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DIPLOMARBEIT

Surface Modification of PLGA Microparticles with β-Galactosidase and WGA: A Biomimetic Strategy for Peroral Enzyme Substitution

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Contents

1. Introduction

Lactose (4-(β-D-galactopyranosyl)-D-glucopyranose) is a disaccharide, which is not only present in milk and dairy products, but also in many pharmaceutical formulations. It is hydrolyzed by the enzyme Lactase (Lactase-phlorizin hydrolase; LPH; ß-galactosidase) into glucose and galactose, which can be absorbed and transported into the blood [1]. LPH is located at the brush border of enterocytes. The inability to metabolize lactose because of the absence of the enzyme lactase is called lactose intolerance. It is estimated that 75 - 90% of birth lactase levels are lost by most people after weaning and the enzyme level decreases continuously during the whole life. The prevalence of lactase deficiency ranges widely with the ethnic background from 2 - 15% among Northern Europeans to 95 - 100% among Asians [2]. The undigested lactose adsorbs water and causes an increase of the osmotic pressure. Furthermore, fermentation of lactose by bacteria in the colon leads to the production of hydrogen, methane, and carbon dioxide. The typical clinical symptoms of lactose intolerance are abdominal pain, distension, flatulence and diarrhea, as well as systemic symptoms caused by the toxic fermentation metabolites [1, 2].

Nowadays, lactose intolerance is usually controlled by strict adherence to a lactose-free or lactose-reduced diet. However, the main disadvantage of avoiding dairy products is to lose a major source of calcium and protein. As an alternative, capsules or tablets containing microbial-derived ß-galactosidase are available. Although they significantly reduce the clinical symptoms of lactose intolerance, the most inconvenient point is that by reason of their short-acting effect, patients have to take those formulations directly before or together with a lactose containing diet [2].

Therefore, the present work aimed at an innovative peroral lactase formulation which enables a longer action and thus offers a better patient compliance by reducing the dosage frequency. At this, microparticles were manufactured from

the biocompatible and biodegradable copolymer poly (D,L-lactide-co-glycolide) (PLGA) [3 - 5] and surface-functionalized with β-galactosidase and wheat germ agglutinin (WGA). Due to their pH optima of 6.0 - 8.0 [6] and 7.0 - 7.5 [7], respectively, β-galactosidase from *Escherichia coli* and *Kluyveromyces lactis* were used, which approach the physiological pH in the target area. The lectin WGA from *Triticum vulgare* was applied as a targeter to prolong the residence time in the gastrointestinal tract by adhering to the gastrointestinal mucus and the glycocalyx of human intestinal epithelial cells due to specific binding to N-acetyl-D-glucosamine and sialic acid [3, 8, 9]. For the presented study, β-galactosidase/WGA-grafted PLGA microparticles were prepared, their enzymatic activity and their binding to Caco-2 cells, which represent a well-established model for the human intestinal epithelium, were assessed.

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2. Background

2.1 Lactose Intolerance

Lactose intolerance is the inability to digest lactose, because of low levels or deficiency of lactase enzyme in the brush border of the intestinal epithelium [1]. It has been recognized since the time of Hippocrates and investigated scientifically since about 50 years [2]. The three main types are primary, secondary, and congenital lactose intolerance. The primary form is a common inherited disorder which is characterized by a continuous decrease of lactase levels after weaning. Secondary intolerance may be caused by injuries of the intestinal mucosa, by reductions of the functional mucosal surface area or by multiple viral syndromes. Congenital lactose intolerance is an extremely rare condition, which is characterized by a lifelong total lack of lactase [1].

In primary lactase deficiency, most people lose 75% - 90% of their birth lactase levels in early childhood, followed by a further decline during lifetime. The prevalence of this type of lactase intolerance depends on the ethnic background. By contrast to 2% - 15% among Northern Europeans, 95% - 100% of Asians are affected [2].

When lactose cannot be broken down in the small intestine, it increases the osmotic pressure, which leads to the attraction of fluid into the bowel lumen. When the undigested lactose reaches the colon, it is fermented by bacteria that produce hydrogen, methane, and carbon dioxide. The most important clinical symptoms of lactose intolerance such as abdominal pain, distension, boborygmi, flatulence and diarrhoea typically occur between 30 minutes and 2 hours after the intake of lactose. Other gastrointestinal symptoms include constipation, nausea and vomiting. Potentially toxic fermentation metabolites may affect ionic signaling pathways in the heart, the nervous system, and the immune system, and induce systemic symptoms such as headaches, light headedness and muscle pain [1, 2].

For diagnosis, the most common lactose tolerance test is the hydrogen breath test. At this, the breath hydrogen levels are measured before and after oral administration of a 50 g bolus of lactose. Moreover, the measurement of stool pH or small-bowel biopsies will also elucidate problems with the digestion of lactose [1].

Primary lactose intolerance is usually controlled by a lactose-free or lactose-reduced diet. However, the elimination of dairy products might provoke a lack of calcium. Marketed lactase preparations such as Lactaid® or Lactrase®, which reduce the gastrointestinal symptoms upon lactose intake, may allow patients to consume dairy products [1].

2.2 ß-Galactosidase

Lactase (Lactase-phlorizin hydrolase; LPH; ß-galactosidase) is localized at the intestinal brush border [3], and catalyses the hydrolysis of lactose into glucose and galactose [4]. The enzyme also possesses a transferase activity and catalyses the formation of galacto-oligosaccharides [5] (Figure 1).

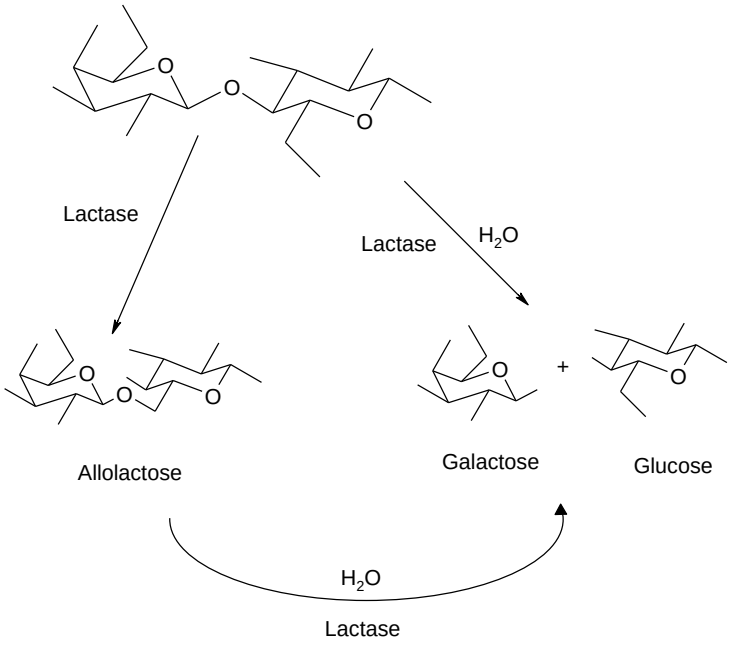

Figure 1: Enzymatic cleavage of lactose by lactase

Lactase has been detected in numerous microorganisms, animals and plants [6]. For the production of ß-galactosidase, microbial sources have numerous advantages, for example easy handling and high production yield [4].

The temperature optimum, pH optimum, and cofactors vary depending on the enzyme source. For example, the pH-optimum of lactase from *K. lactis* is 6.5 - 7.5, and 6.0 - 8.0 for *Escherichia coli*. The temperature optima for *K. lactis* and *E. coli* are 30°C - 35°C and 35°C, respectively [7]. The presence of Mn^{2+} and Mg^{2+} ions increases stability of lactase [6 - 9]. The residual enzyme activity is also influenced by the presence of glucose and galactose [4, 5, 8].The most extensively investigated β-galactosidase is that derived from *E. coli*, a product of the *lacZ* operon, and exhibits a molecular weight of 465000 Da. This enzyme is a tetramer of four identical 1023 - amino acid chains, each consisting of five domains [10]. In contrast to the enzyme from *E. coli*, ß-galactosidase from *Aspergillus niger, A. oryzae, Kluyveromyces lactis*, and *K. fragilis* are used in the food industry because of their GRAS-status (generally recognized as safe) [5]. Among these, the commercial products Lactozym® and Maxilact® from *K. fragilis and K. lactis* are frequently used [9].

ß-Galactosidase is mainly applied in the health and food technology. Firstly, it is used for lactase substitution in lactose intolerant patients. Secondly, the enzyme-aided lactose hydrolysis is applied for preparation of lactose-reduced milk, cheese and yoghurt. Lactase can be also used for lactose hydrolysis in whey, which is a by-product in cheese production [4, 5].

Because of the high costs of ß-galactosidase, several immobilization methods are developed to allow for prolonged or repeated use. Enzyme can either be entrapped in gels or immobilized by physical adsorption or covalent binding to water-insoluble carriers. For the latter approach, the enzyme is conjugated to the carrier via functional groups that are not essential for the catalytic activity. At this, cross-linkers like glutaraldehyde or carbodiimides may be used [4, 5].

2.3 Drug Targeting

The aim of drug targeting is to transport drugs as exclusively as possible to the therapeutic target [11 - 13].

The most important strategies can be divided into three types: passive, active, and physical targeting.

For passive targeting, physiological characteristics of the target tissue are exploited. The most prominent example is the enhanced permeation and retention (EPR) effect, which relies on the increased capillary permeability in quickly proliferating tumors or other types of diseased tissue [12, 13].

For active targeting, the drug or drug carrier is conjugated with a ligand, which specifically binds to certain structures on the cell surface, such as receptors or adhesion proteins [12, 13].

Physical targeting is based on non-physiological pH values or temperatures in the target tissue. For example, it has been observed that tumor tissue often reveals a lower pH or a higher temperature. When the carriers are sensitive to these stimuli, the therapeutic agent is released at its target. A very special example for this type of targeting is magnetic targeting. At this, magnetic carriers are transported to their targets with the aid of an externally applied magnetic field [13].

Wheat Germ Agglutinin

Wheat germ agglutinin (WGA) is the lectin derived from *Triticum vulgare*. Lectins are proteins or glycoproteins, which specifically bind to certain mono- or oligosaccharides. WGA is a dimeric protein, which contains four hevein domains, and specifically binds to sialic acid and N-acetylglucosamine [14, 15].

The biorecognition between lectin-mediated drug delivery systems and glycosylated structures in the intestine may be exploited to improve the efficacy of poorly absorbable drugs. Each mammalian cell possesses a carbohydrate layer, the so-called glycocalyx, which is built up of the oligosaccharide moieties of

proteoglycans, glycolipids and glycoproteins that are anchored in the lipid bilayer of the cell membrane. In the intestine, lectins may bind to carbohydrates in the mucus gel layer, or in the glycocalyx of the underlying cells. WGA-binding is strongly reduced at acidic pH. Therefore, there is very few binding to the gastric mucus. For poorly resorbable drugs, lectin-targeting may shorten the diffusional pathway of the drug and an increased concentration gradient, which is expected to result in improved bioavailability. Interestingly, the glycosylated extracellular domain 3 of the epidermal growth factor (EGF) receptor is involved in WGA-binding. Due to the up regulated expression of EGF-receptors in some tumors, this approach might also be applicable in cancer therapy. The intracellular uptake of WGA is an energy-dependent process. At 4°C, the lectin only binds to the cell surface, while at 37°C the lectin binding to the surface may be followed by internalization. At least 60% of the WGA enter the lysosomal route after uptake in the cells [14 - 17].

Because of the cytoadhesive and cytoinvasive characteristics of this lectin, which offers advantages for drug delivery or vaccination purposes, there are two strategies for the design of WGA-functionalized formulations: i) lectin prodrugs, which are composed of the lectin, a spacer and the drug; ii) lectin-grafted carrier systems, such as microparticles, liposomes or nanoparticles. The latter approach is especially interesting for the presented work which deals with WGA-grafted microparticles [14, 15].

2.4 Poly (D, L-lactide-co-glycolide) Particles

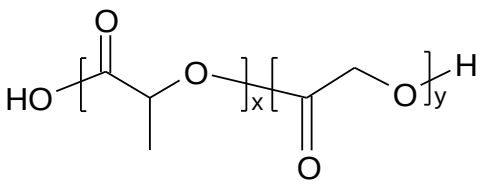

Figure 2: Structure of PLGA

Poly (D,L-lactide-co-glycolide) (PLGA) is a copolymer of lactide and glycolide (Figure 2), which is biocompatible and biodegradable. Moreover, it is approved by the US Food and Drug Administration (FDA) for drug delivery. Thus, numerous active pharmaceutical ingredients such as proteins, peptides, vaccines, genes, antigens, and growth factors have been encapsulated into PLGA-nano/microparticles [18, 19].

The physical, chemical, and biological properties of PLGA affect the mechanical strength, the characteristics of the drug formulation and the drug release from the polymer matrix. Therefore, the intrinsic viscosity related to the molecular weights is usually characterized in commercial products. Furthermore, the crystallinity of the polymers, which depends on the type and the molar ratio of the two monomer components, affects the biodegradation rate and the capacity to undergo hydrolysis. The commonly used PLGA polymers, which contain a 50:50 ratio of lactic and glycolic acid, are hydrolyzed faster than those with a higher proportion of either of the two components. The glass transition temperature of PLGA is slightly above the physiological temperature depending on the copolymer composition and molecular weight. Upon degradation, lactic and glycolic acid are formed. A three phase mechanism of the hydrolytic degradation is proposed: firstly, the molecular weight of PLGA decreases significantly; secondly, soluble oligomeric and monomer products are formed; finally, the polymer is completely solubilized [19].

Three important basic methods for the production of PLGA-particles are known, the solvent evaporation technique, phase separation methods and spray drying. For the solvent evaporation technique, an active pharmaceutical ingredient is either dissolved in an organic solution of PLGA (single emulsion technique) or dissolved in an aqueous solution and dispersed in an organic PLGA phase (double emulsion technique). This organic solution or W/O emulsion is then emulsified in an outer aqueous phase, which results in the formation of an O/W single emulsion or a W/O/W double emulsion, respectively. Subsequently, the organic solvent is allowed to evaporate and the liquid droplets are hardened to

become solid particles. While the solvent evaporation technique is based on the precipitation of PLGA upon solvent extraction, phase separation techniques are based on the addition of a non-solvent. By contrast, spray dried particles are prepared by the atomizing a PLGA solution into a stream of warm air. The liquid droplets are dried in the air stream and the resulting particles are collected [18, 19].

2.5 Conjugation Techniques

2.5.1 Carbodiimide Method

Carbodiimides are probably the most popular zero-length cross-linkers. They mediate the formation of amid linkages between carboxylic and primary amine groups or phosphoramidate linkages between phosphates and amines. Carboxylic groups react with N-substituted carbodiimides to form a highly active O-acylisourea intermediate, which can react with a nucleophile such as a primary amine to form a stable amide bond yielding isourea as a by-product. The optimal reaction medium is at a pH between 4.5 and 7.5.

For the present work, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was used (Figure 3). The reagent is water-soluble, should be dissolved rapidly and used immediately to prevent extensive loss of activity [20].

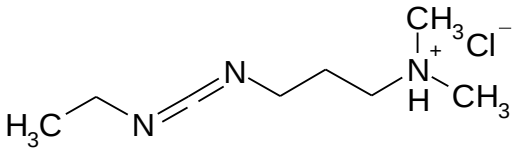

Figure 3: Structure of EDAC

N-Hydroxysuccinimide (NHS) or N-Hydroxysulfosuccinimide (sulfo-NHS) is added to the EDAC-reaction in order to increase the stability of the active intermediate

(O-acylisourea), which is rapidly hydrolyzed in aqueous environment. The hydroxyl group of NHS or sulfo-NHS can easily react with the active-ester to form a NHS-ester intermediate with a longer half-life in the range of some hours. Moreover, when using NHS the reaction can be performed in two steps. In the first step, the carboxylic groups are activated with EDAC/NHS. After removal of excessive coupling reagents by washing, the amine component is added and allowed to react with the active ester. This offers the advantage that the coupling of proteins, which contain carboxylate and amine groups, may be improved by avoiding the cross-linking of protein molecules with each other. Furthermore, the addition of NHS may improve the efficiency of the reaction [20] (Figure 4).

