the solution comprising a lipase or a fragment thereof, a protease or a fragment thereof and a amylase or a fragment thereof, or pancreatin or a solution thereof, to allow 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 lipase on the solid carrier, a protease or a fragment thereof and a 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 lipase or a fragment thereof, a protease or a fragment thereof and a 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 a lipase or a fragment thereof, a protease or a fragment thereof and a 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 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 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 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 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 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 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 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:1, and 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 lipase or a fragment thereof shifts to and/or maintains the open conformation.

- 5 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.

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

- 20 In one embodiment, 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 partially embedded by the protective layer. In a preferred embodiment 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 at least partially embedded by the protective layer. In a more preferred embodiment 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 fully embedded by the protective layer.

- 30 In one embodiment, the protective layer embeds the solid carrier and embeds 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. In one embodiment, the functional constituent immobilized on the surface of the protective layer, is not embedded by the protective layer. Preferably, the protective layer fully embeds the solid carrier and fully embeds the 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. More preferably, the protective layer fully embeds the solid carrier and fully embeds 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 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 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 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 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.

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. Thus, the present invention also provides a composition comprising a solid carrier; pancreatin, wherein the pancreatin comprises a lipase or a fragment thereof wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof and an amylase or a fragment thereof, wherein the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof are immobilized on the surface of the solid carrier; an agent which interacts with the lid domain of the lipase or a fragment thereof; a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof; and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

The protective layer thickness can be measured, by using a microscope such as scanning electron microscope (SEM), transmission electron microscopy (TEM), scanning probe

microscopy (SPM), light scattering methods or by ellipsometry.

The composition of the present invention is usually produced in a reaction vessel like a reactor. The formation of the protective layer is usually carried out by forming the respective
5 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 the building blocks, which can be used to build the protective layer, may depend on the
10 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 protective layer. Structural building blocks which can be used are e.g. tetraethylorthosilicate
15 (designated herein as “TEOS” or “T”). Protective building blocks which can be used are e.g. 3-Aminopropyltriethoxysilane (designated herein as “APTES” or “A”), Propyltriethoxysilane (designated herein as “PTES” or “P”), Isobutyltriethoxysilane (designated as “IBTES”), Hydroxymethyltriethoxysilane (designated herein as “HTMEOS” or H), Benzyltriethoxysilane (designated herein as “BTES”), Ureidopropyltriethoxysilane
20 (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 fragment thereof and the amylase or a fragment
25 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
30 tetraethylorthosilicate, tetra-(2-hydroxyethyl)silane, and tetramethylorthosilicate. Even more preferred protective building blocks are selected from the group consisting of carboxyethylsilanetriol, benzylsilanes, propylsilanes, isobutylsilanes, n-octylsilanes, hydroxysilanes, bis(2-hydroxyethyl)-3 -aminopropylsilanes, aminopropylsilanes,

- ureidopropylsilanes, (N-Acetylglycyl)-3-aminopropylsilanes, hydroxy(polyethyleneoxy)propyl]triethoxysilanes, in particular selected from benzyltriethoxysilane (BTES), propyltriethoxysilane, isobutyltriethoxysilane, n-octyltriethoxysilane, hydroxymethyltriethoxysilane, bis(2-hydroxyethyl)-3 -
- 5 aminopropyltriethoxysilane, 3-Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglycyl)-3-aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutyltrimethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3 -aminopropyltrimethoxysilane, 3-Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglycyl)-3-
- 10 aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutyltrimethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrimethoxysilane, aminopropyltrimethoxysilane, ureidopropyltrimethoxysilane (N-Acetylglycyl)-3-aminopropyltrimethoxysilane or selected from benzyltrihydroxyethoxysilane, propyltrihydroxyethoxysilane,
- 15 isobutyltrihydroxyethoxysilane, n-octyltrihydroxyethoxysilane, hydroxymethyltrihydroxyethoxysilane, bis(2-hydroxyethyl)-3 -aminopropyltrihydroxyethoxysilane, aminopropyltrihydroxyethoxysilane, Ureidopropyltrihydroxyethoxysilane (N-Acetylglycyl)-3-aminopropyltrihydroxymethoxysilane.
- 20 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.
- 25 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
- 30 preferably between 2 and 4 hours, preferably in aqueous solution and preferably at room temperature of about 5 to about 25 °C or at about 20 °C. The formation of the protective layer can be stopped by actively stopping the polycondensation reaction e.g. by removing the non-reacted building blocks e.g. by a washing step or by self-stopping of the polycondensation

reaction caused by a limited amount of building blocks.

