

Functionalized disaccharidase compositions

The field of the invention

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

Background of the invention

Disaccharides are normally split into monosaccharides by disaccharidases located in the brush
15 border of small-bowel enterocytes. Undigested disaccharides cause an osmotic load that
attracts water and electrolytes into the bowel, causing watery diarrhea. Bacterial fermentation
of carbohydrates in the colon produces gases (hydrogen, carbon dioxide, and methane),
resulting in excessive flatus, bloating and distention, and abdominal pain. Diseases which are
related to the defective digestion of disaccharides are e.g. lactase deficiency and congenital
20 sucrase-isomaltase deficiency (CSID). In case of lactase deficiency, lactose malabsorption is
attributable to an imbalance between the amount of ingested lactose and the capacity for
lactase to hydrolyze the disaccharide. It manifests as lactose intolerance, a clinical syndrome
of one or more of the following symptoms: abdominal pain, diarrhea, nausea, flatulence,
and/or bloating after the ingestion of lactose or lactose-containing food substances. The most
25 common cause is primary (hereditary) lactase deficiency attributable to relative or absolute
absence of lactase that develops in childhood at various ages in different racial groups.
Secondary (acquired) lactase deficiency results from small bowel injury, such as acute
gastroenteritis, persistent diarrhea, small bowel overgrowth, cancer chemotherapy, or other
causes of injury to the small intestinal mucosa. Congenital lactase deficiency is an extremely
30 rare, autosomal recessive enzyme defect that prevents lactase expression from birth, requiring
complete lactose avoidance.

Besides lactose avoidance, which could cause significant health issues, enzyme-replacement
therapy with microbial exogenous lactase (β -galactosidase obtained from *Aspergillus oryzae*)

prior to or added to dairy meals represents a possible strategy for primary and secondary lactase deficiency. Results about the exact rate of efficacy are discordant.

CSID is a multifaceted malabsorption disorder of the small intestine with an autosomal recessive mutation in the sucrase-isomaltase gene, characterized by complete, or almost

5 complete lack of sucrose activity and varying degrees of reduction in isomaltase activity.

When this enzyme complex is deficient, nutrients from ingested starch and sucrose cannot be absorbed sufficiently and clinically manifests with diarrhea, abdominal pain and bloating, leading to malnutrition, especially in children. Besides life-long dietary measures, the only effective treatment option is the enzyme replacement with sacrosidase, currently approved in
10 the U.S. by FDA (Sucraid®), but not in Europe. Thus there is a need to provide effective treatments for diseases which are related to the defective digestion of disaccharides.

Summary of the invention

The present invention provides a composition comprising a solid carrier, a disaccharidase or a
15 fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or a fragment thereof by embedding the disaccharidase 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
20 and/or at least one thiol group.

The present invention provides also a method of producing said composition comprising a solid carrier, a disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or a fragment thereof by embedding the
25 disaccharidase 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:

- 30 (a) providing a solid carrier;
(b) immobilizing a disaccharidase or a fragment thereof on the solid carrier;
(c) forming a protective layer on the surface of the solid carrier to protect the disaccharidase or a fragment thereof immobilized on the solid carrier;

(d) immobilizing a functional constituent on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

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It has been surprisingly found by the inventors of the present application that compositions as provided by the present invention if applied therapeutically in the digestion of disaccharides have an unexpected high activity, show low cytotoxicity, and do not disrupt the intestinal barrier if localized in the gastrointestinal tract, thus making them extremely promising for therapeutic use, in particular for therapeutic use in the treatment of lactase deficiency, sucrase-isomaltase deficiency and disaccharidoses intolerances.

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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) a disaccharidase or fragment thereof (indicated as “Protein”) is immobilized on the solid carrier; b) and c) a protective layer grows around the immobilized disaccharidase or fragment thereof embedding the immobilized disaccharidase or fragment thereof; and d) a functional constituent is immobilized on the surface of the protective layer.

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

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

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Figure 4 shows the *in vitro* biocompatibility of a representative model of the nanoparticles on an intestinal barrier. (A) *In vitro* assessment of the integrity of the intestinal barrier by the measurement of the transepithelial electrical resistance (TEER). Differentiated Caco-2/HT29-MTX-E12 co-culture were exposed to NP-1(0.5mg/mL and 1mg/mL) for 16h. TEER data was normalized to the control point consisting in the equilibrium value before the addition NP-1 (defined as control) and set at 100%. The graph represents the time course profile evolution of averaged normalized TEER data over 16h. The dash line represents the untreated condition. (B) *In vitro* evaluation of inflammatory effects on intestinal epithelial barrier. Differentiated Caco2-

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HT29-MTX-E12 and M0-differentiated THP-1 were co-cultured and exposed to NP-1 (1mg/mL) at 37°C for 16h. Lipopolysaccharide (LPS) (50 and 100ug/mL) was used as positive control to induce inflammatory response. TEER data was normalized with the equilibrium value before the addition of NP-1 or LPS set at 100%. The graph represents the time course profile evolution of averaged normalized TEER data over 16h in presence of NP-1.

Figure 5) shows the differences in the size of cecum in rats. Wistar rats were daily dosed intraduodenally with NP-2, NP-1, or the vehicle and immediately gavaged with lactose over a period of 15 days. At termination, the size of the cecum was evaluated. (A) Pictures of the gastrointestinal tract in rats. The circles show the cecum. (B) MRI images of the gastrointestinal tract in rats. The arrows show the cecum. (C) Histogram shows the cecum size in cm³ assessed by MRI imaging. *p<0.05, **p<0.01 by one-way ANOVA test.

Figure 6) shows the *in vitro* digestion of sucrose on a model of intestinal barrier. Differentiated Caco-2/HT29-MTX-E12 co-culture were exposed to different amount of NP-3 (0.5mU or 1mU) in presence of sucrose for 4h at the apical side of the barrier. The hydrolysis of sucrose was evaluated by the quantification of glucose in the basal side of the barrier. The graph shows the time course profile evolution of the accumulation of glucose over 4h.

Figure 7) shows absorbance of nanoparticles NP-2, NP-2(1) and NP-2(2) at 460 nm.

Detailed description of the invention

The present invention relates to a composition comprising a solid carrier, a disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or a fragment thereof by embedding the disaccharidase 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.

10 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.