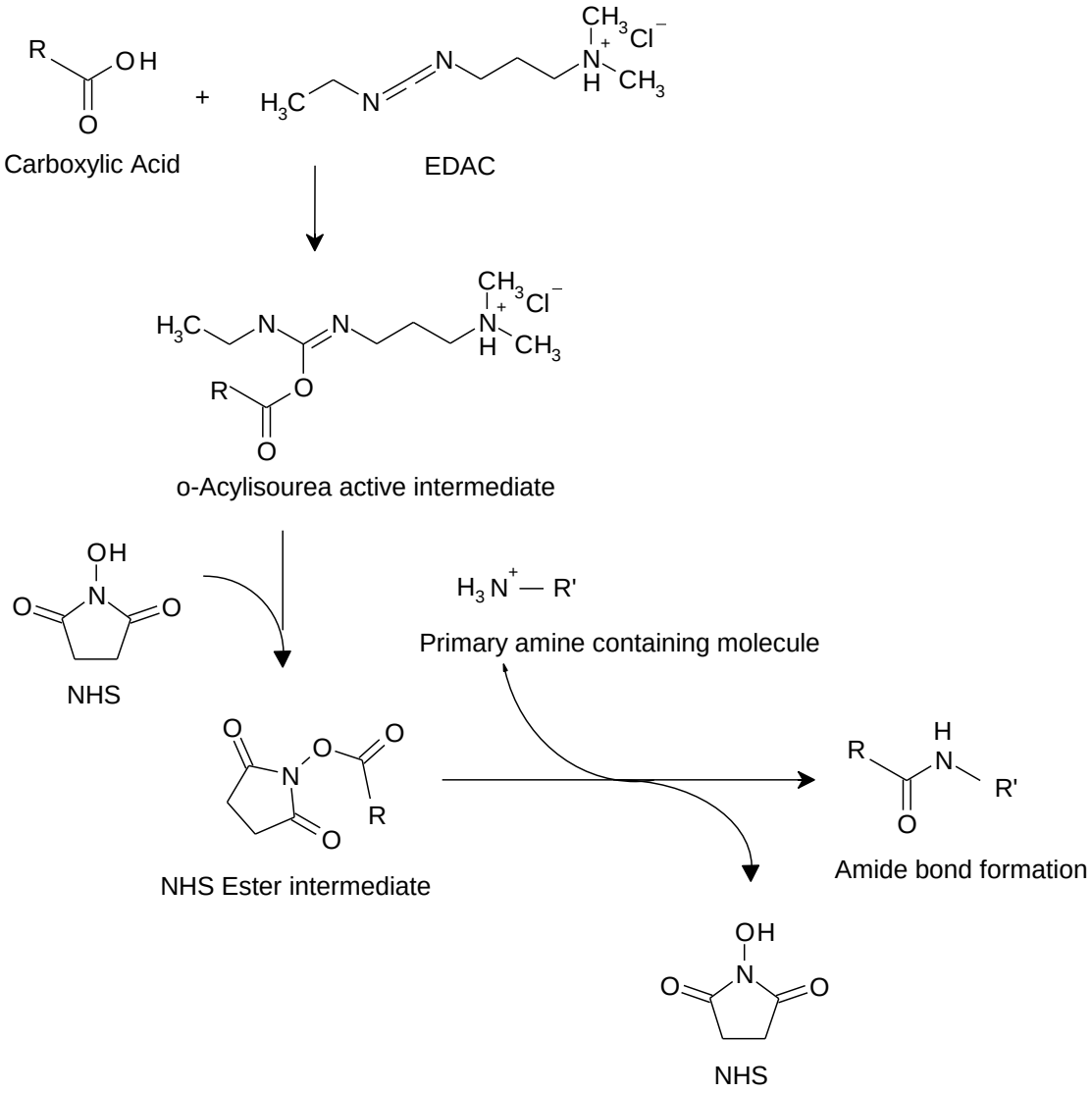

Figure 4: Reaction scheme of the carbodiimide method using EDAC and NHS

2.5.2 Glutaraldehyde Method

Today, glutaraldehyde is the most important bis-aldehyde homobifunctional cross-linker. The cross-linking process between amine-containing molecules is believed to proceed by a number of mechanisms, such as Schiff base formation or a Michael-type addition reaction at points of double-bond unsaturation created by polymerization (Figure 5). In aqueous solutions at alkaline pH, α,β-unsaturated glutaraldehyde polymers are formed by aldol condensation. They are highly reactive towards nucleophiles, especially primary amines. To avoid formation of high-molecular-weight conjugates due to uncontrollable polymerization during the cross-linking process, a two-step procedure is often employed. Firstly, one amine-containing molecule is activated with glutaraldehyde. Upon removal of the excess reagent, the second amine-containing molecule is added to enable the final conjugation [20].

Michael-type addition reaction

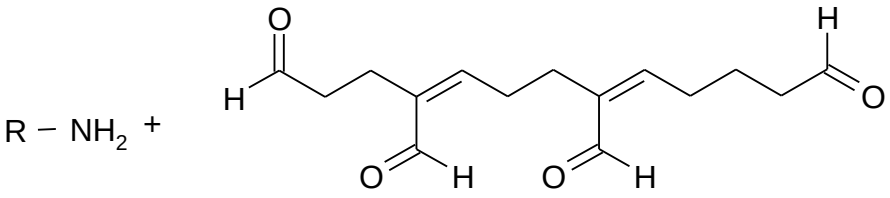

Figure 5: Reaction scheme of glutaraldehyde as a homobifunctional cross-linker

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3. Materials and Methods

3.1 Materials

See Chapter 6 (Attachment A).

3.2 Preparation of PLGA-Microparticles

See Chapter 6 (Attachment A).

3.3 Characterization of PLGA-Microparticles

3.3.1 Laser Diffraction

The particle size distribution was measured within a range of 0.1 µm to 45 µm on a Shimadzu® Laser Diffraction Particle Size Analyzer SALD-1100 (Shimadzu, Kyoto, Japan) applying the Mie theory. A laser light beam crosses a particle suspension. Depending on the different particle sizes, the light is scattered in different angles. Upon detection of the scattered light, the particle size distribution is calculated.

3.3.2 Flow Cytometry

The particle size distribution and the particle granularity were examined using a Coulter® EPICS® XL-MCL™ Flow Cytometer (GMI, Minnesota, USA). The forward scatter (FS) indicated the particle size, while the side scatter (SS) gave information about the particle granularity. 2 µl to 5 µl of the particle suspension were mixed with 1 ml particle-free 0.2% aqueous Tween® 20.

3.3.3 Scanning Electron Microscopy (SEM)

Spray-dried particles were raised on a specimen holder, sputter-coated with gold and imaged in high vacuum with an accelerating voltage 15 kV using a PHILIPS XL-30 ESEM scanning electron microscope.

3.4 Enzymatic Assay of ß-Galactosidase

See Chapter 6 (Attachment A).

3.5 Preparation of Fluorescein-labeled *E.coli* ß-Galactosidase

3.5.1 Coupling of FITC to *E.coli* ß-Galactosidase

2 mg β-Galactosidase from *Escherichia coli* (Grade VI, lyophilized powder, 250-600 units/mg protein, purchased from Sigma Aldrich, Vienna, Austria) were dissolved in 1 ml 0.1M Na_2CO_3 pH 9.0, mixed with 50 µl of a freshly prepared solution of FITC (1 mg/ml in DMSO) and incubated end-over-end overnight at 4°C under light protection. After addition of 10 µl NH_4Cl solution (150 mg/ml) the mixture was incubated for 2 h at 4°C. Finally, excess reagents were removed by gel filtration and affinity chromatography.

3.5.2 Purification

3.5.2.1 Gel Filtration

Gel filtration separates molecules according to differences in size as they pass a gel filtration medium packed in a column. Unlike ion exchange or affinity

chromatography, molecules do not bind to the chromatography medium. Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Purified proteins can be collected in any chosen buffer [1].

Excess FITC was removed using a PD-10 Desalting column (Amersham Biosciences, Buckinghamshire, UK) filled with Sephadex G-25 with a particle size range of 85-260 µm and an exclusion limit of 5 kDa. The matrix is stable in all commonly used buffers between pH 2 and 13.

Initially, the PD-10 column was equilibrated with 20mM HEPES/NaOH pH 7.2 buffer (25 ml). Subsequently, the FITC-ß-galactosidase solution was applied. The enzyme was eluted with 20mM HEPES/NaOH pH 7.2 buffer at a rate of 20 drops per min. The eluate was collected in 20 fractions of 1 ml each.

3.5.2.2 Affinity Chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a molecule and a specific ligand that is coupled to a matrix. The technique offers high selectivity, hence high resolution, and usually high capacity for the molecules of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally high [2].

4-Aminophenyl-β-D-thiogalactopyranoside-Agarose 4B (APTG-agarose; Sigma Aldrich, Vienna, Austria) can be used for the purification of ß-galactosidase [3]. After binding to the substrate analogue APTG the enzyme can be eluted with an appropriate buffer. 1 ml of APTG-agarose suspension was filled into a small chromatography column. After equilibration with 20mM HEPES/NaOH pH 7.2 buffer (10 ml), 500 µl of the sample solution were applied at the head of the column to enable the binding of ß-galactosidase. Contaminants were removed by washing with 20mM HEPES/NaOH pH 7.2 buffer (10 ml). Finally, the enzyme was eluted with an elution buffer (200mM lactose buffer; 0.1M borate/NaOH buffer pH

10; 0.8M Na_2CO_3/NaOH buffer pH 10) and separately collected in fractions of 0.5 ml each.

To enable repeated use, the column was washed with 0.1M Borax pH 10 buffer (5 ml), equilibrated with 20mM HEPES/NaOH pH 7.2 buffer (10 ml), and stored at 4°C.

3.5.2.3 Determination of Non-covalent Binding

To evaluate the purification procedure, an aliquot of the purified sample (1 ml) was incubated with 8M urea solution (1 ml) for 48 h at 4°C. Concentrated urea acts as a chaotropic agent and disrupts all non-covalent interactions. Here, it was used to separate non-covalently bound FITC from ß-galactosidase. Subsequently, the mixture was purified by gel filtration followed by determination of the concentration of both, the fluorophor and protein, in the eluates.

3.5.3 Characterization of FITC-β-Galactosidase

3.5.3.1 Micro BCA™ Assay

The Micro BCA™ assay (Pierce, Illinois, USA) is a colorimetric assay for the quantification of protein. In alkaline environment, Cu^{+2} ions are reduced by protein and form Cu^{+1}. Bicinchoninic acid (BCA) forms a purple-colored chelate with Cu^{+1}, which exhibits a strong absorbance at 562 nm.

Briefly, the sample solution (150 µl/well) was pipetted into a 96-well microplate. Upon addition of 150 µl of working reagent per well the plate was mixed on a plate shaker for 30 seconds and incubated for 2 h at 37°C. Finally, the absorbance was determined at 562 nm [4].

3.5.3.2 Estimation of the Coupling Rate

The coupling rate of FITC modified ß-galactosidase was arbitrary defined as the number of FITC molecules per ß-galactosidase molecule. The FITC-fluorescence was determined at 485nm/525nm (excitation/emission). The amount of immobilized FITC was calculated from a calibration curve and related to the protein content of the solution.

3.6 Covalent Coupling of ß-Galactosidase to PLGA-Microparticles

See Chapter 6 (Attachment A).

3.7 Coupling of F-WGA and ß-Galactosidase to PLGA-Microparticles

In order to prepare WGA-functionalized-galactosidase-grafted PLGA particles, two strategies were assessed. F-WGA was either coupled to already enzyme-modified particles, or the conjugation of WGA and enzyme were performed simultaneously.

3.7.1 Coupling of F-WGA to β-Galactosidase-grafted PLGA-Microparticles

For the covalent immobilization of F-WGA on enzyme-modified particles, the carbodiimide and the glutaraldehyde method were evaluated.

3.7.1.1 Carbodiimide Method

1.25 ml β-Galactosidase-HMD -particle suspension were incubated with 250 µl of the EDAC/NHS solution to activate the carboxylate groups of the surface-coupled enzyme. After purification, 135 µg F-WGA were added and the mixture was incubated end-over-end at 4°C overnight. Subsequently, unreacted binding sites were saturated with glycine and purified by repeated washing. Finally, the particles were re-suspended in 1.25 ml 50mM $MgSO_4$/20mM HEPES/NaOH pH 7.2.

3.7.1.2 Glutaraldehyde Method

12.5 µl 70% Glutaraldehyde solution were added to 1.25 ml β-galactosidase-HMD-particle suspension and incubated for 5 h at 4°C. Excess reagent was removed as described above. After addition of 135 µg F-WGA the suspension was incubated end-over-end at 4°C overnight. Subsequently, unreacted binding sites were saturated with glycine, purified and resuspended as mentioned above (3.7.1.1).

3.7.2 Coupling of β-Galactosidase and F-WGA to HMD-modified Particles

See Chapter 6 (Attachment A).

3.8 Quantification of Particle-bound F-WGA

See Chapter 6 (Attachment A).

3.9 Binding of Modified Microparticles to Caco-2 Cells

See Chapter 6 (Attachment A)

References

[1] GE healthcare Bio-Sciences AB Sweden, Gel Filtration Principles and methods, Handbooks from GE Healthcare, 18-1022-18

[2] GE healthcare Bio-Sciences AB Sweden, Affinity chromatography Principles and methods, Handbooks from GE Healthcare, 18-1022-29

[3] Steers E Jr, Cuatrecasas P, Pollard HB, The purification of beta-galactosidase from Escherichia coli by affinity chromatography, J. biol. Chem. 246(1971) 196-200.

[4] Instruction sheet of MicroBCA™ Protein Assay Kit from Pierce Biotechnology, USA

4. Results and Discussion

4.1 ß-Galactosidase from *E. coli*

4.1.1 Enzymatic Assay

For the determination of the activity of β-galactosidase, a colorimetric assay based on the enzymatic cleavage of ortho-Nitrophenyl-β-galactoside (ONPG) followed by photometric detection of the formed o-nitrophenolate was applied.
For each measurement, a calibration curve was established. For this purpose, aliquots of a stock solution of *E. coli* β-galactosidase were stored at -80°C and processed immediately prior to use. (single data see chapter 7 attachment B figure 20, table 1)

It was necessary to prepare the calibration curves in parallel, because the assay is strongly time dependent. After 60 min of incubation, the absorption approximately doubled as compared to 30 min of incubation (Figure 6). For the applied amounts of *E. coli* β-galactosidase an incubation time of 30 min was sufficient. For the analysis of β-galactosidase from *Kluyveromyces lactis* (Lactozym®) and β-galactosidase-grafted microparticles, however, the incubation time was extended to 120 min in order to increase the sensitivity of the assay.

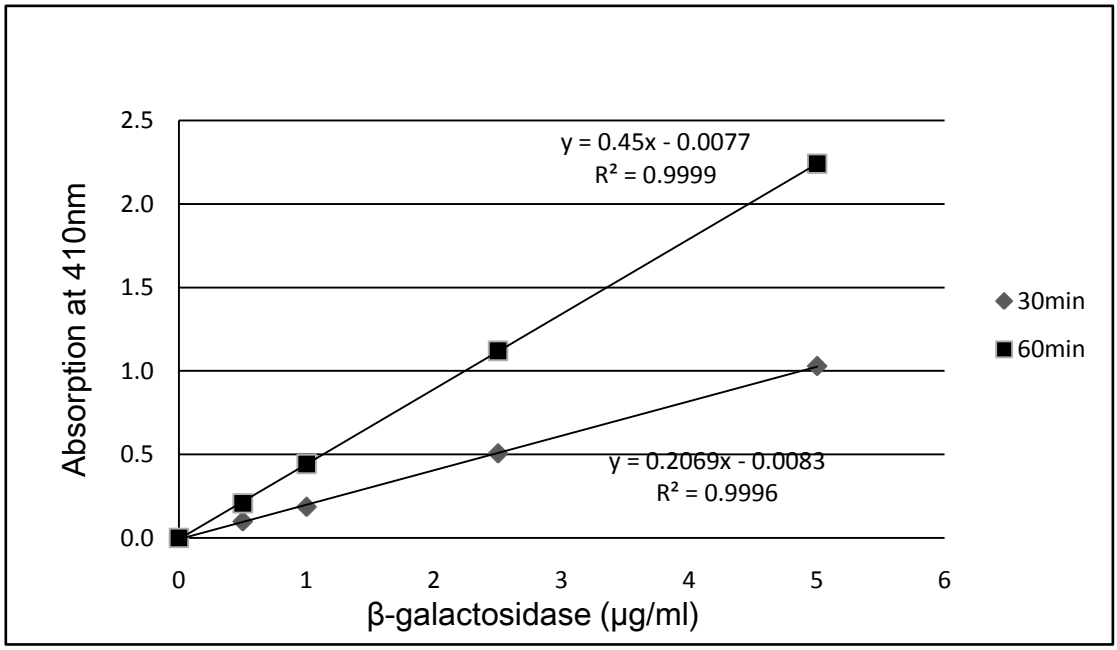


Figure 6: Calibration graph of *E.coli* β-galactosidase after incubation for 30 min or 60 min and readout at 410 nm. (single data see chapter 7 attachment B table 2)

In order to determine the optimum detection wavelength spectral scans of ONPG and o-nitrophenolate were performed (Figure 7). At 410 nm, the o-nitrophenolate exhibited maximum absorption, while the absorption of ONPG was nearly at its minimum. Based on these results, in order to discriminate between the product o-nitrophenolate and the unreacted substrate ONPG, the absorption was determined at 410 nm.