In a further more preferred embodiment the protein 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 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 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 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.

In a preferred embodiment the cross-linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls, sulfhydryl-reactive 2-pyridyldithiol, BSOCOES (Bis[2-(succinimidooxycarbonyloxy)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 3-(2-pyridyldithio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate), SMPT (4-Succinimidylloxycarbonyl-methyl- α -(2-pyridyldithio)toluene), DPDPB (1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane), DTME (Dithio-bismaleimidoethane), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane). More preferably said cross-linker is selected from glutaraldehyde, disuccinimidyl tartrate, disuccinimidyl suberate, bis[sulfosuccinimidyl] suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, 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 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), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3'-dithiobispropionimide·2 HCl), DST (Disuccinimidyl tartrate), BMDB (1,4 bismaleimide-2,3-dihydroxybutane). More preferably said cross-linker is selected from glutaraldehyde, disuccinimidyl tartrate, 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). Most preferred is glutaraldehyde.
- After the protective layer has been formed, the suspension obtained can be washed to remove excess agent which interacts with the lid domain of the lipase or a fragment thereof. 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 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 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 30 hours.

In one embodiment, the functional constituent binds to mucus.

In one embodiment a polymer comprising repeat units wherein each repeat unit comprises at 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.

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 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.

15 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.

25 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.

30 In a further embodiment a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is selected from the group

consisting of a polyglucosamin, a polymerized silane-PEG-NH₂, a polymerized silane 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

5 comprises at least one amino group and/or at least one thiol group is selected from the group consisting of a polyglucosamin selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof; a polymerized silane-PEG-NH₂; a polymerized silane comprising a thiol group, preferably a polymerized MPTS; a polycarbophil-cysteine conjugate; a polymerized silane-PEG-thiol; and

10 a polycysteine. In an even more preferred embodiment the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, is 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,

15 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

20 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

25 comprising a thiol group, which is preferably polymerized MPTS.

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

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

In a more preferred embodiment the polymer comprising repeat units wherein each repeat unit

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

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

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

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

least one thiol group is immobilized on the surface of the protective layer by covalent binding.

In one embodiment the functional constituent is immobilized on the surface of the protective layer using a spacer binding to the surface of the protective layer and the functional
5 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 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, 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
15 reacting the spacer with the functional constituent, so that the spacer binds to the functional constituent and then the functional constituent bound to the spacer is reacted with the the surface of the protective layer.

The immobilization of the functional constituent to the surface of the protective layer is
20 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),
25 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
30 alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). The functional component is then added to the suspension to react usually under stirring with the surface of the protective layer to immobilize the functional

constituent on the surface of the protective layer. Usually such obtained composition is washed and resuspended into water, buffer or non-ionic surfactants or mixtures thereof. Immobilization takes place by non-covalent binding e.g. electrostatic binding or by covalent binding of the functional constituent. The functional constituent may be immobilized by

5 chemically modifying the surface of the protective layer and the functional constituent using e.g. "click chemistry" such as copper-catalyzed click chemistry (Copper-catalysed azide-alkyne cycloaddition, see e.g. Kolb et al. (2001) *Angew. Chem.* 40(11)2004-2021) or by copper free click chemistry (Wittig G, *A Chem Ber*, 1961, 94, 3260), e.g. the solid carrier carrying the protein e.g. the enzyme embedded in a protective layer as described supra is first

10 reacted with a reactive compound like an ethynyl compound and the functional constituent is modified by adding a reactive compound e.g. an azide residue and then both components are reacted to immobilize the functional constituent on the surface of the protective layer.

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

15 as a medicament.