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

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

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

30 The term “linker” or “cross-linker” which are used synonymously herein refers to any linking reagents containing groups, which are capable of binding to specific functional groups (e.g. primary amines, sulfhydryls, etc.). A linker in the context of the present invention usually connects the surface of the solid carrier with the disaccharidase. 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 disaccharidase may be bound to an unoccupied binding-site of the linker.

Alternatively, the linker may firstly bind to the disaccharidase and then the linker bound to the disaccharidase 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
5 known in the art as tags.

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

The term, "disaccharidase or a fragment thereof" includes naturally occurring disaccharidases or a fragment thereof and also includes artificially engineered disaccharidases or a fragment thereof. Disaccharidases are glycoside hydrolases, enzymes that break down certain types of
20 sugars called disaccharides into simpler sugars called monosaccharides. In the human body, disaccharidases are made mostly in an area of the small intestine's wall called the brush border. Disaccharidases includes e.g. lactase, maltase, isomaltase, trehalase and sucrase (which is also named invertase). Artificially engineered disaccharidases or a fragment thereof are e.g. variants or functionally active fragments of the disaccharidase. The terms “fragment
25 of a disaccharidase”, “fragment thereof” in relation to the disaccharidase and “functionally active fragment of the disaccharidase” are thus used synonymously herein. By “variants or functionally active fragments thereof” in relation to the disaccharidase of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the disaccharidase. Such variants
30 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 disaccharidase. A fragment of an disaccharidase as defined herein does usually have the same functional properties as the disaccharidase from which it is
5 derived. A fragment of a disaccharidase contains usually between 100 and 1000 amino acids, preferably between 300 and 800 amino acids, more preferably between 500 and 700 amino acids.

The term “partially embedded disaccharidase” as used herein shall mean that the
10 disaccharidase is not fully covered by the protective layer, thus, the disaccharidase is not fully embedded in the protective layer. In one embodiment less than 50% of the disaccharidase of interest are covered by the protective layer, though typically more at least 70% will be covered, thus improving protection of the disaccharidase. In a preferred embodiment, at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at
15 least 95% of the disaccharidase of interest is covered by the protective layer. In another preferred embodiment, around 70% to around 95%, more preferably around 80% to around 95%, even more preferably around 90% to around 95%, most preferably around 90% to around 95, 96, 97, 98 or 99 % of the disaccharidase of interest are covered by the protective layer. In a particularly preferred embodiment, around 70%, particularly around 80%, more
20 particularly around 90%, most particularly around 95% of the disaccharidase of interest is covered by the protective layer. In a more particularly preferred embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the disaccharidase of interest is covered by the protective layer, wherein the active site is not covered.

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

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

50% to about 100%, more preferably from about 80% to about 100%, even more preferably from about 90% to about 100%, most preferably from about 95% to about 100 %, wherein the active site is preferably covered.

5 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.

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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.

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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. A preferred polymer comprises a number of repeat units (monomers), wherein each repeat unit contains one thiol group.

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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 89(7):901-9.

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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.

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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.

- 5 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. Polyglucosamine contains free amine (-NH₂) groups and may be characterized by the proportion of N-acetyl-D-glucosamine units and D-glucosamine units, which is expressed as the degree of deacetylation (DDA) of the fully acetylated polymer chitin. A preferred
- 10 polyglucosamin of the present invention is selected from the group consisting of chitin, chitosan, polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative thereof. Most preferred is a chitosan or a derivative thereof.

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

- In a first aspect the present invention provides a composition comprising a solid carrier, a
- 25 disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or a fragment thereof by embedding the disaccharidase 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
- 30 least one amino group and/or at least one thiol group.

The disaccharidase or a fragment thereof can be immobilized on the surface of the solid carrier by non-covalent binding or covalent binding. Non-covalent binding includes p-p

(aromatic) interactions, van der Waals interactions, H-bonding interactions, and electrostatic interactions like e.g. ionic interactions. Preferably, the disaccharidase or fragment thereof is immobilized on the surface of the solid carrier by covalent binding or by covalent binding via a linker.

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A solution of a disaccharidase or a fragment thereof usually comprises the protein or a fragment thereof in a buffer solution. Buffers which can be used are usually phosphate, chloride, citrate, MES, MOPS, HEPES, PIPES, ACES or mixtures thereof. The solution may additionally contain sugar alcohols or non-ionic surfactants as described herein. A solution of a disaccharidase or a fragment thereof can be prepared by e.g. dissolving the disaccharidase or a fragment thereof in water to reconstitute the stock buffer of disaccharidase or a fragment thereof.

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In one embodiment the solid carrier is selected from the group of organic particles, inorganic particles, organic-inorganic particles, self-assembling organic particles, silica particles, gold particles, titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP). The particle size is usually measured by measuring the diameter of the particles and is usually between 1 nm and 1000 nm, preferably between 10 nm and 100 nm, particularly about 50 nm. In case the solid carrier is a monodisperse particle, the size is usually between 1 nm and 1000 nm, preferably between 10 nm and 100 nm, particularly about 50 nm. In case the solid carrier is a polydisperse particle, the size is usually between 1 nm and 1000 μ m, preferably between 10 nm and 100 μ m, particularly between 50 nm and 50 μ m. In one embodiment the composition comprises a solid carrier wherein the solid carrier comprises at least 15%, preferably at least 20%, in particular between 15% and 30%, more particular between 20% and 30 % immobilized disaccharidase or fragment thereof per dry weight of the solid carrier.

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

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The solid carrier is usually provided in suspension. Suspension of the solid carrier can be e.g. in water, buffer or non-ionic surfactants or mixtures thereof, preferably in mixtures of water and non-ionic surfactants. Non-ionic surfactants are usually selected from the group consisting of ethoxylated sorbitan esters like PEG-40 sorbitan diisostearate, polysorbate 80 (PS80), polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60); block co-polymers like poloxamer 124, poloxamer 188, poloxamer 331, poloxamer 407, fatty acids ethoxylates like PEG-5 oleate, PEG-8 stearate, polyoxyl 40 stearate, polyoxyl 15 hydroxystearate, fatty alcohol ethoxylates like steareth 40; fatty acid esters like ascorbyl palmitate, beeswax, polyglyceryl 3-oleate, propylene glycol monocaprylate, propylene glycol monolaurate; fatty alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). Buffers which can be used in the method of the present invention are phosphate, piperazine-N,N'-bis(2-ethanesulfonic acid), 2-Hydroxy-3-morpholinopropanesulfonic acid, N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, (3-(N-morpholino)propanesulfonic acid), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3-(N,N-Bis[2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid, N,N-Bis(2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid, N-[Tris(hydroxymethyl)methyl]glycine, Diglycine, 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid, N,N-Bis(2-hydroxyethyl)glycine, N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid.