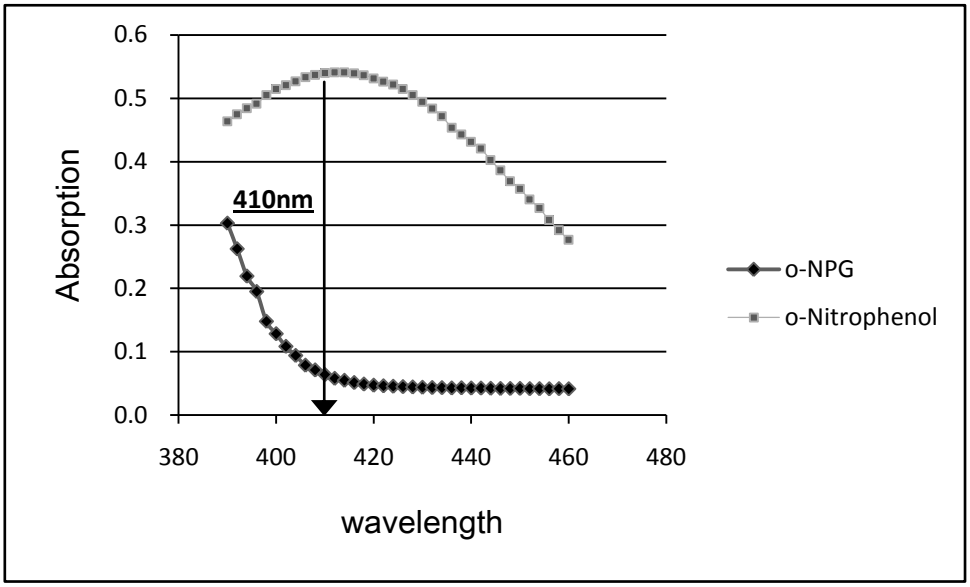


Figure 7: Spectral scan of ONPG and o-nitrophenolate from 390 nm to 460 nm.

4.1.2 Storage Stability

Three different dilutions (10 µg/ml, 5 µg/ml, 1 µg/ml) of *E. coli* ß-galactosidase in 25mM HEPES/NaOH pH 7.2 were prepared. To determine the effect of magnesium on the enzyme stability, three dilutions in 25mM HEPES/NaOH pH 7.2 supplemented with magnesium (50mM $MgSO_4$) were prepared in parallel. After 7 and 14 days at 4°C, the enzyme activity was determined (Figure 8). After two weeks the main part of the enzyme activity was lost in both dilution series, with and without magnesium. Thus, under the employed conditions any beneficial effect of magnesium on the stability could not be observed.

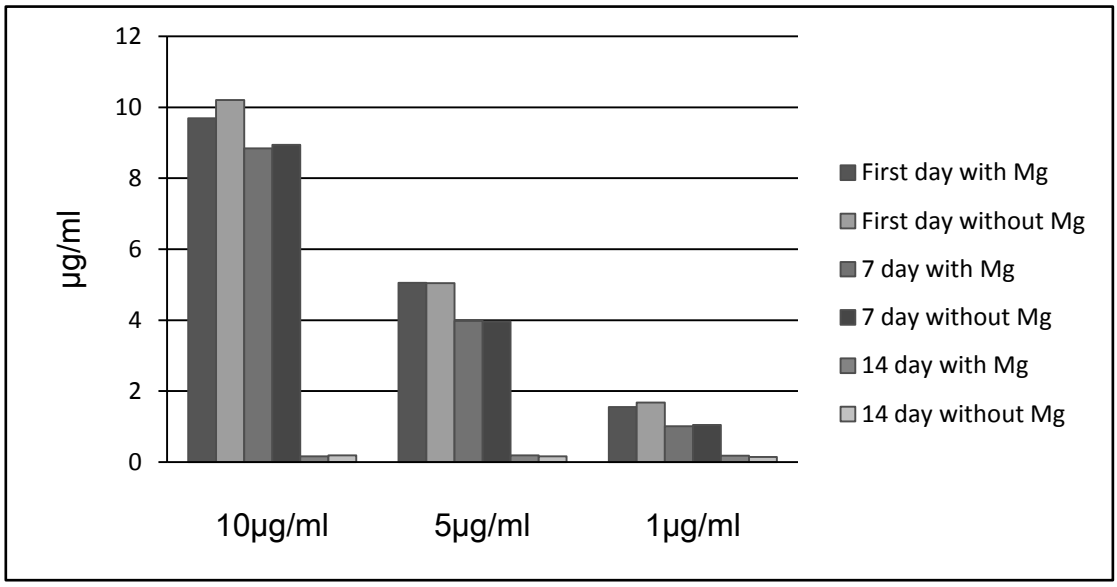


Figure 8: Stability of β-galactosidase from *E. coli* in presence and absence of magnesium at three different enzyme concentrations (10 µg/ml, 5 µg/ml, 1 µg/ml). The enzyme activity is given as µg enzyme/ml. (single data see chapter 7 attachment B table 3)

4.1.3 Protein Quantification

In order to evaluate the FITC-labeling procedure of β-galactosidase, it was necessary to quantify the protein content of the eluates of gel filtration and affinity chromatography. At this, the micro BCA™ assay was applied.

Commonly, BSA is used as a reference in this assay. Since the amino acid composition of a protein influences the extent to which Cu^{2+} is reduced to Cu^{1+} it might be more accurate to use the respective protein as a reference. Indeed, similar amounts (µg/ml) of BSA and ß-galactosidase from *E. coli* gave different results (Figure 9). Therefore, unmodified β-galactosidase was used as a reference for the determination of the enzyme content of the purified solutions after FITC modification.

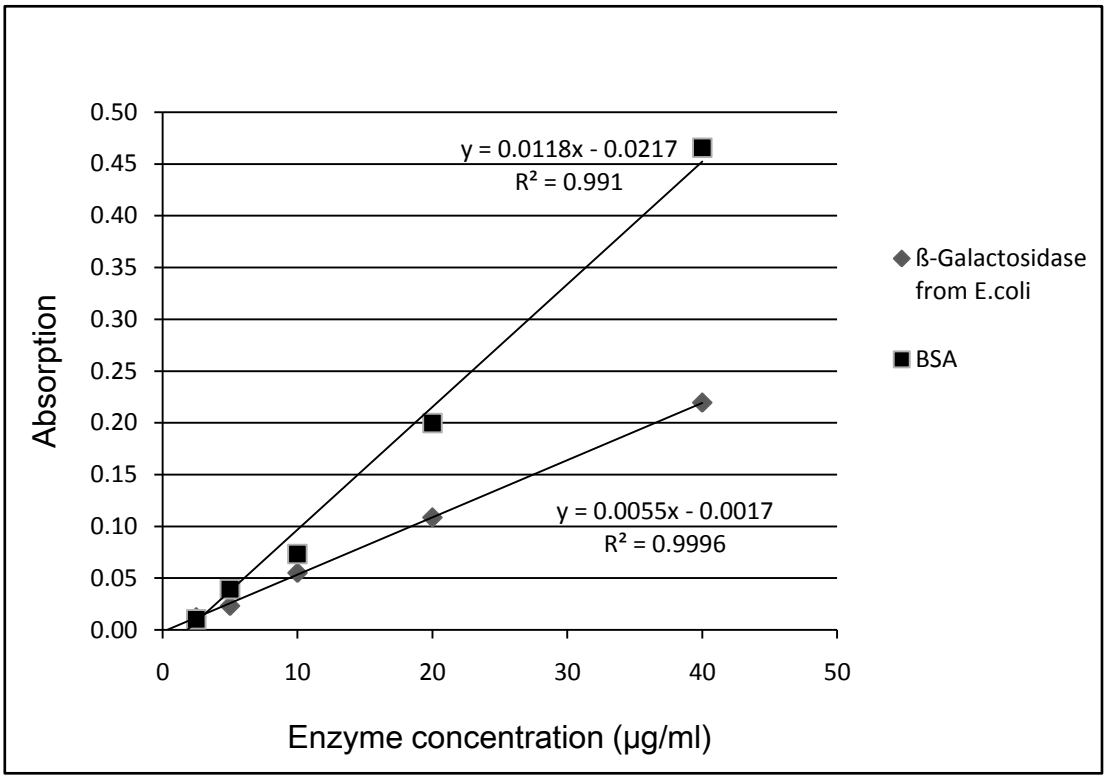


Figure 9: Micro BCA™ assay of known amounts of BSA and *E. coli* ß-galactosidase. (single data see chapter 7 attachment B table 4)

4.1.4 Characterization of FITC-labeled ß-Galactosidase

4.1.4.1 Fluorimetric Analysis

To quantify the amount of enzyme-coupled as well as non-reacted fluorescein, the fluorescence intensity of the eluates was determined at 485 nm/525 nm. For comparison, a calibration curve with fluorescein sodium in 25mM HEPES/NaOH pH 7.2 was prepared. (single data see chapter 7 attachment B figure 21, table 5)

The unbound FITC was removed by gel filtration. The eluate was collected in 20 fractions. The fluorescence intensities in the different fractions are shown in Fig. 10. As large molecules are eluted first, the peak (fractions 3 to 5) was attributed to FITC-modified ß-galactosidase. By contrast, FITC is a small molecule, which is

expected to be retarded. The fluorescence intensities in the later fractions ongoing from fraction 15 increased again, which might be due to free fluorescence.

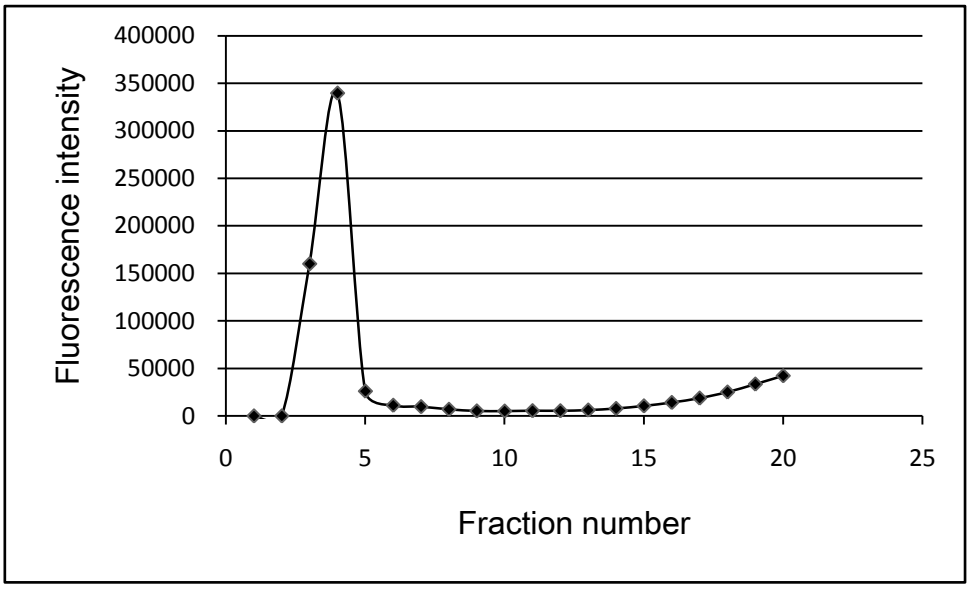


Figure 10: Fluorescence intensities of the eluate (20 fractions) after gel filtration of FITC-labeled ß-galactosidase determined at 485 nm/525 nm.

4.1.4.2 Protein Quantification

The protein content of the fractions was determined by Micro BCA™ assay, and calculated using a calibration curve prepared with *E. coli* β-galactosidase. (4.1.3).

4.1.4.3 F/P Ratio

Ongoing from both assays the ratio between fluorophor and protein (F/P ratio) was calculated which represents the number of immobilized flourescein molecules per ß-galactosidase molecule. On the one hand sufficient FITC should be coupled to the surface of ß-galactosidase to allow the detection of the labeled protein, on the other hand there should remain free coupling sites on the surface of the enzyme sufficient for immobilization at the particles. For this purpose, the optimum F/P ratio should be between one and two FITC molecules per ß-galactosidase molecule. Two batches with different proportions between FITC and

ß-galactosidase were analyzed. The results depicted from Fig. 11 show that a higher FITC concentration (column A) results in a better F/P ratio than the lower concentration (column C).

Magnesium is often added to β-galactosidase preparations to improve the enzyme stability. In order to assess a possible influence of magnesium on FITC-labeling, 50mM $MgSO_4$ was added during the FITC-ß-galactosidase coupling process. However, using the same proportion between FITC and enzyme, the F/P ratio decreased by about 40% in presence of magnesium (column B) as compared to that without magnesium (column A). To conclude, the most appropriate F/P ratio for our purpose was obtained applying coupling process without magnesium and a FITC concentration of 25 µg/mg enzyme.

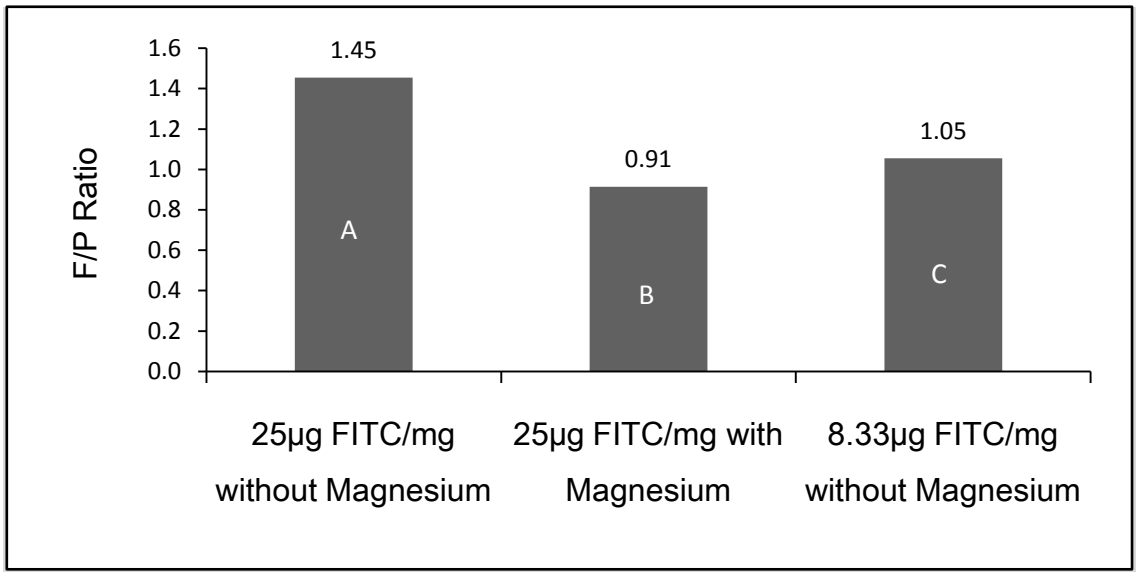


Figure 11: F/P ratio of FITC functionalized ß-galactosidase after gel filtration using two different concentrations of FITC (25 µg/mg, 8.33 µg/mg enzyme) with or without magnesium.

4.1.4.4 Determination of Non-covalent Binding

In order to guarantee stable binding between FITC and ß-galactosidase, a covalent linkage is preferable. To assess contribution of non-covalent binding, the FITC-labeled enzyme was treated with 8M urea, which disrupts non-covalent bonds.

In Fig. 12, three different purification processes are compared. The first column shows the F/P ratio of FITC-modified enzyme after gel filtration. The second column shows the ratio after two consecutive gel filtration processes. In the third column, the ratio is shown for FITC-labeled enzyme that was first purified by gel filtration, then incubated with the chaotropic agent, and finally purified by a second gel filtration. The results demonstrate that one gel filtration process was not sufficient to remove weakly bound FITC since less than one third of the fluorophor was bound covalently after a single size exclusion chromatography.