In a further aspect the present invention provides the composition 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

20 pancreatic insufficiency (EPI).

In a preferred embodiment the present invention provides the composition for use in a method for the prevention, delay of progression 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

25 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

30 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
5 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.

A composition according to the invention is preferably a pharmaceutical composition and
10 comprises a therapeutically effective amount of the composition as described herein and one or more suitable pharmaceutically acceptable carrier. A pharmaceutical composition according to the invention is suitable for oral administration to a subject. If not indicated otherwise, a pharmaceutical composition according to the invention is prepared in a manner known per se.
15 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 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
20 conveniently be administered in unit dosage forms.

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
25 well within the capability of those skilled in the art, especially in light of the detailed disclosure 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
30 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, 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
5 will be appreciated that when a medicament is administered to a patient to treat a disease, the outcome may not always be effective treatment.

As used herein, "delay of progression" means increasing the time to appearance of a symptom of or slowing the increase in severity of a symptom. Further, "delay of progression" as used
10 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
15 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
20 will depend on the severity and course of the disease, previous therapy, the subject's health status and response to the drugs, and the judgment of the treating physician.

In the case wherein the subject's condition does not improve, the pharmaceutical combination of the invention may be administered chronically, which is, for an extended period of time, including throughout the duration of the subject's life in order to ameliorate or otherwise
25 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
30 the pharmaceutical combination of the invention is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, is optionally reduced, as a function of the symptoms, to a level at which the improved disease is retained.

In a further aspect the present invention provides a method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:

- (a) providing a solid carrier;
- (b) providing a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof;
- (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);
- (e) immobilizing the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier;
- (f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the solid carrier;
- (g) 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.

In a preferred embodiment, pancreatin comprising a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof is provided in step b).

Step (a) is usually carried out by providing the solid carrier in suspension in water or a buffer, preferably in water, non-ionic surfactants or a buffer or mixtures thereof, preferably in buffer,

more preferably in suspension in water and/or non-ionic surfactants, even more preferably in suspension in water and/or non-ionic surfactants wherein no buffer is present in the suspension, in particular in suspension in mixtures of water and non-ionic surfactants, more particular in suspension in mixtures of water and non-ionic surfactants wherein no buffer is present in the suspension. In a preferred embodiment, pancreatin is provided in step b). Step b) and c) are usually performed separately or can be performed at once e.g. lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof and agent, or pancreatin and agent, can be provided in one solution. The immobilization of the protein 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 in step b) of the present method is usually carried out by adding a solution of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, or pancreatin or a solution thereof, to the suspension of the solid carrier. Preferably a linker to connect the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is added to the suspension of the solid carrier prior to adding the solution of the protein to the suspension of the solid carrier. In a preferred embodiment the immobilization of the protein 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 adding a solution of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, or pancreatin or a solution thereof, wherein the suspension with the added solution of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, or pancreatin or a solution thereof is incubated to allow the protein 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 the protein 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 in step (e) is carried out by i) adding a linker to the solid carrier provided in step (a), preferably adding a linker to a suspension of the solid carrier provided in step a), and ii) adding the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), preferably adding a solution of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), to the solid carrier and the linker or to the

suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e). In one embodiment, a building block of the protective layer, preferably a monomer of a building block of the protective layer, more

5 preferably an organosilane, even more preferably a triethoxysilane, in particular APTES, is added to the solid carrier and the linker or to the suspension comprising the solid carrier and the linker, prior to adding the solution 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 a preferred embodiment the surface of the solid carrier is at least partly modified to
10 improve immobilization of the protein 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. In particular, the surface of the solid carrier is at least partly modified before the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof is immobilized. The surface of the solid carrier can be at least partly modified by adding a
15 molecule as anchoring point for the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof to the surface of the solid carrier as described supra.

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 the molecule as
20 anchoring point, the solid carrier and the linker and, the solid carrier comprising the linker and the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, respectively, so that the the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof connects the solid carrier, preferably the surface of the solid carrier, with the the protein e.g.
25 the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof via the linker, preferably by covalent binding, thereby immobilizing the protein 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.