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 disaccharidase or for the linker connecting the disaccharidase 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 disaccharidase 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.

- 5 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.
- 10
- 15 In one embodiment, the disaccharidase or fragment thereof is partially embedded by the protective layer. In a preferred embodiment the disaccharidase or fragment thereof is at least partially embedded by the protective layer. In a more preferred embodiment the disaccharidase or fragment thereof is fully embedded by the protective layer.
- In one embodiment, the protective layer embeds the solid carrier and embeds the disaccharidase or fragment thereof 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 disaccharidase or fragment thereof immobilized on the surface of the solid carrier. More preferably, the protective layer fully embeds the solid carrier and fully embeds the disaccharidase or fragment thereof immobilized on the surface of the solid carrier and the functional constituent immobilized on the surface of the protective layer is not embedded by the protective layer. If the protective layer fully embeds the solid carrier and fully embeds the disaccharidase or fragment thereof immobilized on the surface of the solid carrier, the disaccharidase or 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.
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In a preferred embodiment the disaccharidase or a fragment thereof is selected from the group consisting of lactase or a fragment thereof, maltase or a fragment thereof, isomaltase or a fragment thereof, trehalase or a fragment thereof and invertase or a fragment thereof or mixtures thereof, more preferably selected from the group consisting of lactase or a fragment thereof, isomaltase or a fragment thereof, and invertase or a fragment thereof or mixtures thereof. Thus the composition also comprises more than one e.g. two, three or four different disaccharidases or fragments thereof immobilized on the surface of the solid carrier and embedded. When more than one disaccharidase or a fragment thereof is used, preferably two different disaccharidases, more preferably invertase and isomaltase are used. Thus in a particular embodiment, a mixture of isomaltase or a fragment thereof and invertase or a fragment thereof is immobilized on the surface of the solid carrier of the composition of the present invention.

In a particular preferred embodiment the disaccharidase or a fragment thereof is selected from the group consisting of lactase or a fragment thereof, and invertase or a fragment thereof and mixtures thereof. Most preferred is invertase or a fragment thereof.

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

The composition of the present invention is usually produced in a reaction vessel like a reactor. The formation of the protective layer is usually carried out by forming the respective protective layer by building blocks, wherein the building blocks build the protective layer in a polycondensation reaction. The polycondensation can be effected in different solvents, preferably in aqueous solution. Polycondensation can be easily controlled and stopped if appropriate, allowing achievement of a defined thickness of the protective layer. The choice of the building blocks, which can be used to build the protective layer, may depend on the known structure of the disaccharidase in order to adapt the affinity of the protective layer according to optimal and/or desired parameters. As building blocks for the protective layer usually structural building blocks and protective building blocks are used to build the protective layer. Structural building blocks which can be used are e.g. tetraethylorthosilicate (designated herein as "TEOS" or "T"). Protective building blocks which can be used are e.g.

- 3-Aminopropyltriethoxysilane (designated herein as “APTES” or “A”), Propyltriethoxysilane (designated herein as “PTES” or “P”), Isobutyltriethoxysilane (designated as “IBTES”), Hydroxymethyltriethoxysilane (designated herein as “HTMEOS” or H), Benzyltriethoxysilane (designated herein as “BTES”), Ureidopropyltriethoxysilane (designated as “UPTES”), or Carboxyethyltriethoxysilane (designated herein as “CETES”).
- Structural building blocks are usually precursors of inorganic silica, capable of forming 4 covalent bonds in the layer formed. Protective building blocks are usually organosilanes, bearing an organic moiety endowed with the ability to interact with the disaccharidases. Preferred structural building blocks are tetravalent silanes, in particular tetra-alkoxy-silanes.
- Preferred protective building blocks are trivalent silanes, in particular tri-alkoxy-silanes. More preferred structural building blocks are mixtures of tetravalent silanes and trivalent silanes, in particular mixtures of tetra-alkoxy-silanes and tri-alkoxy-silanes. Even more preferred structural building blocks are selected from the group consisting of tetraethylorthosilicate, tetra-(2-hydroxyethyl)silane, and tetramethylorthosilicate. Even more preferred protective building blocks are selected from the group consisting of carboxyethylsilanetriol, benzylsilanes, propylsilanes, isobutylsilanes, n-octylsilanes, hydroxysilanes, bis(2-hydroxyethyl)-3 -aminopropylsilanes, aminopropylsilanes, ureidopropylsilanes, (N-Acetylglycyl)-3-aminopropylsilanes, hydroxy(polyethyleneoxy)propyl]triethoxysilanes, in particular selected from benzyltriethoxysilane, propyltriethoxysilane, isobutyltriethoxysilane, n-octyltriethoxysilane, hydroxymethyltriethoxysilane, bis(2-hydroxyethyl)-3 -aminopropyltriethoxysilane, 3-Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglycyl)-3-aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutyltrimethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3 -aminopropyltrimethoxysilane, aminopropyltrimethoxysilane, ureidopropyltrimethoxysilane (N-Acetylglycyl)-3 -aminopropyltrimethoxysilane or selected from benzyltrihydroxyethoxysilane, propyltrihydroxyethoxysilane, isobutyltrihydroxyethoxysilane, n-octyltrihydroxyethoxysilane, hydroxymethyltrihydroxyethoxysilane, bis(2-hydroxyethyl)-3 -aminopropyltrihydroxyethoxysilane, aminopropyltrihydroxyethoxysilane, Ureidopropyltrihydroxyethoxysilane (N-Acetylglycyl)-3-aminopropyltrihydroxymethoxysilane.