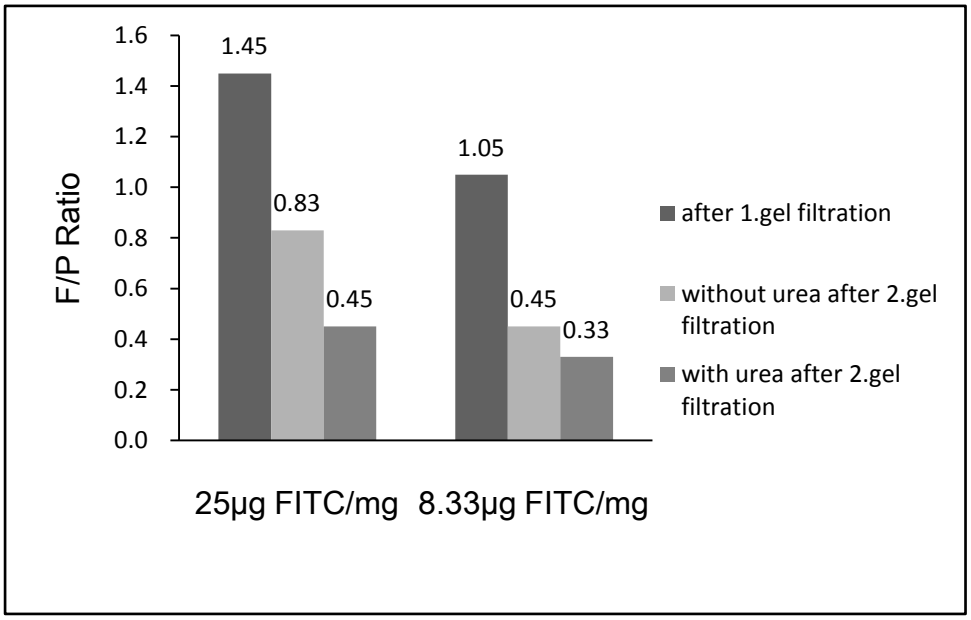


Figure 12: Influence of the purification procedure on the amount of ß-galactosidase bound fluorescein (F/P-ratio) of two batches with different initial amounts of fluorophor (25 µg/mg, 8.33 µg/mg enzyme).

In summary, β-galactosidase was successfully labeled with FITC. For an optimal F/P ratio between one and two, which seems most appropriate for the further coupling steps, a FITC concentration of 25 µg/mg enzyme yielded favorable results. Interestingly, the addition of magnesium reduced the coupling efficiency. However, the subsequent purification procedure needs further improvements. To remove the non-covalently bound FITC one or two gel filtration steps were not sufficient as demonstrated by the use of the chaotropic agent. Moreover, as a main drawback, the enzyme solution is more and more diluted by multiple gel filtration steps, which makes the sole application of this chromatographic technique inappropriate for further coupling to the particle surface.

4.2 ß-Galactosidase from *Kluyveromyces lactis* (Lactozym®)

In contrast to β-galactosidase from *E.coli*, the enzyme from *Kluyveromyces lactis* (Lactozym®) is generally recognized as safe (GRAS) by the FDA (USA) [1]. Therefore, β-galactosidase from *Kluyveromyces lactis* (Lactozym®) was used for the further work.

4.2.1 Enzymatic Assay

According to the specifications provided by the manufacturer, the Lactozym® solution contained ≥ 3000 U/ml. For all experiments with *K. lactis* lactase performed in this diploma thesis, the activity of the original solution was set to be 3000 U/ml and dilutions thereof were used for calibration. (single data see chapter 7 attachment B figure 22, table 6)

Evaluation of a possible impact of PLGA-microparticles on the enzymatic assay

Unmodified PLGA-Microparticles were tested (see chapter 6 Attachment A) using three different concentrations (4 mg/ml, 2 mg/ml, 1 mg/ml) in order to detect an eventual influence on the enzyme assay. Actually, the presence of PLGA particles exerted no influence on the test. (single data see chapter 7 attachment B table 7)

4.2.2 Protein Assay

Lactozym® solution represents a stabilized preparation of β-galactosidase, which is stored at 4°C. Types and concentrations of stabilizers as well as total protein content are not communicated by the manufacturer. Traditional stabilization strategies involve the addition of glycerine, different metal salts, and protein. When Lactozym® solution is used for carbodiimide- or glutaraldehyde-mediated coupling, other proteins might also react and compete with the enzyme. Therefore, the total protein content of the original solution has to be quantified by a micro BCA™ assay. Additionally it is very likely that glycerol forms part of the commercial Lactozym® solution. Regarding the rather high viscosity of the preparation it might even be present in higher concentrations. However, glycerol is known to interfere with the micro BCA™ assay [2].

Therefore, different purification strategies were assessed to remove glycerol from the original solution.

4.2.3 Purification of Lactozym®

In order to remove glycerol, the original Lactozym® solution was purified by gel filtration, dialysis, and affinity chromatography and the resulting purified enzyme

solutions were characterized in terms of protein content and enzymatic activity.

Gel filtration

0.5 ml Lactozym® solution was passed through a PD-10 desalting column using 25mM HEPES/NaOH pH 7.2 for elution.

Dialysis

1 ml Lactozym® solution was dialyzed against 25mM HEPES/NaOH pH 7.2 buffer (500 ml). Within one week, the external buffer was changed five times.

Affinity chromatography

Different strategies are reported for elution of β-galactosidase from an APTG-agarose affinity matrix [3]. To meet optimum conditions, three different eluting buffers have been tested. Each time, 0.5 ml Lactozym® solution was applied onto 1 ml of APTG gel suspension filled in a column. For elution, 200mM lactose solution, borate buffer pH 10, and carbonate buffer pH 10 were assessed.

a) Elution with 200mM lactose buffer

β-Galactosidase binds to the substrate analogue APTG, which is immobilized on the matrix. As APTG and lactose compete for enzyme binding, high concentrations of lactose are apparently useful for enzyme elution.

However, the enzymatic activity assay of the eluates revealed that there was no β-galactosidase activity detectable. One possible reason might be that the eluting effect of lactose was not strong enough. To elucidate this issue, yellow-colored FITC-β-galactosidase (*E. coli*) was loaded on the APTG matrix and eluted with the same lactose solution. While the gel matrix was visibly yellow-colored, the lactose eluates did not exhibit relevant fluorescence. Thus, this rather high amount of lactose seems to be not strong enough to disrupt the interaction between APTG-agarose and β-galactosidase.

b) Elution with 0.1M borate/NaOH buffer pH 10

Another possibility to elute β-galactosidase from the APTG-matrix is the use of alkaline buffers. Upon elution with 0.1M borate/NaOH pH 10 the collected fractions have to be quickly neutralized in order to prevent protein denaturation. For this purpose, three different neutralization methods were assessed. The eluates were either supplemented with 4M HCl (35 µl acid per 0.5 ml fraction) immediately after elution, or diluted 100-fold with 25mM HEPES/NaOH buffer pH 7.2, or directly collected in test tubes containing 1M HEPES/NaOH buffer pH 7.2. Unfortunately, for neither one of the neutralization methods enzyme activity was detectable in the eluates. It was assumed that boric acid destroys the active center of β-galactosidase. In order to evaluate a potentially harmful influence of the borate buffer on ß-galactosidase activity, five different dilutions of the original enzyme solution with the same buffer steps（500-, 1000-, 2000-, 5000-, and 10,000- fold） were kept for two hours at 4°C, and finally neutralized with 4M HCl. Subsequently, the enzyme activity was totally lost.

c) Elution with 0.8M Na_2CO_3/NaOH buffer pH 10

According to the harmful effect of borate buffer on enzymatic activity, 0.8M Na_2CO_3/NaOH buffer pH 10 with an equal buffer capacity as boric acid was used for elution. The eluates were neutralized immediately by the use of test tubes that contained 1M HEPES/NaOH pH 7.2 buffer. Once again, no enzymatic activity was detected in the eluates.

Not only the enzymatic activity, but also the protein content of the eluates was assessed by the Micro BCA™ test. However, most of the samples were not directly applicable for that assay. Lactose represents a strong reducing carbohydrate and therefore disturbs the assay. Additionally, high HEPES concentrations are not compatible with the Micro BCA™ test. Consequently, the enzyme eluates neutralized with 1M HEPES/NaOH pH 7.2 were diluted 40-fold with distilled water prior to the protein assay in order decreed the compatible concentration of 100mM

HEPES [2].

There were pronounced differences in the protein recovery (mg protein per ml applied Lactozym® solution) between with the various purification methods. Affinity chromatography yielded the lowest recovery rate. While elution with borate buffer resulted in a recovered protein amount of 3.63mg per ml Lactozym®, carbonate buffer yielded even less (1.03mg per ml Lactozym®). An explanation for the low recovery rate might be overloading of the matrix with the applied amounts of enzyme so that part of the enzyme initially could not bind and was washed away. Although gel filtration showed the highest recovery rate with 21.9mg per ml Lactozym®, the enzyme solution was diluted at least threefold, which was not advantageous for further works such as coupling. Additionally, it should be mentioned that the dilutions necessary for the protein assay and the subsequent recalculation might multiply the error of the assay. Dialysis yielded 12.49 mg per ml Lactozym®. Nevertheless, no enzymatic activity was detectable after dialysis. This might be attributed to the lack of appropriate stabilizers in the exchange buffer.

In summary, the presented experiments cannot provide a satisfying answer to the basic question about the total protein content of the original Lactozym® solution. Furthermore, the purification methods were not effective enough to prepare pure and active enzyme for coupling. This will be the subject of future studies.

4.2.4 Storage Stability

The stability of β-galactosidase from *Kluyveromyces lactis* was tested at 6 different concentrations (20 U/ml, 15 U/ml, 10 U/ml, 5 U/ml, 2 U/ml, and 1 U/ml). The original solution (Lactozym®) was diluted with 50mM $MgSO_4$ in 20mM

HEPES/NaOH pH 7.2, which is the same buffer as in β-galactosidase modified PLGA-suspension. The analysis of the enzyme activities on day 0, 1, 2, 7 and 28 revealed a similar decrease as already observed in case of the β-galactosidase-PEI or -HMD modified particle suspension (see chapter 6 Attachment A) (Figure 13).

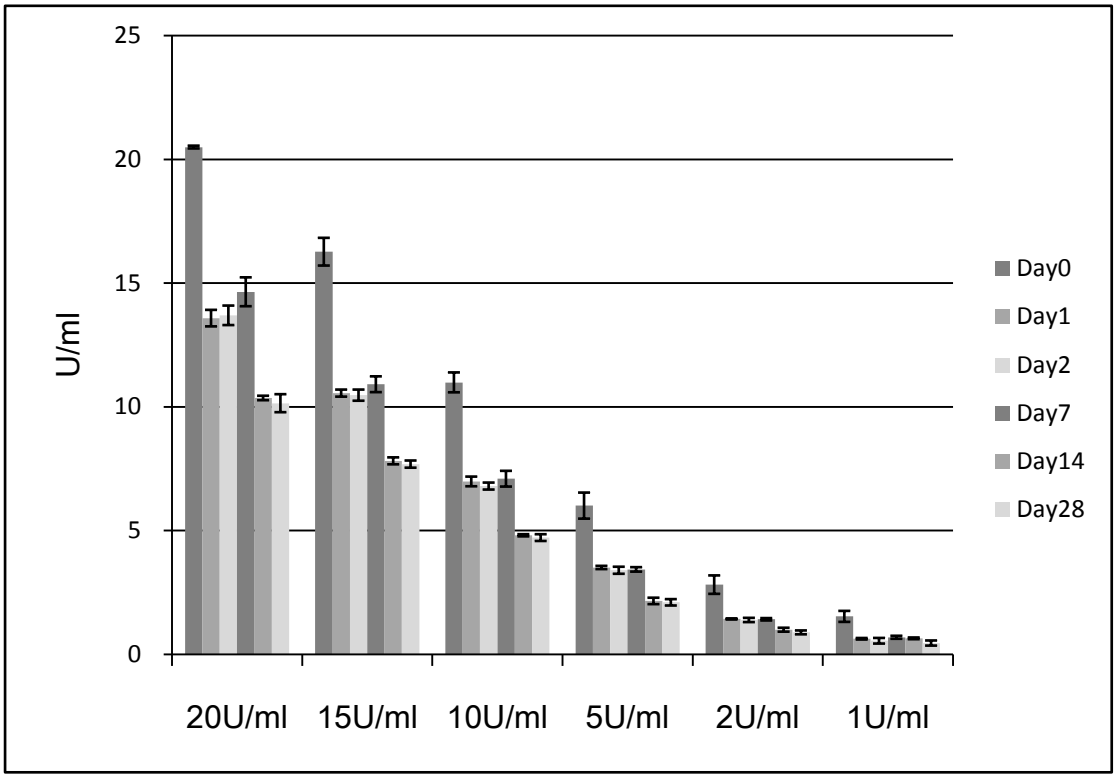


Figure 13: Stability of β-galactosidase from *Kluyveromyces lactis.* The β-galactosidase activity in given in units (U) per ml solution of diluted original product (Lactozym®). (single data see chapter 7 attachment B table 8)

4.3 Preparation and Characterization of PLGA-Microparticles

4.3.1 Preparation of PLGA-Microparticles

In the present diploma thesis, PLGA microparticles were prepared by spray-drying (see chapter 6 Attachment A). The yield of this process amounted to only 10 to 40% and there was a considerable proportion of aggregated particles. The drawbacks of spray-drying PLGA are the low glass transition temperature of PLGA, which is slightly above body temperature, and the low boiling points of appropriate solvents. PLGA is soluble in organic solvents like ethyl acetate, ethyl formate, acetone or methylene chloride. For the present study, the solvent with the lowest boiling point (40°C), methylene chloride, was used. However, the attempt to avoid temperatures higher than the glass transition temperature resulted in particles that still contain residual solvent when they reach the cyclone separator. These non-hardened particles can stick to the glass walls and reduce the yield. Moreover, they tend to form aggregates. When the temperature was increased in spite of that, the particles adhered to the walls as well. Elevating the temperature above the glass transition temperature seemed to produce sticky particles as well. However, those problems of poor yield might be overcome by using another type of spray dryer. A drying tower with a larger diameter might allow for more complete drying at moderate temperatures.

SEM images of PLGA microparticles reveal a broad size distribution and the presence of some aggregates (Figure 14).

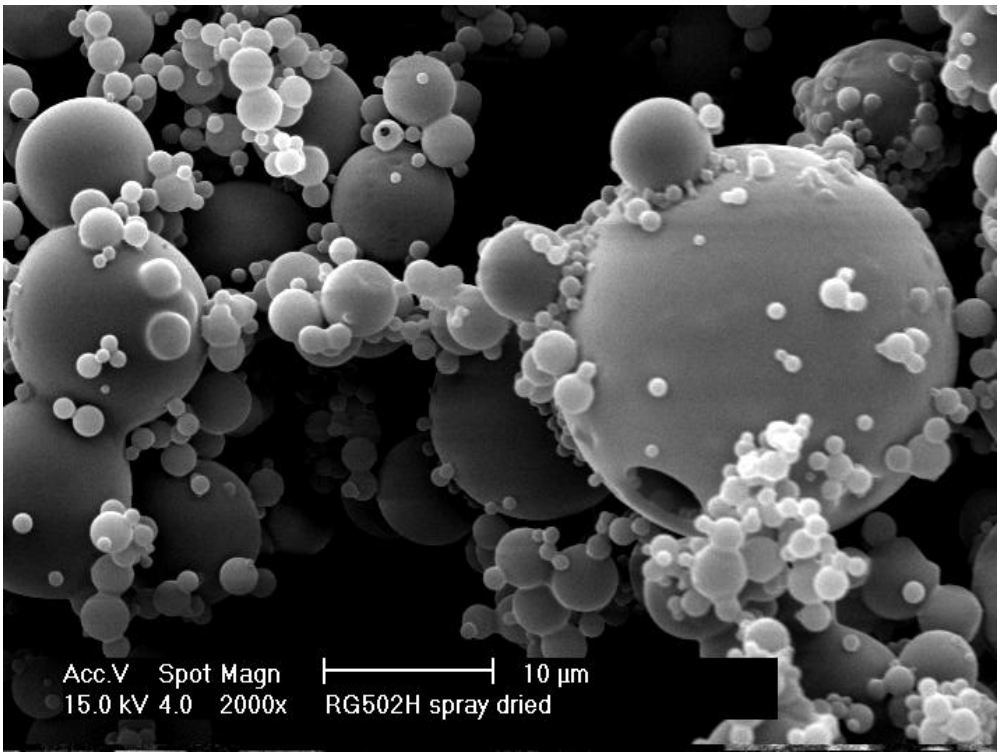


Figure 14: SEM image of spray dried PLGA-microparticles prior size fractionation.

Out of this reasons, the particles were fractionated according to their size prior to surface modification (Figure 15) (see chapter 6 Attachment A).