30 In one embodiment in step (e) the protein 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 solid carrier by connecting the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof via a linker, preferably

by connecting the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof via a linker, wherein the solid carrier is connected with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by covalent binding between the

5 linker and the solid carrier and between the linker and the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof. Preferably i) a linker is added to solid carrier provided in step (a), and ii) the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b) is added to the solid carrier and the linker, wherein the linker connects the

10 solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e). The linker used is as described supra and connects the surface of the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by preferably covalent binding. More preferably the linker is added to the solid carrier provided

15 in step (a), in a molar excess to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), preferably the linker is added to the solid carrier in step (b), in a 1 fold to 1000 fold molar excess to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), more preferably the linker is added to the

20 solid carrier in step (b), in a 2 fold to 300 fold molar excess to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), even more preferably the linker is added to the solid carrier in step (b), in a 4 fold to 250 fold molar excess to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), in

25 particular the linker is added to the solid carrier in step (b), in a nine fold molar excess to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b).

In a preferred embodiment the linker which has not connected the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the

30 amylase or a fragment thereof in step (e), is present during formation of a protective layer on the surface of the solid carrier in step (f). In a more preferred embodiment the linker which has not connected the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e), or a part

therof, covalently binds the protective layer to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (f). In a furthermore preferred embodiment the linker which has not connected the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e) is not removed in step (e) or step (f) or in between step (e) and (f). In a particular embodiment the linker which has not connected the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e) is not removed in step (e) or step (f) or in between step (e) and (f) and the linker which has not connected the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e), or a part thereof, covalently binds the protective layer to the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (f). The amount of the linker which has not connected the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e) 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 (e). In one embodiment there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the protein 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 and the linker in ii). In one embodiment there is no washing step between any of steps (a) to (f). In one embodiment there is no washing step between adding the linker to the solid carrier provided in step (e) in (i) and adding the protein 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 and the linker in ii) and there is no washing step between any of steps (a) to (f).

In one embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls, sulfhydryl-reactive 2-pyridyldithiol, BSOCOES (Bis[2-(succinimidooxycarbonyloxy)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 3-(2-pyridyldithio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate), SMPT (4-Succinimidylloxycarbonyl-methyl- α -[2-pyridyldithio]toluene), DPDPB (1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane), DTME (Dithio-bismaleimidoethane), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane) and is preferably glutaraldehyde.

In a preferred embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOCOES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3'-dithiobispropionimide·2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane) and is preferably glutaraldehyde.

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

In one embodiment the present invention provides a method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the

surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:

- (a) providing a solid carrier;
- 5 (b) providing a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof;
- (c) immobilizing the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier;
- (d) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- 10 (e) allowing the lipase or a fragment thereof of (b) to interact with the agent of (d);
- (f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the solid carrier;
- (g) 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. In a preferred embodiment, pancreatin comprising a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof is provided in step b). Steps (a) to (g) can be carried out analogously to steps (a) to (g) of the
- 20 method provided in the further aspect of the present invention described supra, considering that step (c) of the above method corresponds to step (e) of the method provided in the further aspect of the present invention described supra.

In one embodiment the protective layer is formed by building blocks, wherein as building

25 blocks structural building blocks and protective building blocks are used to form the protective layer, wherein the structural building blocks are precursors of inorganic silica, capable of forming 4 covalent bonds in the layer formed and the protective building blocks are organosilanes as described above.

30 In one embodiment the protective layer embeds from about 30% to about 100% 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 one embodiment the solid carrier is selected from the group of organic particles, inorganic particles, organic-inorganic particles, self-assembled organic particles, silica particles, gold particles, magnetic particles and titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP).

5

- A preferred method of the present invention is a method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:
- (a) providing a solid carrier, wherein the solid carrier is provided in suspension, preferably wherein the solid carrier is provided in suspension in buffer, 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) providing a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof;
 - (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
 - (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);
 - (e) immobilizing a protein 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, wherein preferably the surface of the solid carrier is at least partly modified before the protein 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 solid carrier, wherein i) a linker is added to the suspension of the solid carrier provided in step (a) or i) a linker is added to the suspension of the solid carrier provided in step (a) after the at least partly modification of the surface of the solid carrier and

ii) the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b), preferably a solution of the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b) is added to the suspension of the solid carrier and the linker,

5 wherein the linker connects the solid carrier with the protein e.g. the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e);

(f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier

10 with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof;

(g) 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. Alternatively steps (a) to (g) can be carried out analogously to steps (a) to (g) of the method described supra considering that step (e) of the above method corresponds to step (c) of the method described supra.