Particular preferred building blocks are TEOS as structural building block and APTES, PTES,

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

- 5 The reaction time of the building blocks with the solid carrier depends on the length of the linker, if a linker is used, and the size of the disaccharidase. The reaction is usually carried out for a time period of between 0.5 to 10 hours, preferably between 1 and 5 hours, more preferably between 1 and 4 hours, even more preferably between 2 and 4 hours, preferably in aqueous solution and preferably at room temperature of about 5 to about 25 °C or at about 20
- 10 °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.
- 15 In a furthermore preferred embodiment the disaccharidase 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 disaccharidase and by using a linker, preferably a cross-linker binding to the anchoring point and the disaccharidase.
- 20 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
- 25 glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, 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'-
 - 30 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-Succinimidyl-oxycarbonyl-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
- 5 glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls (e.g. sulfhydryl-reactive 2-pyridyldithio). 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
- 10 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 bismaleimidyl-2,3-dihydroxybutane).
- 15 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.
- 20
- After the protective layer has been formed, the solid carrier comprising the disaccharidase 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 disaccharidase
- 25 and the protective layer is stored at a constant temperature between 2 to 25 °C. In a further preferred embodiment, the solid carrier comprising the disaccharidase and the protective layer is stored 5 to 48 hours, preferably 10 to 30 hours. More preferably the solid carrier comprising the disaccharidase and the protective layer is stored at a constant temperature between 2 to 25 °C, preferably at room temperature for 10 to 30 hours.
- 30
- 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.

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.

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

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

In a further embodiment a polymer comprising repeat units wherein each repeat unit comprises 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, most particular chitosan or a derivative thereof.

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

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

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 constituent. Thus in one embodiment the present invention comprises a composition

comprising a solid carrier, a disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or a fragment thereof by embedding the disaccharidase 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 functional constituent is immobilized on the surface of the protective layer by a spacer. Examples of such a spacer include a polyethylene such as PEG4, PEG2000, PEG5000. A functional constituent immobilized on the surface of the protective layer, by a spacer is usually produced by firstly reacting the spacer with the functional constituent, so that the spacer binds to the functional constituent and then the functional constituent bound to the spacer is reacted with the the surface of the protective layer.

The immobilization of the functional constituent to the surface of the protective layer is usually carried out in a reaction vessel like a reactor by suspending the solid carrier carrying the disaccharidase embedded in a protective layer as described supra in e.g. in water, buffer or non-ionic surfactants or mixtures thereof, preferably in mixtures of water and non-ionic surfactants. Non-ionic surfactants are usually selected from the group consisting of ethoxylated sorbitan esters like PEG-40 sorbitan diisostearate, polysorbate 80 (PS80), polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60); block co-polymers like poloxamer 124, poloxamer 188, poloxamer 331, poloxamer 407, fatty acids ethoxylates like PEG-5 oleate, PEG-8 stearate, polyoxyl 40 stearate, polyoxyl 15 hydroxystearate, fatty alcohol ethoxylates like steareth 40; fatty acid esters like ascorbyl palmitate, beeswax, polyglyceryl 3-oleate, propylene glycol monocaprylate, propylene glycol monolaurate; fatty alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). The functional component is then added to the suspension to react usually under stirring with the surface of the protective layer to immobilize the functional constituent on the surface of the protective layer. Ususally such obtained composition is washed and resuspended into water, buffer or non-ionic surfactants or mixtures thereof. Immobilization takes place by non-covalent binding e.g. electrostatic binding or by covalent binding of the functional constituent. The functional constituent may be immobilized by chemically modifying the surface of the protective layer and the functional constituent using

e.g. “click chemistry” such as copper-catalyzed click chemistry (Copper-catalysed azide-alkyne cycloaddition, see e.g. Kolb et al. (2001) Angew. Chem. 40(11)2004-2021) or by copper free click chemistry (Wittig G, A Chem Ber, 1961, 94, 3260) ., e.g. the solid carrier carrying the disaccharidase embedded in a protective layer as described supra is first reacted
5 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.

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

In a further aspect the present invention provides the composition for use in a method for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances. In one embodiment the present invention
15 provides the composition for use in a method for the prevention, delay of progression or treatment of lactase deficiency or sucrase-isomaltase deficiency. Lactase deficiency includes primary (hereditary) lactase deficiency, secondary (acquired) lactase deficiency and congenital lactase deficiency and is preferably secondary lactase deficiency. Sucrase-isomaltase deficiency includes Congenital Sucrase-Isomaltase Deficiency (CSID) which is
20 preferred.

Also provided is the use of the composition as described herein for the manufacture of a medicament for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances in a subject. Also provided
25 is the use of the composition as described herein for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances in a subject. Also provided is a method for the prevention, delay of progression or treatment of lactase deficiency, sucrase-isomaltase deficiency, and/or disaccharidoses intolerances in a subject, comprising administering to said subject a therapeutically effective
30 amount of the composition as described herein.

A composition according to the invention is preferably a pharmaceutical composition and comprises a therapeutically effective amount of the composition as described herein and one

or 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.

- 5 An exemplary treatment regime entails administration once daily, twice daily, three times daily, every second day, twice per week, once per week. The composition, e.g. the pharmaceutical composition of the invention is usually administered on multiple occasions. Intervals between single dosages can be, for example, less than a day, daily, every second day, twice per week, or weekly. The composition, e.g. the pharmaceutical composition of the
- 10 invention may be given as a continuous uninterrupted treatment. The composition, e.g. the pharmaceutical composition of the invention may also be given in a regime in which the subject receives cycles of treatment interrupted by a drug holiday or period of non-treatment. Thus, the composition, e.g. the pharmaceutical composition of the invention may be administered according to the selected intervals above for a continuous period of one week or
- 15 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. Units ("U") of enzyme activity can be described in terms of weight or mass of substrate hydrolyzed per unit time. Units ("U") can be described in terms of μmol substrate converted per minute (or $\mu\text{mol}/\text{min}$). In an exemplary treatment regime between 500 U to 20,000 U of disaccharidase comprised by the composition, e.g. the pharmaceutical composition of the invention can be administered per
- 25 day.

- 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
- 30 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 mammal and especially a human, that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; (2) inhibiting the state, disorder or condition (e.g. arresting, 5 reducing or delaying the development of the disease, or a relapse thereof in case of maintenance treatment, of at least one clinical or subclinical symptom thereof); and/or (3) relieving the condition (i.e. causing regression of the state, disorder or condition or at least one of its clinical or subclinical symptoms). The benefit to a patient to be treated is either statistically significant or at least perceptible to the patient or to the physician. However, it 10 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 e.g. treatment of lactase deficiency, sucrase-isomaltase deficiency or disaccharides 15 intolerance or a mark associated with e.g. treatment of lactase deficiency, sucrase-isomaltase deficiency or disaccharides intolerance or slowing the increase in severity of a symptom of e.g. a disaccharides intolerance. Further, "delay of progression" as used herein includes reversing or inhibition of disease progression. "Inhibition" of disease progression or disease complication in a subject means preventing or reducing the disease progression and/or disease 20 complication in the subject.