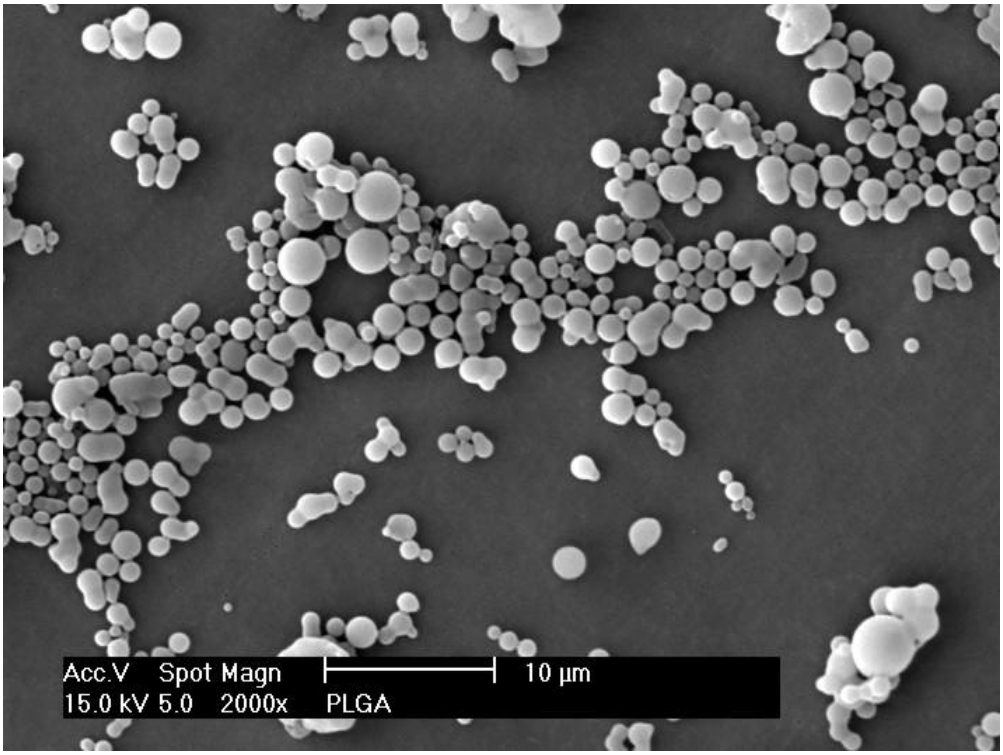
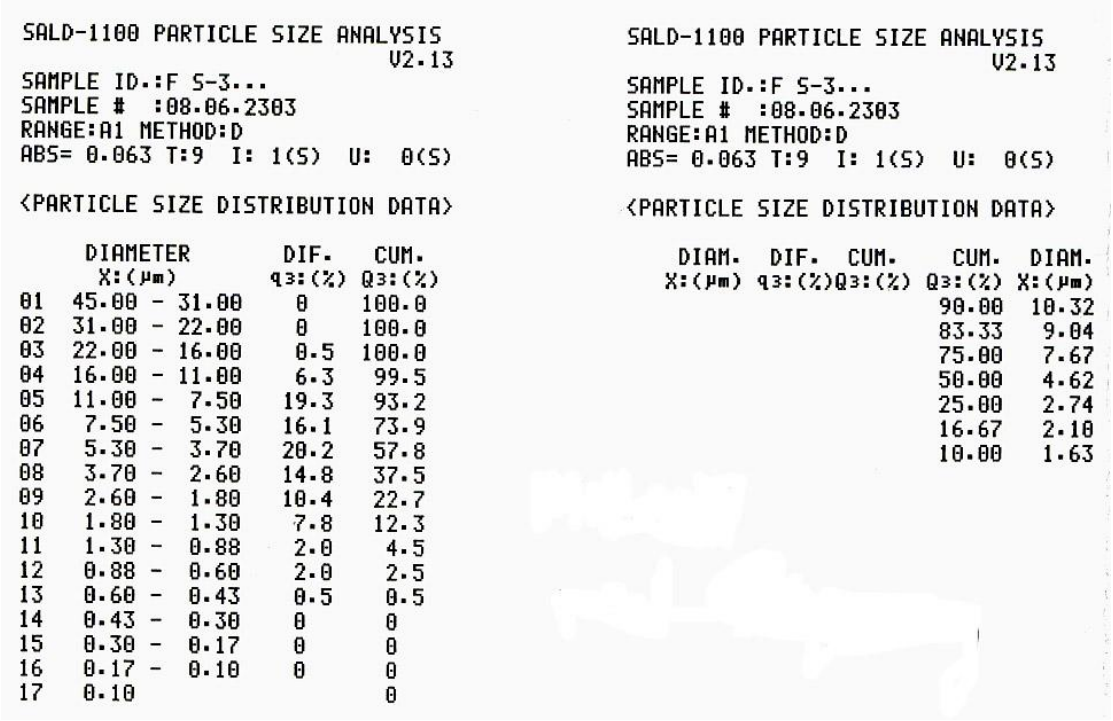


Figure 15: SEM image of spray-dried PLGA-microparticles after size fractionation.

4.3.2 Characterization of the PLGA-Microparticles

The mean particle size and the size distribution can be determined by different methods. Image evaluation of microscopic images (see chapter 6 Attachment A) revealed a particle size of about 2 - 4 µm. This was confirmed by flow cytometric analyses (data not shown), whereas laser diffractometry pointed to bigger particles (Figure 16). According to laser diffraction analyses, about 50% of the particles were smaller than 4.6 µm and 90% were smaller than 10.3 µm.



SALD-1100 PARTICLE SIZE ANALYSIS V2.13
SAMPLE ID.:F S-3...
SAMPLE # :08.06.2303
RANGE:A1 METHOD:D
ABS= 0.063 T:9 I: 1(S) U: 0(S)

<PARTICLE SIZE DISTRIBUTION DATA>

	DIAMETER X:(µm)	DIF. q3:(%)	CUM. Q3:(%)
01	45.00 - 31.00	0	100.0
02	31.00 - 22.00	0	100.0
03	22.00 - 16.00	0.5	100.0
04	16.00 - 11.00	6.3	99.5
05	11.00 - 7.50	19.3	93.2
06	7.50 - 5.30	16.1	73.9
07	5.30 - 3.70	20.2	57.8
08	3.70 - 2.60	14.8	37.5
09	2.60 - 1.80	10.4	22.7
10	1.80 - 1.30	7.8	12.3
11	1.30 - 0.88	2.0	4.5
12	0.88 - 0.60	2.0	2.5
13	0.60 - 0.43	0.5	0.5
14	0.43 - 0.30	0	0
15	0.30 - 0.17	0	0
16	0.17 - 0.10	0	0
17	0.10		0

SALD-1100 PARTICLE SIZE ANALYSIS V2.13
SAMPLE ID.:F S-3...
SAMPLE # :08.06.2303
RANGE:A1 METHOD:D
ABS= 0.063 T:9 I: 1(S) U: 0(S)

<PARTICLE SIZE DISTRIBUTION DATA>

DIAM. X:(µm)	DIF. q3:(%)	CUM. Q3:(%)	CUM. Q3:(%)	DIAM. X:(µm)
			90.00	10.32
			83.33	9.04
			75.00	7.67
			50.00	4.62
			25.00	2.74
			16.67	2.10
			10.00	1.63

Figure 16: Size distribution after size fractionation as determined by laser diffractometry.

However, the results obtained by the laser diffraction technique are volume-distributed. Therefore, smaller particles are underrepresented despite their potentially large number. By contrast, image evaluation and flow cytometry are based on measuring the characteristics of many single particles.

4.4 Coupling of ß-Galactosidase to PLGA-Microparticles

4.4.1 Immobilization of *K. lactis* ß-Galactosidase Using Different Spacers

See Chapter 6 (Attachment A).

4.4.2 Effect of Coupling Reagent and Incubation Time

In order to optimize ß-galactosidase coupling to HMD-modified particles (PLGA-HMD-c-Gal), different concentrations of EDAC/NHS and different incubation times were tested (Figure 17).
The highest particle-bound enzymatic activity amounted to 12 U per ml particle suspension. It was achieved by using the highest concentration of EDAC/NHS ("100%") and incubating overnight. Applying only one-tenth of the coupling reagents decreased the result by 25% (9 U/ml). A further reduction of activation reagents to only 1% of the highest amount resulted in a very low activity of only 1 U/ml. Shortening the incubation time from overnight to 2 h resulted in a decrease of particle-bound enzymatic activity by two thirds (4 U/ml). The lowest activity (1 U/ml) was observed after overnight incubation with 1% EDAC/NHS. Taken together, the best results were obtained with the highest concentration of reagents and the longest incubation time.

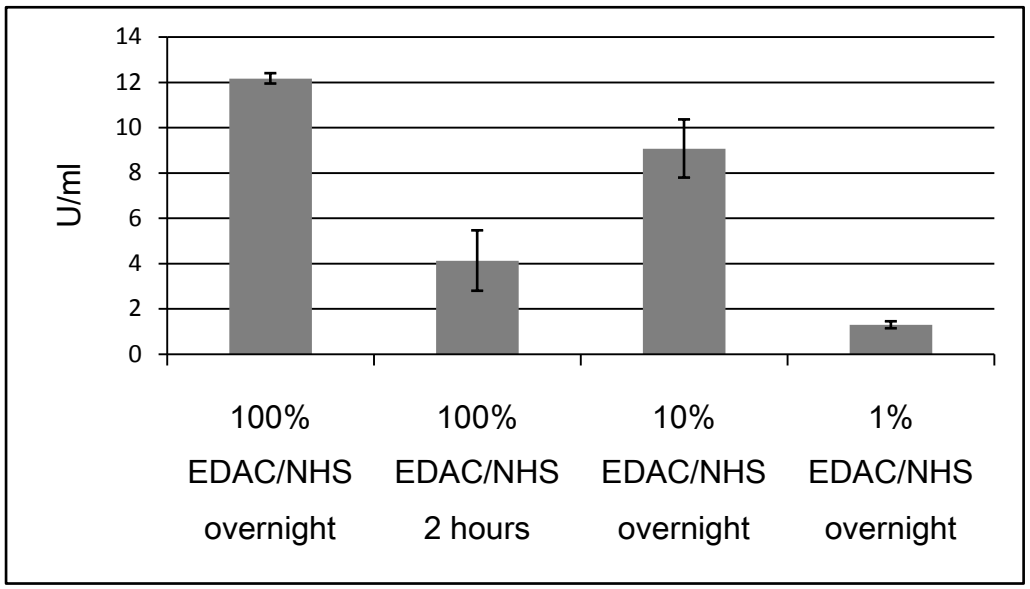


Figure 17: ß-Galactosidase activity in units (U) per ml suspension of PLGA-HMD-c-Gal particles. PLGA-HMD particles were activated at three different concentrations of EDAC/NHS and two different incubation times; 100% EDAC/NHS corresponds to 45 mg EDAC and 1.875 mg per 625 µl ß-galactosidase and 1.25 ml particle suspension each. (single data see chapter 7 attachment B table 9)

4.5 Coupling of F-WGA and ß-Galactosidase to the PLGA-Microparticles

4.5.1 Immobilization of F-WGA on β-Galactosidase-grafted PLGA-Microparticles

4.5.1.1 Carbodiimide Method

Fluorescein-labeled WGA was coupled to ß-galactosidase-HMD-modified PLGA-microparticles (PLGA-HMD-c-Gal) via the carbodiimide method (Figure 18). While the particle-bound activity amounted to 9.9 U/ml prior to F-WGA conjugation, it was only about 1 U/ml after the additional modification step. During the coupling procedure, the particle-bound enzyme was lost for the most part. Thus, F-WGA coupling using the carbodiimide reaction impaired the activity of ß-galactosidase.

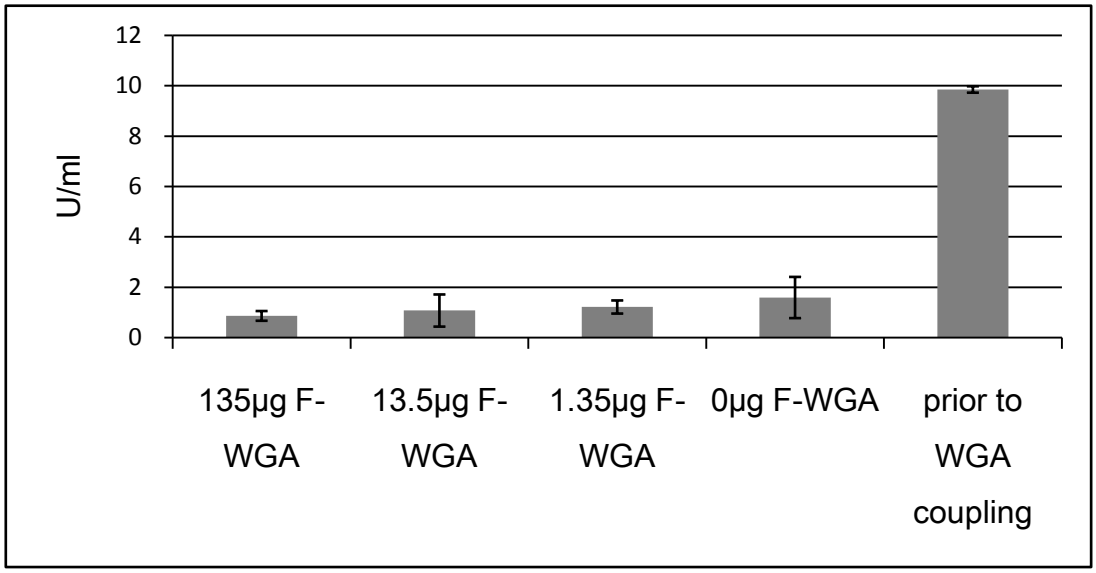


Figure 18: ß-Galactosidase activity in units (U) per ml modified PLGA-microparticle suspension of F-WGA-PLGA-HMD-c-Gal microparticles. PLGA-HMD-c-Gal particles before and after carbodiimide-mediated F-WGA conjugation using four different F-WGA concentrations (135 µg, 13.5 µg, 1.35 µg, 0 µg F-WGA per 625 µl ß-galactosidase and 1.25 ml particle suspension each). (single data see chapter 7 attachment B table 10)

4.5.1.2 Glutaraldehyde Method

As carbodiimide-mediated coupling of F-WGA impaired the activity of the particle-bound enzyme, F-WGA conjugation via glutaraldehyde was assessed. While the particle-bound activity was 8.6 U/ml before coupling, it decreased to 0.6 U/ml after F-WGA coupling (Figure 19). In summary, this method was also not applicable for F-WGA-coupling to the enzyme-grafted microparticles.

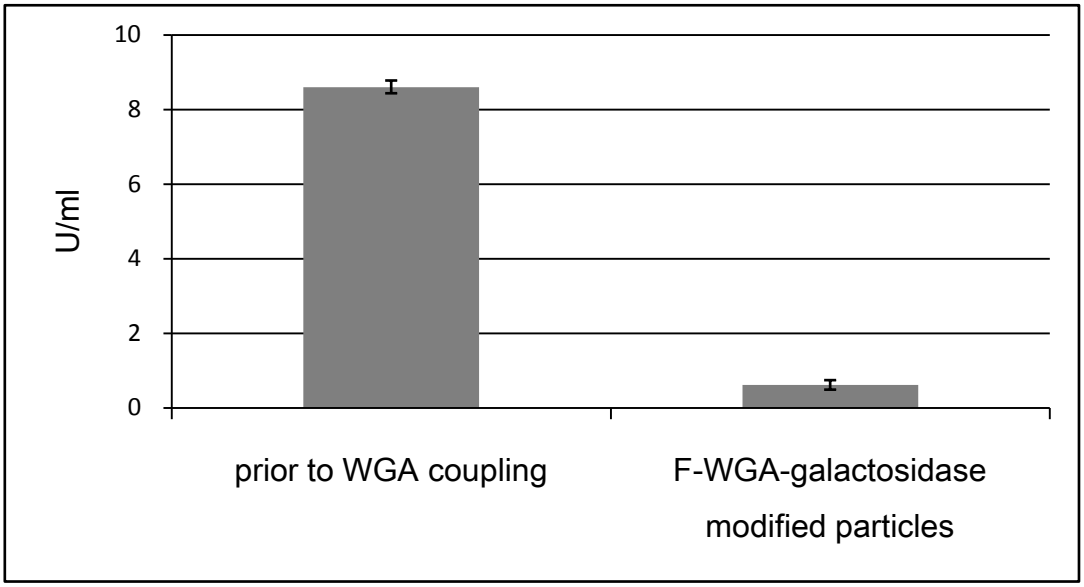


Figure 19: ß-Galactosidase activity in units (U) per ml modified PLGA-microparticle suspension of WGA-PLGA-HMD-c-Gal microparticles. PLGA-HMD-c-Gal particles before and after glutaraldehyde-mediated F-WGA conjugation using 135 µg per 625 µl ß-galactosidase and 1.25 ml particle suspension each (single data see chapter 7 attachment B table 11).