20

Also provided is a composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier,

25 an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent

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

Examples

Material and Methods:

Reagents:

- Tetraethyl orthosilicate 99% (TEOS), (3-aminopropyl)-triethoxysilane (APTES), ammonium hydroxide (ACS grade, 28–30%), ethanol (ACS grade, anhydrous), glutaraldehyde (grade I, 25% in water), 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, 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
- Indium chloride (¹¹¹In) was purchased from Curium
- Animal diet Altromin 1324 and Altromin 9033 were purchased at Altromine 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 (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), Dulbecco's Phosphate Buffered Saline DPBS (1X), 0.25% Trypsin-EDTA (1X), RPMI 1640 Medium, DMEM were purchased from Gibco.
- Antizonula occludens 1 (ZO-1) antibody, goat anti-rabbit IgG conjugated to Alexa Fluor 488 and mounting medium containing DAPI were purchased at ThermoFischer Scientific.

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
5 using the image analysis software Olympus stream motion.

Enzyme shielding and protein shielding, and surface functionalization

- Production of NP-1:

To SNPs (10 mg/mL, 56 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.8 mM). The
10 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,
15 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
20 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,
25 400 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 NP-2:

30 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 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 centrifuged 5 min at 20000 rcf and washed 3 times in NaCl (0.9%)/PS80 (8 mg/L). NP-2 was cured overnight in a water bath at 20°C.

- *Production of NP-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. 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 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 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.

- *Production of 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
5 reaction 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
10 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 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.
15 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-4. NP-4 was cured overnight in a water bath at 20°C.

- Production of NP-5:

20 To SNPs (10 mg/mL, 58 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.7 mM). The 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,
25 protease and/or a fragment thereof and amylase and/or a fragment thereof 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 (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
30 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 NP-3 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, comparative experiment, enzyme immobilisation and formation of the protective layer were carried out according to WO2015/014888 A1 to produce 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, 15 400 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 25 cured overnight in a water bath at 20°C.

In a second experiment, 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” 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, 30 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 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 pancreatin and the organosilica layer. The absorbance of nanoparticles NP-3(1) and NP-3 at 460 nm was measured after the organosilica layer formation and final particles washing (see Figure 9), 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 NP-2” and “Production of 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 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 measuring the amount of sodium hydroxide added to the reaction mixture to maintain the pH at 9 for 10 minutes.

Pancreatic protease activity assay:

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

Pancreatic amylase activity assay:

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

20 ***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:***

- 25 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
30 had access *ad libitum* to drinking water.

○ *Pancreatic duct ligation (PDL) and 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. 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 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.

○ *Evaluation of NP-3 efficacy:*

PDL rats were dosed through the duodenum with either NP-4 (7mg) or 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 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:*

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. 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).

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.

5 - **Minipigs:**

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

 ○ *Diet and drinking water:*

10 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

15 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 scannings (Clinical D670 SPECT/CT, GE) were initiated 15 min, 3, 8, 24, 48 and
20 72 hours post dosing (+/- ½ h) of the In-111-labeled nanoparticles. For the control animal no SPECT/CT scannings 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
25 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
30 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.

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

- 5 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
- 10 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.

- 15 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.

20

○ *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.

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

○ *Evaluation of NP-3 efficacy:*

▪ *Single dosing:*

- 30 PDL minipigs were dosed through the intra duodenal catheter with either NP-4 (1g) or 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 x g), and a minimum of 500 uL plasma was transferred into Eppendorf tubes and stored at -80°C until analysis for triglycerides content.