Preventive treatments comprise prophylactic treatments. In preventive applications, the pharmaceutical combination of the invention is administered to a subject suspected of having, or at risk for developing the above mentioned diseases or disorders e.g. treatment of lactase 25 deficiency, sucrase-isomaltase deficiency or disaccharides intolerance. In therapeutic applications, the pharmaceutical combination is administered to a subject such as a patient already suffering from the above mentioned diseases or disorders e.g. treatment of lactase deficiency, sucrase-isomaltase deficiency or disaccharides intolerance, in an amount sufficient to cure or at least partially arrest the symptoms of the disease. Amounts effective for this use 30 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 control or limit the symptoms of the subject's disease or condition.

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

In a further aspect the present invention provides a method of producing a composition as described supra, e.g. a composition comprising a solid carrier, a disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or a fragment thereof by embedding the disaccharidase 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) immobilizing a disaccharidase or a fragment thereof on the solid carrier;
- (c) forming a protective layer on the surface of the solid carrier to protect the disaccharidase or the fragment thereof immobilized on the solid carrier;
- (d) immobilizing a functional constituent on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

Step (a) is usually carried out by providing the solid carrier in suspension in water, non-ionic surfactants or a buffer or mixtures thereof, preferably in suspension in water and/or non-ionic surfactants, more preferably in suspension in water and/or non-ionic surfactants wherein no buffer is present in the suspension, even more preferably in suspension in mixtures of water and non-ionic surfactants in particular in suspension in mixtures of water and non-ionic

surfactants wherein no buffer is present in the suspension. The suspension can be stirred e.g. at 400 rpm, 20°C for 30 min. The immobilization of the disaccharidase on the solid carrier in step b) of the present method is usually carried out by adding a solution of the disaccharidase to the suspension of the solid carrier. Preferably a linker to connect the solid carrier with the disaccharidase is added to the suspension of the solid carrier prior to adding the solution of the disaccharidase to the suspension of the solid carrier. In a preferred embodiment the immobilization of the disaccharidase on the solid carrier is carried out by providing a suspension of the solid carrier and adding a solution of the disaccharidase, wherein the suspension with the added solution of the disaccharidase is incubated to allow the enzyme to bind on the surface of the solid carrier. In a more preferred embodiment the immobilization of the disaccharidase or a fragment thereof on the solid carrier in step b) is carried out by i) adding a linker to the solid carrier provided in step (a), preferably adding a linker to a suspension of the solid carrier provided in step a), and ii) adding the disaccharidase or a fragment thereof, preferably adding a solution of the disaccharidase or a fragment thereof, 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 disaccharidase or a fragment thereof. In one embodiment, a building block of the protective layer, preferably a monomer of a building block of the protective layer, more preferably an organosilane, even more preferably a triethoxysilane, in particular APTES, is added to the solid carrier and the linker or to the suspension comprising the solid carrier and the linker, prior to adding the solution of the disaccharidase or a fragment thereof. In a preferred embodiment the surface of the solid carrier is at least partly modified to improve immobilization of the disaccharidase on the solid carrier. In particular, the surface of the solid carrier is at least partly modified before the disaccharidase is immobilized. The surface of the solid carrier can be at least partly modified by adding a molecule as anchoring point for the disaccharidase 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 anchoring point, the solid carrier and the linker and, the solid carrier comprising the linker and the disaccharidase or a fragment thereof, respectively, so that the disaccharidase or a fragment thereof connects the solid carrier, preferably the surface of the solid carrier, with the

disaccharidase or a fragment thereof via the linker, preferably by covalent binding, thereby immobilizing the disaccharidase or a fragment thereof on the solid carrier.

In one embodiment in step (b) the disaccharidase or a fragment thereof is immobilized on the solid carrier by connecting the solid carrier with the disaccharidase or a fragment thereof via a linker, preferably by connecting the solid carrier with the disaccharidase or a fragment thereof via a linker, wherein the solid carrier is connected with the disaccharidase or a fragment thereof by covalent binding between the linker and the solid carrier and between the linker and the disaccharidase or a fragment thereof. Preferably in step b), i) a linker is added to the solid carrier provided in step (a), and ii) the disaccharidase or a fragment thereof is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the disaccharidase or a fragment thereof. The linker used is as described supra and connects the surface of the solid carrier with the disaccharidase by preferably covalent binding. More preferably the linker is added to the solid carrier in step (b), in a molar excess to the disaccharidase or a fragment thereof, preferably the linker is added to the solid carrier in step (b), in a 1 fold to 1000 fold molar excess to the disaccharidase or a fragment thereof, more preferably the linker is added to the solid carrier in step (b), in a 2 fold to 300 fold molar excess to the disaccharidase or a fragment thereof, even more preferably the linker is added to the solid carrier in step (b), in a 4 fold to 250 fold molar excess to the disaccharidase or a fragment thereof, in particular the linker is added to the solid carrier in step (b), in a 25 fold molar excess to the disaccharidase or a fragment thereof.

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

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

In one embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated thiols, thiol-reactive 2-pyridylthiol, BSOCOES (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), Sulfo-LC-SMPT (4-Sulfosuccinimidyl-6-methyl-α-(2-pyridylthio)toluamido]hexanoate), SPDP (N-Succinimidyl 3-(2-pyridylthio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridylthio]-propionamido)hexanoate), SMPT (4-Succinimidylcarbonyl-methyl-α-[2-pyridylthio]toluene), DPDPB (1,4-Di-[3'-(2'-pyridylthio)-propionamido]butane), DTME (Dithio-bismaleimidoethane), BMDDB (1,4 bismaleimido-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-(succinimidocarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate),

DTBP (Dimethyl 3,3'-dithiobispropionimidate-2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane) and is preferably glutaraldehyde.

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

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

15

In one embodiment the protective layer embeds from about 30% to about 100% of the disaccharidase.

20 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).