4.5.2 Coupling of β-Galactosidase and F-WGA to HMD-modified Particles

The particle-bound enzyme activity as well as the amount of immobilized targeter was determined and the interaction of the targeted microparticles with Caco-2 cell monolayers was examined (see chapter 6 Attachment A).

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5. Conclusion

Aiming at a new bioadhesive lactase preparation, biodegradable polymeric microparticles were prepared and functionalized with microbial-derived ß-galactosidase and wheat germ agglutinin.

At this, poly (D,L-lactide-co-glycolide) (PLGA) microparticles were manufactured by spray drying. To eliminate aggregates and submicron particles, a method for size fractionation was established, which relies on a combination of subsequent suspension and centrifugation steps. The resulting mean diameter of the particles used for surface modification amounted to 2.78 ± 1.05 µm.

For surface modification, ß-galactosidase from *Escherichia coli* and *Kluyveromyces lactis* were tested. First of all, a colorimetric assay for the quantification of the enzymatic activity was established, which is based on the hydrolytic cleavage of 2-nitrophenyl β-D-galactopyranoside (ONPG) and the detection of the yellow-colored product o-nitrophenolate. A bicinochoninic acid assay was used for protein quantification. Fluorescent-labeled *E. coli* ß-galactosidase was prepared by FITC conjugation. Despite gel filtration, rather high amounts of non-covalently bound FITC remained in the enzyme fractions. An examination of the storage stability of *E. coli* ß-galactosidase in 25mM HEPES/NaOH pH 7.2 buffer revealed full loss of enzymatic activity within two weeks. Moreover, no stabilizing effect of magnesium was detected.

Searching for a more stable alternative, lactase from *K. lactis* was assessed, which has the additional advantage to be generally recognized as safe (GRAS) by the FDA. At this, the commercial preparation Lactozym® was used. To remove any additives that potentially impair the coupling to the particle surface, affinity chromatography with APTG agarose was investigated. However, elution with a concentrated lactose solution was not successful, and elution with alkaline buffers was not applicable due to harmful effects on the enzyme activity. The identification

of an appropriate purification method that combines high recovery rates and conserved enzyme activities will be the topic of future studies.

Thus, untreated Lactozym® solution was applied for coupling to the surface of PLGA-microparticles. Three different spacers, i.e. hexamethylene diamine, 6-aminocaproic acid, and branched polyethylene imine, were examined as well as two different types of coupling techniques, namely carbodiimide and glutaraldehyde chemistry. The highest particle-bound enzyme activities were obtained with hexamethylene diamine amounting to 1470 U per g PLGA and with polyethyleneimine amounting to 970 U per g PLGA, which compares favorably with commercial products. Upon particle storage at 4°C, 50% of the activity was retained after one week, and 25% (HMD) or 20% (PEI) of the initial activity directly after coupling were still preserved after four weeks. Under the same conditions, dilutions of the original Lactozym® solution showed a similar decrease. Thus, future work will focus on the evaluation of appropriate additives to the storage buffer to guarantee long-term stability.

To prepare targeted enzyme-loaded microparticles, F-WGA was first coupled to already enzyme-functionalized particles. However, the resulting enzyme activity was severely impaired. Alternatively, WGA and enzyme were coupled simultaneously to hexamethylene diamine-modified PLGA particles. The resulting microspheres displayed an enzymatic activity of 780 U per g PLGA. Moreover, an in-vitro cell binding assay using Caco-2 cell monolayers showed significantly increased particle-cell interaction of WGA-decorated particles.

The present study demonstrates the feasibility of PLGA microparticles that are surface-modified with active β-galactosidase and with WGA as a functional active targeter to enable an interaction with enterocytes and thereby an enhanced intestinal residence time. On this basis, a more convenient management of lactose intolerance might be possible.

6. Attachment A Manuscript

Author's contributions

I hereby declare to have significantly contributed to the present manuscript. As part of my diploma thesis, I was involved in the experiments, the analysis and interpretation of the data.

TARGETED PLGA-MICROPARTICLES AS A NOVEL AND CONVENIENT STRATEGY TO TREAT LACTOSE INTOLERANCE

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KEYWORDS: Lactose intolerance, β-galactosidase, lactase, PLGA, microparticles, WGA, targeting, Caco-2.

Abstract

Background: Peroral β-galactosidase preparations for the management of lactose intolerance need to be administered in huge amounts (1500 to 6000 Unit) immediately before or together with a lactose-containing meal.

Aim: Therefore, this work was aimed to develop an innovative long-acting formulation based on biodegradable polymeric microcarriers that are functionalized with β-galactosidase and targeted with wheat germ agglutinin (WGA) for bioadhesion and thus prolonged residence time in the small intestine.

Methods: Spray-dried poly (D,L-lactide-co-glycolide) (PLGA) particles with 2.78 ± 1.05 µm in diameter were functionalized with β-galactosidase from *Kluyveromyces lactis* and WGA using different types of spacers (polyethyleneimine, hexamethylene diamine, 6-aminocaproic acid) and coupling methods (carbodiimide, glutaraldehyde). The particle-bound enzyme activity was determined and the interaction with Caco-2 cell monolayers was assessed.

Results: Up to 1470 Units β-galactosidase per gram PLGA were immobilized. The best results were obtained with hexamethylene diamine as a spacer applying the carbodiimide method. At this, a nearly 6-fold increase in enzyme activity was obtained as compared to particles without spacer. Upon targeting with WGA, binding to artificial human intestinal epithelium was increased considerably.

Conclusions: WGA-targeted PLGA microparticles for the delivery of β-galactosidase were prepared, which are promising candidates for a convenient biomimetic treatment regimen of lactose intolerance.

1 INTRODUCTION

Globally, the majority of people continuously lose the ability to produce lactase (lactase-phlorizin hydrolase; LPH; β-galactosidase) after weaning. The prevalence of lactase deficiency coincides with the ethnic background reaching from 2-15% among northern Europeans to nearly 100% among Asians.[1] LPH is located at the brush border of enterocytes[2], where it catalyzes the hydrolytic cleavage of β-galactose-1,4-glucose (lactose) to galactose and glucose. In absence of enzyme, lactose cannot be digested in the small intestine and it reaches the colon, where it increases the osmotic pressure and pulls water into the gut lumen. Moreover, it is fermented by colon bacteria, which may lead to increased production of intestinal gas and potentially toxic metabolites. When lactose ingestion induces clinical symptoms, which are gut-related such as abdominal pain, bowel distension, and flatulence or even systemic like headache, loss of concentration, and muscle pain, lactose maldigestion is referred to as lactose intolerance.[1,3]

Lactose is not only present in milk at 5% (w/v), but also in a broad range of dairy products and processed foods, such as ice cream or even salad sauces and sausages, as well as pharmaceuticals. Milk and whey can be industrially treated with lactase in order to reduce their lactose content.[4-5] Although an increasing variety of such pre-treated low-lactose dairy products is available, it is still often inconvenient for affected persons to avoid the uptake of lactose in amounts that might provoke symptoms of intolerance. Moreover, omitting dairy products has the disadvantage of losing a major source of calcium and proteins. A well-established strategy for the management of lactose

intolerance is peroral lactase substitution with capsules or tablets containing microbial-derived β-galactosidase preparations. They have proven successful in reducing the severity of symptoms caused by lactose uptake in a dose-dependent manner.[6-7] However, they are only effective when administered immediately before or together with the lactose-containing meal.

Therefore, the aim of this work was to develop an innovative long-acting alternative formulation, which allows for a reduced dosing frequency and higher patient compliance. For this purpose, microparticles from the biocompatible, biodegradable and FDA-approved polymer poly (D,L-lactide-co-glycolide) (PLGA) were prepared[8] and functionalized with β-galactosidase and wheat germ agglutinin (WGA). The choice of the microbial source for β-galactosidase mainly depends on the required pH optimum of the enzyme[4] and the pH in the target region. As the physiological pH in the small intestine ranges from slightly acidic conditions to about pH 7.5, the enzyme from *Kluyveromyces lactis* was chosen, which exhibits optimum activity at pH 7.0-7.5.[9] Moreover, it is generally recognized as safe by the FDA.[10] The dietary lectin WGA, which is derived from *Triticum vulgare*, was used as a targeter. It specifically binds to N-acetyl-D-glucosamine and sialic acid, which form part of the gastrointestinal mucus and the glycocalyx of human intestinal epithelial cells.[11-12] WGA-grafted particles are already known to attach to the mucus and the epithelium of the small intestine.[8,13-14] For the work presented, WGA-targeted β-galactosidase-loaded carriers were prepared, which should be retained near the brush border, i.e. in exactly those regions of the gastrointestinal tract where physiological lactase would occur and exerts its action.

2 MATERIALS AND METHODS

2.1 Materials

Resomer RG502H (PLGA, 50:50 lactide/glycolide) was purchased from Boehringer Ingelheim (Ingelheim, Germany). β-Galactosidase from *Kluyveromyces lactis* (Lactozym®; ≥ 3000 U/ml), and glutaraldehyde solution (70% in H_2O) were obtained from Sigma Aldrich (Vienna, Austria). Fluorescein-labeled wheat germ agglutinin (F-WGA, molar ratio fluorescein/protein = 2.9) was bought from Vector Laboratories (Burlingame, USA). Polyethyleneimine (PEI, branched, MW 1200) was purchased from Polysciences (Warrington, USA). All other chemicals were of analytical purity and obtained from Sigma Aldrich.

2.2 Preparation of PLGA microparticles

PLGA microparticles were prepared by spray-drying as previously described[8,14] and fractionated according to their size. Briefly, a 6.5% (w/v) solution of PLGA in methylene chloride was spray-dried with a Mini Spray Dryer B-191 (Buechi Labortechnik AG, Flawil, Switzerland). 100 mg spray-dried particles were suspended in 10 ml distilled water by ultrasonication. Remaining aggregates and larger particles were spun down for 2 min at 400 rpm. To remove submicron particles, the supernatant was further centrifuged for 10 min at 3200 rpm and the pellet was re-suspended in 5 ml distilled water. The PLGA

content was determined gravimetrically after lyophiliziation of an aliquot. For further processing the suspension was centrifuged again (3500 rpm, 10 min, 4 ℃) and the pellet was re-suspended in 20mM HEPES/NaOH pH 7.0 at a concentration of 10 mg PLGA/ml. The mean particle size was determined by measuring the diameter of 200 particles after microscopic inspection using Lucia Gv5.0 software for image evaluation.

2.3 Covalent coupling of β-galactosidase and F-WGA

Following different strategies, the surface of PLGA microparticles was functionalized with β-galactosidase and fluorescent-labeled WGA. Each approach was assessed in triplicate.

2.3.1 Activation of PLGA carboxylate groups via the carbodiimide method

1.25 ml particle suspension (10 mg/ml in 20mM HEPES/NaOH pH 7.0) were activated by addition of 250 µl of a freshly prepared solution of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) (180 mg/ml EDAC and 7.5 mg/ml NHS in 20mM HEPES/NaOH pH 7.0) and end-over-end incubation for 2 h at room temperature. Excess reagents were removed by centrifugation (3500 rpm, 10 min, 4 ℃) followed by re-suspension in 1.875 ml of 15mM lactose/50mM $MgSO_4$/20mM HEPES/NaOH pH 7.4. After a second centrifugation step, the pellet was suspended in 625 µl of fresh buffer.

2.3.2 Modification of the particle surface with different spacers

For the introduction of spacers, 625 µl PEI (10 mg/ml in 1M HEPES/NaOH pH 7.2), 625 µl hexamethylene diamine (HMD; 20 mg/ml in 1M HEPES/NaOH pH 7.2), or 625 µl 6-aminocaproic acid (ACA; 10 mg/ml in 50mM $MgSO_4$/20mM HEPES/NaOH pH 7.2) solution were added to the activated particle suspension (2.3.1) and incubated end-over-end overnight at room temperature. Excess spacer was removed by three washing steps including centrifugation (3500 rpm, 10 min, 4 ℃) and re-suspension in 1.875 ml 20mM HEPES/NaOH pH 7.0. Subsequently, the pellet was suspended in 625 µl of the same buffer.

2.3.3 Coupling of β-galactosidase to the particle surface without a spacer

After addition of 625 µl Lactozym® solution, the activated microparticles (2.3.1) were allowed to react overnight at 4 ℃. Unreacted binding sites were saturated by incubation with 250 µl of 100 mg/ml aqueous glycine solution for 30 min at 4 ℃. Subsequently, the suspension was purified by repeated centrifugation (3500 rpm, 10 min, 4 ℃) and re-suspension in fresh buffer. For the first two washing steps 1.875 ml 15mM lactose/50mM $MgSO_4$/20 mM HEPES/NaOH pH 7.2 were used as a buffer. In order to avoid any interference with the enzyme activity assay, lactose free buffer was applied for the last two washing steps. Finally, the particles were suspended in 1.25 ml of 50mM $MgSO_4$/20mM HEPES/NaOH pH 7.2 and stored at 4 ℃ until use.

2.3.4 Coupling of β-galactosidase to ACA-modified particles via the carbodiimide method

The carboxylate groups of the immobilized 6-aminocaproic acid (2.3.2) were activated using 250 µl of the EDAC/NHS solution as described above (2.3.1). After purification, 625 µl Lactozym® solution were added for conjugation via enzyme amine groups, and the suspension was further treated as mentioned above (2.3.3).

2.3.5 Coupling of β-galactosidase to PEI- and HMD-modified particles via the carbodiimide method

In order to activate enzyme carboxylate groups, 625 µl Lactozym® solution were mixed with 250 µl EDAC/NHS solution (2.3.1) at 4 ℃. After addition to the spacer-grafted microparticles (2.3.2) the mixture was rotated overnight at 4 ℃ to allow for a reaction with the amine groups of the spacer. Finally, excess binding sites were blocked, and the suspension was purified (see 2.3.3).

2.3.6 Coupling of β-galactosidase to PEI- and HMD-modified particles via glutaraldehyde

12.5 µl 70% aqueous glutaraldehyde were added to PEI- and HMD-coupled particles (2.3.2) and incubated for 5 h. After removal of excess reagent as described above (2.3.1) 625 µl Lactozym® solution were added to the suspension and the mixture was kept overnight at 4 ℃ on a rotator, saturated with glycine and purified as above (2.3.5).

2.3.7 Coupling of β-galactosidase and F-WGA to HMD-modified particles

Different amounts of Lactozym® (625 µl; 312.5 µl) were mixed with 27 µl F-WGA (5 mg/ml), adjusted to 652 µl with 20mM HEPES/NaOH pH 7.2 and combined with 250 µl EDAC/NHS solution (2.3.1). This solution was added immediately to HMD-grafted PLGA microparticles (2.3.2) and incubated end-over-end at 4 ℃ overnight. Subsequently, remaining binding sites were blocked and the suspension was purified extensively (2.3.5). Reference samples were prepared accordingly but omitting WGA.

2.4 Activity assay of β-galactosidase

The β-galactosidase activity was determined by a modified assay based on the enzymatic cleavage of 2-nitrophenyl β-D-galactopyranoside (ONPG) and the colorimetric detection of the resulting yellow o-nitrophenolate.[15] Each well of a 96-well plate was filled with 5 µl of a solution containing β-galactosidase and 50 µl working buffer (5 mM $MgSO_4$/20mM HEPES/NaOH pH 7.2). Upon addition of 100 µl ONPG solution (14mM in distilled water) the mixture was incubated for 2 h at 37 ℃. Finally, 50 µl stop buffer (250mM HEPES/NaOH pH 9) were added and the absorption was read at 410 nm. Particle-bound enzyme activity was determined by end-over-end incubation of 200 µl particle suspension, 20 µl working buffer, and 400 µl ONPG solution (14mM in distilled water) in a test tube for 2 h at 37 ℃. After addition of 200 µl stop buffer, the suspension was centrifuged (5 min, 10000 rpm, 4 ℃), 205 µl of the supernatant were transferred into a 96-well plate and the absorption was determined at 410 nm. Each time, freshly prepared

dilutions of β-galactosidase in working buffer were measured in parallel, and the resulting calibration curve was used for calculation. The enzymatic activity of the particle suspension was determined immediately and after storage at 4 ℃ for 1, 2, 7, 14, and 28 days. For comparison, the activity of β-galactosidase (1 - 20 U/ml) kept in the same buffer at 4 ℃ was determined at the same intervals.

2.5 Quantification of particle-bound F-WGA

Aliquots (35 µl) of the F-WGA modified particle suspensions were hydrolyzed with 315 µl 0.2M NaOH. In a microplate, a triplicate of 100 µl/well was analyzed at 485/525 nm and the amount of immobilized F-WGA was determined using a calibration curve established with soluble F-WGA.