5 ▪ *Chronic dosing:*

PDL minipigs were dosed through the intra duodenal catheter with either NP-4 (1g) or NP-3 (1g; 1365U) on a daily basis twice a 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
10 kept at -20°C until analysis.

 ○ *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.

15 ○ *Measurement of fecal fat content of minipigs*

The quantification of fecat fat content was assessed by Near Infra-Red spectroscopy (NIR) using the Impana FT 9700 (Perkin Elmer). Stools were weighed, homogenised and placed in open glass Petri dish for analysis by NIR (700-2500nm). For each sample, measurement were read at three different spots. Results were expressed as the mean fat concentration of the three
20 spot specimens (grams of fat per 100 g of stool wet weight).

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
25 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 (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
30 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.

5

Confocal microscopy

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 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, Japan).

15

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, non-functionalized-radiolabelled nanoparticles (NP-2) and chitosan-functionalized radiolabelled nanoparticles (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 2A. 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. 2B). 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.

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25

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Example 2: Biocatalytic activities of 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
5 pancreatic enzyme product (lipase, amylase, protease) from porcine origin.

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 3A and Figure 3C. Enzymatic activity of protease of NP-5 is displayed in Figure 3B.

10 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 NP-3 in PDL-rats

Given the importance of fat digestion for patients with exocrine pancreatitis insufficiency
15 (EPI), the validation of 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 NP-3 or 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.4).
20 This result demonstrates the ability of NP-3 to restore lipase digestive function in an EPI animal model.

Example 4: *In vivo* activity of 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
25 assess the efficacy of 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 NP-3 showed a similar kinetic with a maximal level 3 hours after feeding (Fig.5A). The calculation of the area under the curve (AUC) demonstrates
30 an extremely surprising digestion rate of 62% with NP-3 compared with a healthy minipig (Fig.5B). These results confirm the ability of NP-3 to restore lipase digestive function in an EPI animal model.

Example 5: *In vivo* therapeutic efficacy of NP-3 in PDL-minipigs

To further point-out the ability of NP-3 to restore lipase digestive functions, PDL-minipigs receiving a diet enriched with fat were daily dosed with 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 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.6A). Importantly, the level of fecal fat content decreases in PDL-minipigs treated with NP-3 compared to the untreated PDL-minipigs (Fig.6A). In fact, PDL-minipigs that received daily doses of NP-3 were able to digest 53.4% of the fat intake (Fig.6B). These results clearly demonstrate the therapeutic efficacy of 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).

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, 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. 6B). 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 NP-3 for lipase digestion and highlight the therapeutic potential of NP-3 for EPI patients.

Example 6: *In vitro* biocompatibility of 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 NP-5, we focused on the maintenance of the intestinal barrier integrity in presence of the nanoparticles and compared it to pancreatin (Fig. 7A). 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
10 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.7B). When cultured in presence of NP-5, ZO-1 staining shows a continuous ring appearance at the cell borders while in presence of pancreatin, the staining of ZO-1 reveals
15 a diffused punctuated protein distribution around intestinal cells. These morphological data confirmed the safety of NP-5 for gastrointestinal application and highlights the added value of our technology for pancreatic enzyme replacement therapies.

Example 7: Enhancing Enzyme Stability through Covalent Attachment to the protective layer

20 In a first experiment, 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, nanoparticles NP-3 were produced in non-buffered conditions without any intermediate washing steps (*i.e.* unreacted glutaraldehyde still present in the reaction mixture during layer growth).

25 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 (NP-3) increases the enzyme immobilization yield by a factor of 2 (Fig. 8A), resulting in a doubling of enzyme loading per dry weight of SNP (Fig. 8B) compared to buffered conditions
30 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. 8C). 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

5 buffered conditions where glutaraldehyde is removed by washing steps (Fig. 8D). In summary, covalent attachment of the protective layer to the enzyme surface unexpectedly enhances its load, stability and enzyme specific activity compared to enzymes protected with an organosilica layer via electrostatic interactions only.