25 A preferred method of the present invention is a method of producing a composition, the composition comprising a solid carrier, a disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or the fragment thereof by embedding the disaccharidase or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:

30

(a) providing a solid carrier, wherein the solid carrier is provided in suspension, preferably wherein the solid carrier is provided in suspension in water and/or non-ionic surfactants, more

preferably wherein the solid carrier is provided in suspension in mixtures of water and non-ionic surfactants;

(b) immobilizing a disaccharidase or a fragment thereof on the solid carrier, wherein preferably the surface of the solid carrier is at least partly modified before the disaccharidase or a fragment thereof is immobilized on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier or i) a linker is added to the suspension of the solid carrier after the at least partly modification of the surface of the solid carrier and ii) the disaccharidase or a fragment thereof, preferably a solution of the disaccharidase or a fragment thereof is added to the suspension of the solid carrier and the linker, wherein the linker connects the solid carrier with the disaccharidase or a fragment thereof;

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

(d) immobilizing a functional constituent on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group.

Also provided is a composition comprising a solid carrier, a disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or the fragment thereof by embedding the disaccharidase or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group, wherein the composition is obtainable by the methods, in particular by the preferred method of the invention as described supra.

Examples

Examples

Material and Methods:

Reagents:

- 5 - Tetraethyl orthosilicate 99% (TEOS), (3-aminopropyl)-triethoxysilane (APTES), ammonium hydroxide (ACS grade, 28–30%), ethanol (ACS grade, anhydrous), glutaraldehyde (grade I, 25% in water), polysorbate 80, acetic acid, lactase (USP reference standard), invertase, bovine serum albumin (BSA), invertase activity assay kit, lactose, lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. BSA, lactase and
10 invertase were dissolved in water to reconstitute the stock buffer.
- Chitosan 95/500 P was purchased from Heppe Medical Chitosan GmbH
Caco-2 (human colorectal adenocarcinoma cell line) and HT29-MTX-E12 (human colon cancer cell line) was purchased from the European Collection of Authenticated Cell Cultures (ECACC).
- 15 - THP-1 (human acute monocytic leukemia cell line) was purchased from LGC.
- ThinCert™ cell culture insert plates (1.0µm membrane) were purchased from Greiner bio-one.
- Fetal Bovine Serum, Penicillin/Streptomycin (10'000 U/ml Penicillin/10'000 µg/ml Streptomycin), MEM Non-Essential Amino Acids (100x), L-Glutamine 200mM (100x),
20 Dulbecco's Phosphate Buffered Saline DPBS (1X), 0.25% Trypsin-EDTA (1X), RPMI 1640 Medium, DMEM, HEPES, Sodium pyruvate, D-glucose, β-mercaptoethanol were purchased from Gibco.
- Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free was purchased from Corning.
- 25 - Animal diet Altromin 1319 and AIN 93G modified 200g polysaccharides were purchased at Altromin international
- The catheters were purchased at Instech Laboratories
- Glucose meter TB100 Holtex kit was bought from MediSafe
- Oligo-α-1,6-Glucosidase 13A from Bifidobacterium adolescentis, Recombinant (Isomaltase)
30 was purchased from Creative Enzymes at a concentration of 1mg/mL in 35 mM NaHepes buffer, pH 7.5, 750 mM NaCl, 200 mM imidazol, 3.5 mM CaCl₂, 0.02% sodium azide and 25% (v/v) glycerol.
- .

Synthesis of silica nanoparticles:

Silica nanoparticles (50 nm) have been synthesized following the original Stöber process as described in WO2015/014888 A1. Briefly, ethanol, distilled water (6 M) and ammonium hydroxide (0.13 M) were mixed and stirred at 400 rpm for 1 h. TEOS (0.28 M) was added, and the solution was stirred at 400 rpm at 20°C for 22h. The solution was then centrifuged at 20000 g for 20 min and washed successively with ethanol and water. Particle size measurement was carried out on SEM micrographs acquired at a magnification of 150000x using the image analysis software Olympus stream motion.

Enzyme shielding and surface functionalization***- Production of NP-2:***

To SNPs (10 mg/mL, 55 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.9 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.9 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.9 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Lactase (7 mg/mL, 0.1 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lactase using APTES (8.4 mM) and TEOS (125.9 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 121 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in NaCl (0.9%)/PS80 (8 mg/L). NP-2 was cured overnight in a water bath at 20°C.

- NP-2 variants:

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

In a first experiment, nanoparticles (NP-2(1)) were produced in H₂O/PS80 (8 mg/L).

Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.3 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). Then, glutaraldehyde (3.3 mM)

was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A priming was performed by adding APTES (3.3 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). Lactase (5.2 mg/mL, 0.1 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lactase using APTES (6.5 mM) and TEOS (93 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). NP-2(1) were cured overnight in a water bath at 20°C.

In a second, comparative experiment, enzyme immobilisation and formation of the protective layer were carried out according to WO2015/014888 A1 to produce nanoparticles (NP-2(2)) in buffer. Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) was added APTES (3.3 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Then, glutaraldehyde (3.3 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). A priming was performed by adding APTES (3.3 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Lactase (5.2 mg/mL, 0.1 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lactase using APTES (6.5 mM) and TEOS (93 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). NP-2(2) were cured overnight in a water bath at 20°C.

In a third experiment, nanoparticles (NP-2) were produced in H₂O/PS80 (8 mg/L) according to the section headed "Production of NP-2" above. To keep the excess amount of glutaraldehyde which has not linked the solid carrier to the lactase in the reaction mixture, the nanoparticles were not washed between each chemical step. Therefore, glutaraldehyde was still present during

layer growth and caused a covalent bonding of the protective layer to the lactase. The covalent binding of the protective layer to lactase can be observed by the appearance of a yellow/orange 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 lactase and the organosilica layer. The absorbance of nanoparticles NP-2(1), NP-2(2), and NP-2 at 460 nm was measured after the organosilica layer formation and final particles washing i.e. after the organosilica layer was formed and the particles were washed 3 times in H₂O/PS80 and resuspended in H₂O/PS80 as described the section headed "Production of NP-2" above, showing an higher absorbance at 460 nm for NP-2 than for NP-2(1) and NP-2(2) (see Figure 7). NP-2(1) and NP-2(2) still show absorbance to some degree at this wavelength, as imine bonds are also formed during enzyme immobilization. However, the absorbance of NP-2 is significantly higher indicating an additional formation of imine bonds caused by covalent binding of the protective layer to lactase.