2.6. Binding of surface-modified microparticles to Caco-2 cells

160 µl/well cell suspension each containing 17 000 Caco-2 cells (DSMZ, Braunschweig, Germany) were seeded into a 96-well microplate. After 7 days, the confluent monolayers were incubated with 100 µl of a suspension containing either HMD/β-galactosidase-modified particles or HMD/β-galactosidase/F-WGA-functionalized carriers in 20mM isotonic HEPES/NaOH pH 7.4 for 1 h at room temperature under moderate shaking. Subsequently, the cells were washed five times with 200 µl of 20mM isotonic HEPES/NaOH pH 7.4 each, followed by addition of 50 µl of the same buffer to

each well. To assess the optical density of the cell layer with associated particles, the absorption at 600 nm was determined using a microplate reader (Infinite 200, Tecan Group Ltd., Grödig, Austria). Finally, these cells were imaged using light microscopy.

3 RESULTS AND DISCUSSION

3.1 Preparation of PLGA microparticles

After size fractionation the mean diameter of the spray-dried PLGA microparticles amounted to 2.78 ± 1.05 µm. This seems to be a reasonable particle size range for the required application: small enough to offer a large total surface area for high enzyme loading, and big enough to remain in the intestinal lumen and avoid cellular uptake. As to Desai et al., submicron particles are absorbed to a much higher extent than carriers in the micrometer range.[16] For these reasons, significantly larger and smaller particles were removed in advance. Another advantage of administering microparticles is their short gastric residence time. Particles below 3 mm leave the stomach together with the fluid stream without remarkable retention time. Therefore, they are exposed to the acidic gastric juice for only a very short time.

3.2 Immobilization of β-galactosidase using different spacers

In order to guarantee the interaction between the enzyme and its substrate, β-galactosidase was not incorporated into the particle matrix, but immobilized at the particle surface. At this, the enzyme was coupled to PLGA carboxylate groups either directly or via different spacers (Figure 1). For enzyme immobilization at the particle surface without any spacer, the PLGA carboxylates were activated with carbodiimide and

allowed to react with the primary amine groups of β-galactosidase. Thereby, a particle-bound enzyme activity of 2.5 U per ml particle suspension was obtained (PLGA-*c*-Gal). However, these results were outperformed up to 6-fold when using a spacer: The linear spacer hexamethylene diamine (PLGA-HMD-*c*-Gal) increased the particle-bound activity nearly 6-fold (14.7 U/ml), while the increase was 4-fold (9.7 U/ml) in case of PEI (PLGA-PEI-*c*-Gal). Only 6-aminocaproic acid (PLGA-ACA-*c*-Gal) yielded a lower activity than without spacer. Besides different spacers, two different chemical procedures for the immobilization of β-galactosidase involving different sites of the enzyme were applied. While the highest activities were found when the enzyme was coupled via its carboxylate group to the amine groups of surface-immobilized HMD or PEI using the carbodiimide method, there was no benefit in using a spacer when the enzyme was conjugated via its amine groups. Neither coupling of β-galactosidase to the carboxylate groups of ACA-modified particles via the carbodiimide method (PLGA-ACA-*c*-Gal), nor conjugation to the amine groups of HMD (PLGA-HMD-*g*-Gal) or PEI (PLGA-PEI-*g*-Gal) applying glutaraldehyde was successful. Thus, either the carboxylate groups of β-galactosidase seem to be better accessible for conjugation than its amine groups or the activity of the enzyme might be affected to a lesser extent by the coupling procedure.

Interestingly, the activity of β-galactosidase coupled PEI-modified microparticles (PLGA-PEI-*c*-Gal) was lower than that of HMD-particles (PLGA-HMD-*c*-Gal), although branched polyethyleneimine offers multiple primary amine groups per molecule whereas hexamethylene diamine provides only one free amine group for coupling. Thus, the lower

activity in case of PEI might not be due to a smaller amount of coupled β-galactosidase, but to a harmful microenvironment that restricts the activity of the immobilized enzyme. The pH optimum of β-galactosidase from *Kluyveromyces lactis* is in the neutral range, but due to its numerous amine groups PEI might create an alkaline microenvironment thus constraining the enzyme activity.[9]

The stability of the carrier-bound β-galactosidase was examined by enzyme assays after storage at 4 ℃ for defined time intervals. For PEI- (PLGA-PEI-*c*-Gal) and HMD- (PLGA-HMD-*c*-Gal) modified particles, the activity decreased by about a third after one day. After one week, about half of the initial activity was retained, and after 4 weeks, it decreased to one fifth (PEI) or one quarter (HMD) of the initial values. Concerning storage stability, most favourable results were obtained without any spacer (PLGA-*c*-Gal). For comparison, the original β-galactosidase solution (Lactozym®) was diluted in the same buffer that had been used for storage of the particles. Thereby, the activity of the dilutions decreased by a similar extent as observed for the particle suspensions (data not shown).

To elicit a therapeutic effect, high amounts of active β-galactosidase are necessary. Single doses of lactase usually contain between 1500 and 6000 U.[6] The highest activity obtained in the presented study was 14.7 U/ml for a suspension that contained 10 mg/ml PLGA particles. This corresponds to 1470 U/g polymer, which compares favorably with commercial products.

3.3 Immobilization of β-galactosidase and F-WGA

For targeted delivery to the small intestine, wheat germ agglutinin was used which offers the advantage to lose its carbohydrate-binding capacity in acidic environment.[12] Therefore, the gastric passage of the particles will be unhampered, and they can rapidly attain the small intestine, where WGA binds to the mucus and the glycocalyx of enterocytes. Furthermore, it is most unlikely that WGA interacts with nutritional components. In preliminary assays, WGA was conjugated to β-galactosidase grafted microparticles (PLGA-HMD-*c*-Gal). However, the resulting particles exerted negligible enzymatic activity. As an alternative approach, both fluorescent labeled WGA and β-galactosidase were activated concurrently and conjugated to HMD-grafted PLGA microcarriers. Fluorimetric analysis revealed that 0.5765 ± 0.1040 µg/ml F-WGA were immobilized together with β-galactosidase. Reducing the amount of β-galactosidase for coupling to 50% doubled the lectin-coupling efficiency yielding 1.1607 ± 0.1634 µg/ml F-WGA. Whereas application of half of the enzyme resulted in half enzyme immobilisation efficiency, there were only slight differences between the enzyme activities in presence and absence of WGA. In addition, there was no negative influence of WGA on the storage stability of the enzyme.

Furthermore, it is most likely that the proteins were not only coupled to the spacer, but also crosslinked. As long as their functionality is not impaired by chemical modification of the binding site or by steric hindrance, this represents no drawback. Lactose is a small molecule, which can diffuse to the active sites of β-galactosidase even if the enzyme is

not antenarry exposed at the microparticle. By contrast, the WGA coupling position is essential for targeting, because it has to interact with the corresponding carbohydrate moieties of the mucus or the glycocalyx. While fluorimetry allowed to quantify the amount of immobilized fluorescent-labeled WGA, its effectiveness as a targeter was assessed by cell interaction studies.

3.4 Cytoadhesion

To study the cytoadhesive properties of the modified particles, Caco-2 cells were used, which represent a recognized model for the human intestinal epithelium. Caco-2 monolayers were incubated with carriers that were either decorated with β-galactosidase alone or with the enzyme and WGA. After removing unbound particles by repeated washings, the optical density of the monolayers was determined at 600 nm. While plain monolayers yielded an OD_{600} of 0.0998 ± 0.0006, enzyme-functionalized particles enhanced the OD_{600} by 0.0424 ± 0.0097. In case of WGA-functionalized particles the increase amounted to 0.0791 ± 0.0127. The almost doubled OD_{600} values point to a considerable increase of cell-bound particles in presence of WGA. This observation of significantly higher binding of WGA-grafted particles to epithelial cells as compared to non-targeted particles was also supported by microscopic images (Figure 3).

4 CONCLUSION AND OUTLOOK

β-Galactosidase was covalently conjugated to the surface of biodegradable polymeric microparticles with a mean diameter of 2.78 ± 1.05 µm. As highest particle-bound enzymatic activities were observed when using hexamethylene diamine (1470 U/g PLGA) and polyethyleneimine (970 U/g PLGA) as a spacer, it might be concluded that immobilization via enzyme carboxylate groups is preferable. After storage for one week at 4 °C, about half of the activity was retained, and after four weeks, the beads yielded still one quarter (HMD) or one fifth (PEI) of the original activity. However, marketed products tested under the same conditions revealed similar results. Thus, improved storage stability might be achieved by addition of appropriate stabilizers. Immobilisation of both β-galactosidase and WGA significantly enhanced particle binding to Caco-2 cell monolayers, which represent a model for the human intestinal epithelium.

To conclude, the presented drug delivery system is a promising approach towards a more convenient management of lactose intolerance, since it mimics the physiological situation in healthy individuals. Similar to the natural localization of LPH at the brush border of enterocytes, WGA might mediate binding of the enzyme preparation to the mucus or the glycocalyx of the enterocytes, which should significantly prolong the residence time in the intestine. Ongoing from the presented data this biomimetic regimen merits further examination by *in vivo* studies.

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FIGURE LEGENDS

Figure 1.

β-Galactosidase activity in units (U) per ml modified PLGA microparticle suspension. The particles were grafted with (PEI, HMD, ACA) or without spacer, β-galactosidase was immobilized via the carbodiimide method (c) and the glutaraldehyde (g). (n=3, mean ± SD)

Figure 2.

β-Galactosidase activity in units (U) per ml modified PLGA microparticle suspension. The particles were coupled with HMD as a spacer and β-galactosidase at two different concentrations was immobilized in presence or absence of F-WGA. (n=3, mean ± SD)

Figure 3.

Caco-2 cell monolayers incubated with β-galactosidase- (A) and β-galactosidase/WGA- (B) functionalised PLGA microparticles.

FIGURES

Figure 1

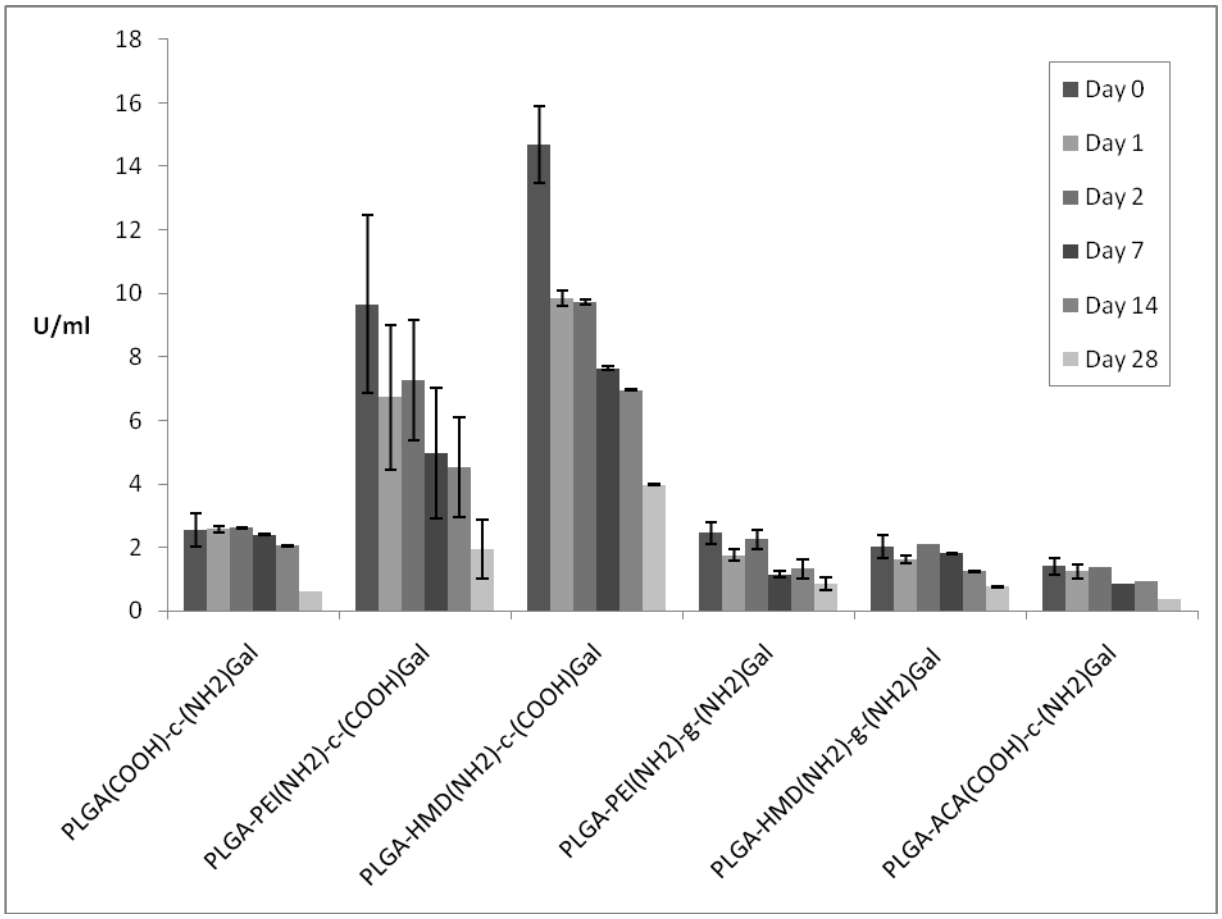


Figure 2

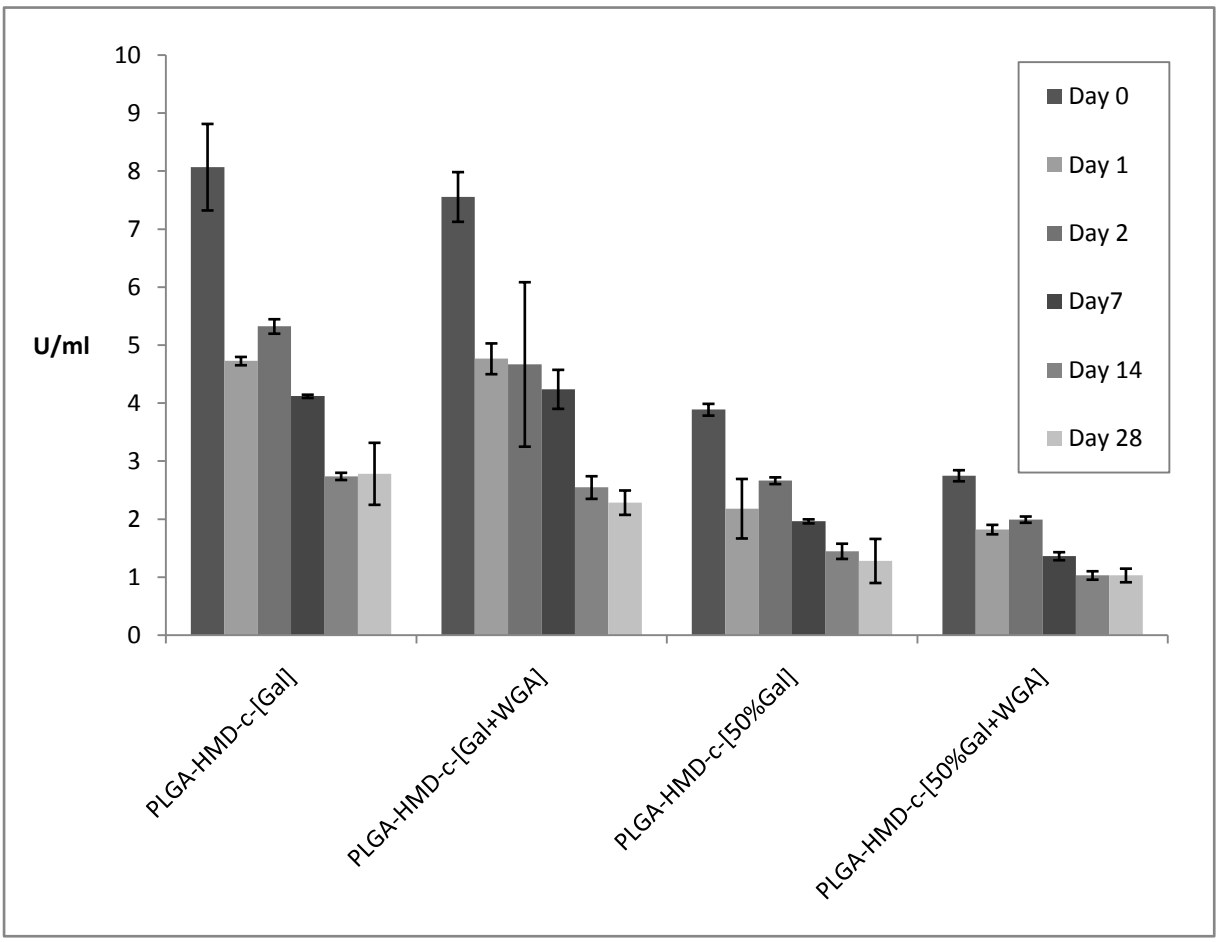
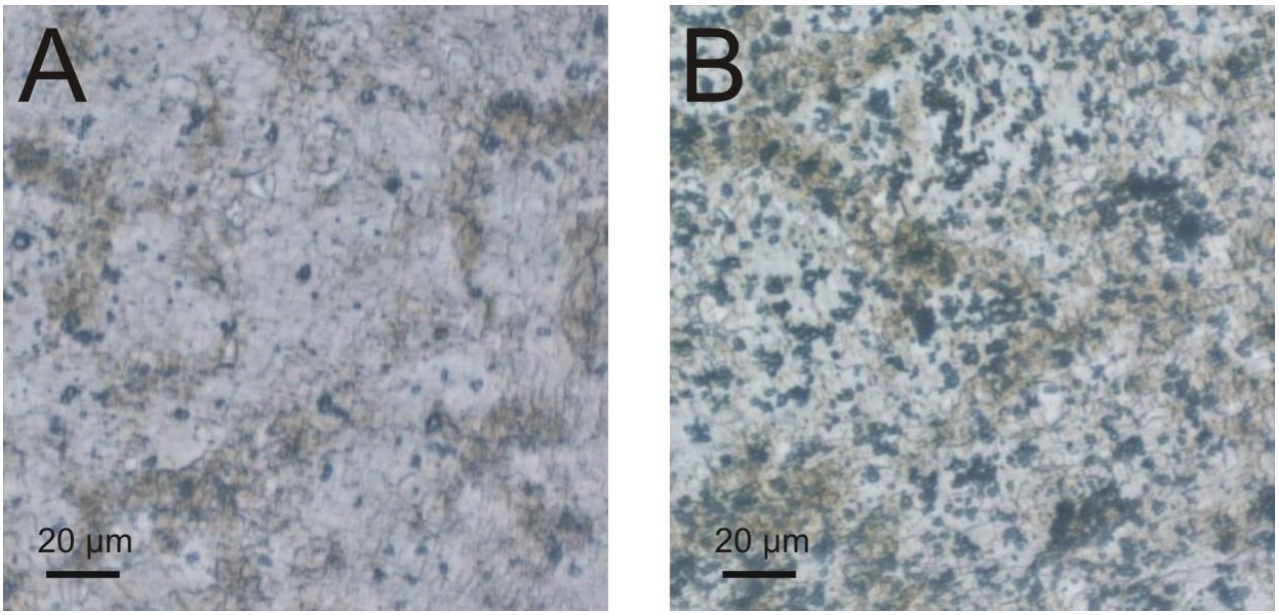