Claims

1. A composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.
2. The composition according to claim 1, wherein 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.
3. The composition according to claim 1 or 2, wherein the agent interacts specifically with the lid domain of the lipase or a fragment thereof so that the lipase or a fragment thereof shifts to and/or maintains the open conformation.
4. The composition according to any one of claims 1-3, wherein about 100% of the lipase or a fragment thereof immobilized on the surface of the solid carrier is in the open conformation.
5. The composition according to any one of claims 1-4, wherein the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is a polyglucosamin selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a

derivative thereof.

- 5 6. The composition according to any one of claims 1-4, wherein the polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group is chitosan or a derivative thereof.
- 10 7. The composition according to any one of claims 1-6, wherein the functional constituent is immobilized on the surface of the protective layer by non-covalent binding or by covalent binding.
- 15 8. The composition according to any one of claims 1-7, wherein the composition comprises a solid carrier; pancreatin, wherein the pancreatin comprises a lipase or a fragment thereof wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof and an amylase or a fragment thereof, wherein the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof are immobilized on the surface of the solid carrier; an agent which interacts with the lid domain of the lipase or a fragment thereof; a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof; and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.
- 20 9. The composition of any one of claims 1-8, wherein the protective layer embeds the solid carrier, embeds the lipase or a fragment thereof immobilized on the surface of the solid carrier, embeds the protease or a fragment thereof immobilized on the surface of the solid carrier and embeds the amylase or a fragment thereof immobilized on the surface of the solid carrier.
- 25 30

10. The composition of any one of claims 1-9, wherein the functional constituent immobilized on the surface of the protective layer is not embedded by the protective layer.
- 5 11. The composition of any one of claims 1-10, for use as a medicament.
12. The composition of any one of claims 1-10, 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).
- 10 13. A method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:
- 15 20 25 30
- (a) providing a solid carrier;
 - (b) providing a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof;
 - (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
 - (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);
 - (e) immobilizing the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier;

- (f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the solid carrier;
- (g) immobilizing a functional constituent on the surface of the protective layer,
- 5 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.
14. A method of producing a composition, the composition comprising a solid carrier, a
- 10 lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the
- 15 lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units
- 20 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;
- (b) providing a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof;
- 25 (c) immobilizing the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof on the solid carrier;
- (d) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (e) allowing the lipase or a fragment thereof of (b) to interact with the agent of (d);
- 30 (f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof immobilized on the solid carrier;

(g) 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

15. The method of claim 14 or 15, wherein in step b) pancreatin comprising a lipase or a fragment thereof, a protease or a fragment thereof and an amylase or a fragment thereof is provided.

10

16. The method of claim 13, wherein i) a linker is added to the solid carrier provided in step (a), and ii) the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b) is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e).

15

17. The method of claim 16, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e), is present during formation of a protective layer on the surface of the solid carrier in step (f).

20

18. The method of claim 16, wherein there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof to the solid carrier and the linker in ii).

25

19. The method of any one of claims 13-18, wherein there is no washing step between any of steps (a) to (f).

30

20. The method of any one of claims 13-19, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (e), or a part thereof, covalently

binds the protective layer to the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (f).

21. The method of claim 14, wherein i) a linker is added to the solid carrier provided in
5 step (a), and ii) the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof provided in step (b) is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (c).
10
22. The method of claim 21, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (c), is present during formation of a protective layer on the surface of the solid carrier in step (f).
15
23. The method of claim 21, wherein there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof to the solid carrier and the linker in ii).
20
24. The method of any one of claims 14 and 21-23, wherein there is no washing step between any of steps (a) to (f).
25. The method of any one of claims 14 and 21-24, wherein the linker which has not
25 connected the solid carrier with the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (c), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof in step (f).
- 30 26. The method of any one of claims 13-25, 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-

5 dinitrobenzene, BSO COES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3 '-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3 '-dithiobispropionimidate·2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane).

27. The method of any one of claims 13-25, wherein the linker is glutaraldehyde.
- 10 28. A composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, a protease or a fragment thereof immobilized on the surface of the solid carrier, an amylase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, a protective layer to protect the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof by embedding the lipase or a fragment thereof, the protease or a fragment thereof and the amylase or a fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group, wherein the composition is obtainable by the method of any one of claims 17-20 or 22-25.
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