15 - *Production of NP-3:*

To SNPs (10 mg/mL, 56 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.8 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.8 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Invertase (1.726 mg/mL, 0.03 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized invertase using APTES (5.4 mM) and TEOS (81.3 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 115 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in NaCl (0.9%)/PS80 (8 mg/L). NP-3 was cured overnight in a water bath at 20°C.

25 - *Production of NP-4:*

30 To SNPs (10 mg/mL, 59 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.6 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.6 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.6 mM) and stirring the reaction mixture for 10 min at 20°C,

400 rpm. Isomaltase (3.55 mg/mL, 0.05 mM) was added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized invertase using APTES (5.8 mM) and TEOS (88 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 82 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O/PS80 (8 mg/L). NP-4 was cured overnight in a water bath at 20°C.

10 - *Production of NP-5:*

To SNPs (10 mg/mL, 59 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.6 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.6 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.6 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Isomaltase (1.77 mg/mL, 0.025 mM) and invertase (4.05 mg/mL, 0.07 mM) were added, and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized enzymes using APTES (5.8 mM) and TEOS (88 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. The particles were washed 3 times (by centrifugation during 5 min at 20000 rcf) in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 82 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O/PS80 (8 mg/L). NP-5 was cured overnight in a water bath at 20°C.

20 *Production of NP-1:*

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

resuspended in H₂O/PS80 (8 mg/L). A solution of chitosan in acetic acid (0.1 M) was added to the particle suspension to achieve a final chitosan concentration of 121 µg/mL. The reaction mixture was allowed to react for 30 min at 20°C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in NaCl (0.9%)/PS80 (8 mg/L). NP-1 was cured overnight in a water bath at 20°C. SNPs-BSA-AT were cured overnight at 20°C.

Disaccharidases activity assays:

- Lactase activity assay

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

- Invertase activity assay

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

- Isomaltase activity assay

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

- Co-immobilized isomaltase-invertase activity assay

NP-5 isomaltase activity was assessed using a glucose meter. An isomaltose solution (25 µL, 100 mM) was equilibrated for 5 minutes at 37°C, 700 rpm. Then, to the isomaltose solution was added NP-5 (25 µL, 67 µg) in phosphate buffer 50 mM, pH 6.8. The reaction mixture was

incubated for 10 min at 37°C, 700 rpm. Samples were collected after 2 min, 5 min and 10 min and glucose concentrations were determined using a glucose meter.

NP-5 invertase activity was assessed using a glucose meter. A sucrose solution (25 µL, 100 mM) was equilibrated for 5 minutes at 37°C, 700 rpm. Then, to the sucrose solution was added
5 NP-5 (25 µL, 67 µg) in phosphate buffer 50 mM, pH 6.8. The reaction mixture was incubated for 10 min at 37°C, 700 rpm. Samples were collected after 2 min, 5 min and 10 min and glucose concentrations were determined using a glucose meter.

Cell culture:

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

Caco2 (human colorectal adenocarcinoma cell line) and HT29-MTX-E12 (human colon cancer cell line) cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 1% non-essential amino-acid and 100 U/mL penicillin/streptomycin. THP-1 (Human monocytic leukaemia cell line) cells were maintained in culture in RPMI 1640
15 supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, and 100 U/mL penicillin/streptomycin.

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

- Intestinal barrier model

For the development of the intestinal barrier model, cells were seeded at a density of 2.6 x
25 10⁵cells/cm² in transwell PET inserts (1µm pore size). All cell models were used for experiments on day 21. For the co-culture, Caco-2 and HT-29-MTX-E12 cells were used at a ratio 75%-25%.

- Immunocompetent intestinal barrier model

For the development of the immunocompetent intestinal barrier model, M0 differentiated THP-
30 1 were added to the intestinal barrier model at day 21. Immune cells were attached to the posterior side of the Transwell membrane contained the previously differentiated co-culture with a 25% Matrigel solution *via* the drop method.

Transepithelial electrical resistance

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

In vitro digestion of sucrose

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

Animals:

All animal experimentations were carried out under a license approved by the National Animal Experiments Inspectorate under the Ministry of Food, Agriculture and Fisheries of Denmark. The study was performed in male Wistar rats (8 weeks of age) of the stock from Janvier, France.

- Diet and drinking water:

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

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

- Duodenum catheterization

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

- Dosing and lactose administration

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

5 - *Cecum analysis*

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

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

Results:

15 **Example 1: *Enhancing Enzyme Loading through Covalent Attachment to the protective layer***

In a first experiment, nanoparticles NP-2(1) were produced in non-buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth).

20 In a second experiment, nanoparticles NP-2(2) were produced in buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth).

In a third experiment, nanoparticles NP-2 were produced in non-buffered conditions without any intermediate washing steps (*i.e.* unreacted glutaraldehyde still present in the reaction mixture during layer growth).

Protein quantification was performed on the reaction supernatants to determine lactase immobilization yield at the surface of NP-2(1), NP-2(2) and NP-2. The results show that surprisingly enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-2) increases the enzyme immobilization yield by a factor of twenty-six (Fig. 2A), resulting in a 24-fold increase of enzyme loading per dry weight of SNP (Fig. 2B) compared to buffered conditions where glutaraldehyde is removed by washing steps (NP-2(2)).

30 Similarly, enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-2) results in a 2 times higher enzyme loading per dry weight of SNP (Fig. 2B) compared to unbuffered conditions where glutaraldehyde is removed by washing steps (NP-2(1)).

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

5 **Example 2: Disaccharidases activity of NP-2, NP-3, NP-4 and NP-5**

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

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

15 **Example 3: Biocompatibility of the nanoparticles for gastrointestinal applications**

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

20 The *lamina propria* includes a diffuse lymphoid tissue constituted by immune cells that maintain homeostasis or respond to a breakdown of epithelial protection.

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

25 To evaluate the safety of the nanoparticles, we first focused on the maintenance of the intestinal barrier integrity in presence of NP-1 (Fig. 4A). The transepithelial electrical resistance (TEER) measurements across Caco2-HT29-MTX-E12 cell monolayers shows that the integrity of the intestinal epithelial barrier remains intact when in contact with NP-1 for 24h. This result demonstrates the *in vitro* biocompatibility of NP-1.

30 To further characterize the impact of the nanoparticles on the intestinal barrier, we evaluated the ability of NP-1 to trigger an inflammatory response. To this purpose, we developed an immunocompetent intestinal barrier model and monitored its integrity. Figure 4B shows a decrease in integrity of the barrier in a dose dependant manner when treated with LPS, a

proinflammatory component. This lost of integrity reveals the recruitments of macrophages from the basal to the apical side of the barrier.

Most importantly, the TEER measurements showed that the integrity of the epithelial barrier remained intact when in contact with NP-1 (Fig. 4B). NP-1 does not stimulate the recruitment of macrophages at the apical side of the intestinal barrier, and it can be concluded that NP-1 does not induce inflammation.

Altogether, these results demonstrate the *in vitro* safety of the nanoparticles for gastrointestinal applications.

10 **Example 4: *In vivo* efficacy of NP-2**

Lactose malabsorption is attributable to an imbalance between the amount of ingested lactose and the capacity for lactase to hydrolyze the disaccharide. Digestion and absorption of lactose takes place in the small intestine. In case of lactose malabsorption, undigested lactose reaches the large intestine and comes into contact with the intestinal microbiota. The bacterial lactose fermentation leads to the production of short chain fatty acids and gasses resulting notably in an enlargement of the cecum.

In rats daily gavaged with high dose of lactose for 15 days, greater substrate availability for fermentation in the large intestine led to an enlargement of the cecum compared to rats fed with a normal diet without additional intake of lactose (Conditions “Vehicle” vs “No lactose” respectively) (Fig. 5). Importantly, a significant cecum size reduction is reported in rats dosed with NP-2, while the administration of inactive nanoparticles (NP-1) has no impact on cecum size reduction. These results demonstrate the *in vivo* biocatalytic activity of NP-2 for the digestion of lactose.

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

In Figure 5, the *in vivo* efficacy of NP-2 is demonstrated with a dose of 97 units for 3g of lactose. When compared to the dosage of currently available lactase preparation, this dose surprisingly corresponds to a 14.5 lower dose. So, this set of data demonstrate the high value of NP-2 for

lactose digestion and highlights the therapeutic potential of NP-2 for patients with defective disaccharide digestion like patients with lactose malabsorption.

Example 5: *In vitro* efficacy of NP-3

- 5 Congenital Sucrase-Isomaltase Deficiency (CSID) is characterized by complete, or almost complete lack of sucrose activity and varying degrees of reduction in isomaltase activity. The efficacy of NP-3 to digest sucrose was assessed on a model of intestinal barrier. Differentiated Caco2-HT29-MTX-E12 cell monolayers were exposed at their apical side to NP-3 in presence of its substrate for 4h. The quantification of the product of sucrose
- 10 hydrolysis in the basal compartment of the barrier is shown on Figure 6. The graph reports a dose dependant accumulation of glucose in the basolateral side of the barrier in presence of increasing amount of NP-3 while no glucose is detected in the untreated intestinal barrier. This result demonstrates the *in vitro* efficacy of NP-3 and underlines the use of NP-3 for therapeutic applications.

Claims

1. A composition comprising a solid carrier, a disaccharidase or a fragment thereof
immobilized on the surface of the solid carrier, a protective layer to protect the
5 disaccharidase or the fragment thereof by embedding the disaccharidase or the
fragment thereof, and a functional constituent immobilized on the surface of the
protective layer, wherein the functional constituent immobilized on the surface of the
protective layer is a polymer comprising repeat units wherein each repeat unit
comprises at least one amino group and/or at least one thiol group.
10
2. The composition according to claim 1, wherein the polymer comprising repeat units
wherein each repeat unit comprises at least one amino group and/or at least one thiol
group is a polyglucosamin selected from the group consisting of chitin, chitosan,
polyglucosaminoglycans, chondroitin, heparin, keratan and dermatan or a derivative
15 thereof.
3. The composition according to anyone of claims 1-2, 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.
20
4. The composition according to anyone of claims 1-3, wherein the functional constituent
is immobilized on the surface of the protective layer by non-covalent binding or by
covalent binding.
- 25 5. The composition according to anyone of claims 1-4, wherein the disaccharidase or a
fragment thereof is selected from the group consisting of lactase or a fragment thereof,
maltase or a fragment thereof, isomaltase or a fragment thereof, trehalase or a
fragment thereof and invertase or a fragment thereof or mixtures thereof.
- 30 6. The composition according to anyone of claims 1-4, wherein the disaccharidase or a
fragment thereof is selected from the group consisting of lactase or a fragment thereof
and invertase or a fragment thereof or mixtures thereof.

7. The composition according to anyone of claims 1-4, wherein the disaccharidase or a fragment thereof is invertase or a fragment thereof
- 5 8. The composition of anyone of claims 1-7, wherein the protective layer embeds the solid carrier and embeds the disaccharidase or a fragment thereof immobilized on the surface of the solid carrier.
- 10 9. The composition of anyone of claims 1-8, wherein the functional constituent immobilized on the surface of the protective layer is not embedded by the protective layer.
10. The composition of anyone of claims 1-9, for use as a medicament.
- 15 11. The composition of anyone of claims 1-9, for use in a method of treatment of lactase deficiency, sucrase-isomaltase deficiency and disaccharidoses intolerances.
- 20 12. A method of producing a composition, the composition comprising a solid carrier, disaccharidase or a fragment thereof immobilized on the surface of the solid carrier, a protective layer to protect the disaccharidase or the fragment thereof by embedding the disaccharidase or the fragment thereof, and a functional constituent immobilized on the surface of the protective layer, wherein the functional constituent immobilized on the surface of the protective layer is a polymer comprising repeat units, wherein each repeat unit comprises at least one amino group and/or at least one thiol group, the method comprising the following steps:
 - 25 (a) providing a solid carrier;
 - (b) immobilizing a disaccharidase or a fragment thereof on the solid carrier;
 - (c) forming a protective layer on the surface of the solid carrier to protect the disaccharidase or the fragment thereof immobilized on the solid carrier;
 - (d) immobilizing a functional constituent on the surface of the protective layer,
30 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.

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

20. A composition comprising a solid carrier, an disaccharidase or a fragment thereof
immobilized on the surface of the solid carrier, a protective layer to protect the
disaccharidase or the fragment thereof by embedding the disaccharidase or the
5 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 14-19.

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