U/ml
10
9
8
7
6
5
4
3
2
1
0
Day 0
Day 1
Day 2
Day7
Day 14
Day 28
PLGA-HMD-c-[Gal]
PLGA-HMD-c-[Gal+WGA]
PLGA-HMD-c-[50%Gal]
PLGA-HMD-c-[50%Gal+WGA]

Figure 3



7. Attachment B

B 1. ß-Galactosidase from *E. coli*

B 1.1 Enzymatic Assay

Table 1: Calibration curve at 410 nm after30 min incubation.

Enzyme concentration (µg/ml)	Absorption Units
5	1.0295
2.5	0.5061
1	0.1857
0.5	0.0987
0	0.0000

Figure 20: Calibration graph at 410nm upon with 30 min incubation.

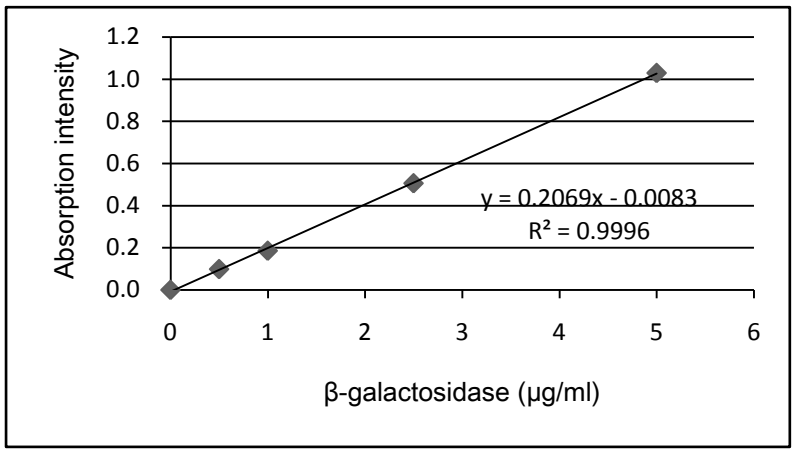


Table 2: Calibration curve at 410 nm after 30 min and 60min incubation.

Enzyme concentration (µg/ml)	**Absorption units (30 min)**	**Absorption units (60 min)**
5	1.0295	2.2415
2.5	0.5061	1.1205
1	0.1857	0.4424
0.5	0.0987	0.2068
0	0.0000	0.0000

B 1.2 Storage Stability

Table 3: Stability of ß-galactosidase in presence and absence of magnesium as indicated by the enzyme activity (U/ml).

Storage time		**ß-Galactosidase concentration (µg/ml)**		
		10µg/ml	**5µg/ml**	**1µg/ml**
First day	**with Mg**	9.69	5.05	1.55
	without Mg	10.2	5.04	1.68
7 day	**with Mg**	8.84	3.98	1.01
	without Mg	8.94	3.96	1.05
14 day	**with Mg**	0.16	0.19	0.18
	without Mg	0.19	0.16	0.14

B 1.3 Protein Quantification

Table 4: Calibration of the Micro BCA™ Assay using BSA (bovine serum albumin) and ß-galactosidase from *E. coli* (562nm).

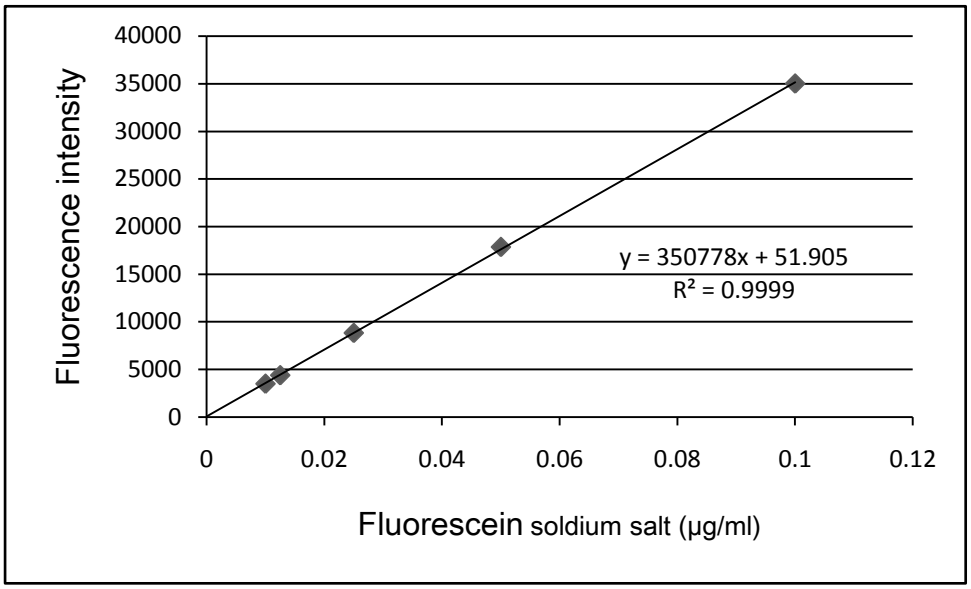
Protein concentration (µg/ml)	**Absorption units (*E.coli*)**	**Absorption units (BSA)**
40	0.2195	0.4656
20	0.1085	0.1997
10	0.0550	0.0734
5	0.0231	0.0394
2.5	0.0121	0.0103
0	0.0000	0.0000

B 1.4 Characterization of FITC-labeled ß-Galactosidase

Table 5: Calibration of fluorescein-Na diluted in 25mM HEPES/NaOH pH 7.2 at 485 nm/525 nm.

Fluorescein-Na (µg/ml)	**Fluorescence intensity**
0.1	35005.0
0.05	17856.3
0.025	8834.6
0.0125	4393.0
0.01	3501.3
0	0.0

Figure 21: Calibration graph of fluorescein-Na in 25mM HEPES/NaOH pH 7.2 at 485 nm/525 nm.



B 2 β-Galactosidase from *Kluyveromyces lactis* (Lactozym®)

B 2.1 Enzymatic Assay

Table 6: Calibration of ß-Galactosidase upon incubation for 120 min and reading at 410nm.

ß-Galactosidase (µg/ml)	Absorption units
24	1.0180
12	0.3105
6	0.0984
3	0.0327
1.5	0.0111
0.6	0.0051
0.3	0.0019
0	0.0000

Figure 22: Calibration graph of ß-Galactosidase upon incubation for 120 min incubation and readout at 410nm.

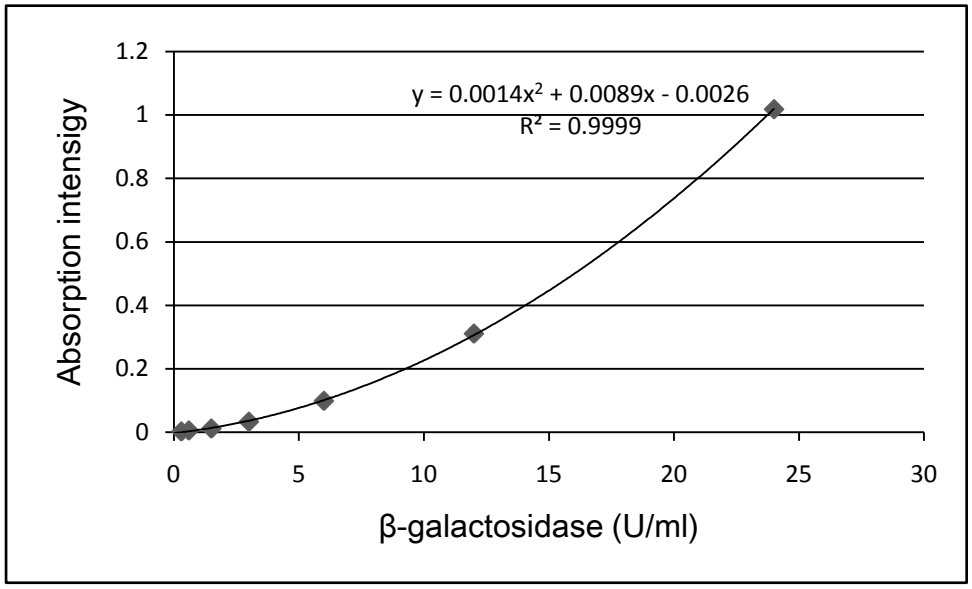


Table 7: Evaluation of a possible impact of PLGA-microparticles on the enzymatic assay.

	Absorption units at 410nm	
Concentration of particle suspension	**60 min incubation**	**120 min incubation**
4mg/ml	0.0911±0.0068	0.0693±0.0077
2mg/ml	0.0709±0.0035	0.0685±0.0037
1mg/ml	0.0679±0.0084	0.0754±0.0003
Blank	0.0803±0.0097	0.0667±0.0102

B 2.2 Storage Stability

Table 8: Stability of β-galactosidase Lactozym®.

β-galactosidase Lactozym® **(U/ml)**	**Day0**	**Day1**	**Day2**	**Day7**	**Day14**	**Day28**
20	20.49	13.58	13.69	14.65	10.36	10.15
15	16.27	10.55	10.47	10.91	7.82	7.69
10	10.99	6.98	6.80	7.10	4.81	4.72
5	6.01	3.51	3.40	3.43	2.16	2.10
2	2.82	1.43	1.39	1.42	1.00	0.89
1	1.53	0.63	0.55	0.69	0.65	0.46

B 3 Coupling of ß-Galactosidase to PLGA-Microparticles

Table 9: Influence of coupling reagent and incubation time on enzyme activity (U/ml).

	U/ml particle suspension	**standard deviation**
100% EDAC/NHS overnight	12.17	0.23
100% EDAC/NHS 2 hours	4.13	1.33
10% EDAC/NHS overnight	9.07	1.28
1% EDAC/NHS overnight	1.30	0.15

B 4 Coupling of F-WGA and ß-Galactosidase to PLGA-Microparticles

Table 10: Carbodiimide method – influence of WGA on enzyme activity (U/ml).

	U/ml particle suspension	**Standard deviation**
100% F-WGA	0.86	0.19
10% F-WGA	1.08	0.64
1% F-WGA	1.22	0.26
0% F-WGA	1.59	0.82
blank	9.85	0.13

Table 11: Glutaraldehyde method – influence of WGA on enzyme activity (U/ml).

	U/ml particle suspension	**Standard deviation**
Blank	8.6051	0.1709
F-WGA-galactosidase modified particles	0.6155	0.1274

8. German Abstract - Zusammenfassung

Ausgangspunkt: Perorale β-Galactosidase-Zubereitungen zur Behandlung der Laktoseintoleranz müssen in hohen Dosen (1500 bis 6000 Unit) unmittelbar vor oder gemeinsam mit Laktose-haltiger Nahrung verabreicht werden.

Ziel: Das Ziel der vorliegenden Arbeit war daher die Entwicklung einer innovativen Formulierung mit länger anhaltender Wirkung basierend auf bioabbaubaren Polymer-Mikropartikeln, die mit β-Galactosidase und mit Weizenkeimagglutinin (WGA) als bioadhäsivem Targeter gekoppelt werden, um eine längere Verweilzeit im Darm zu erreichen.

Methoden: Sprühgetrocknete Poly(D,L-lactid-co-glycolid) (PLGA) Partikel mit einem Durchmesser von 2,78 ± 1,05 µm wurden mithilfe verschiedender Spacer (Polyethylenimin, Hexamethylendiamin, 6-Aminocapronsäure) und verschiedener Kopplungsmethoden (Carbodiimid, Glutaraldehyd) mit β-Galactosidase von *Kluyveromyces lactis* und WGA funktionalisiert. Die partikelgebundene Enzymaktivität sowie die Interaktion mit Caco-2-Zell-Monolayern wurde untersucht.

Ergebnisse: Es wurden bis zu 1470 Unit β-Galactosidase pro Gramm PLGA immobilisiert. Die besten Ergebnisse wurden unter Verwendung von Hexamethylendiamin als Spacer mit der Carbodiimidmethode erreicht. Dabei wurde beinahe sechsmal so viel aktives Enzym gekoppelt wie in Abwesenheit eines Spacers. Durch das immobilisierte WGA wurde die Bindung an künstliches humanes Darmepithel deutlich erhöht.

Zusammenfassung: Als vielversprechender neuer Ansatz für eine patientenfreundliche biomimetische Behandlung der Lactoseintoleranz wurden WGA-gekoppelte β-Galactosidase-beladene PLGA-Mikropartikel erfolgreich hergestellt.

9. Curriculum vitae

Personal Dates:

Name: WANG, Xue Yan

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School:

09/1997 - 07/2003	Hui Wen High School, Peking
11/1992 - 07/1997	Fang Cao Di Primary School, Peking
09/1991 - 11/1992	No.4 Jin Song Primary School, Peking

Education:

03/2008 - 02/2009	Research for the diploma thesis at the Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Austria (Supervisor: Univ.-Prof. Mag. Dr. Franz Gabor)
02/2004 - 09/2009	Studies of Pharmacy, University of Vienna, Austria
09/2003 - 02/2004	Studies of Pharmacy, School of Pharmacy, Beijing University, China

Practice:

08/2007 Practice student in the Food Bio-safety Laboratory of the Institute of Food Safety of Chinese Academy of Inspection and Quarantine (CAIQ)

07/2007 Internship student in the Department of Scientific and Regulatory Affairs of Shenzhen Sanofi Pasteur Biological Products Co., Ltd. Beijing Branch

Awards:

(1) National Chemistry Competition of Senior High Schools 2002
(Provincial/Municipal Matching Region)
The second grade award, awarded by China Chemistry Society

(2) National Chemistry Competition of Senior High Schools 2002
(Selective Matching)
The first grade award, awarded by Peking Chemistry Society

(3) The fifth Peking Competition of Application of Acknowledge of Mathematics of Senior High Schools
The first grade award, awarded by Organizing Commission of Peking Competition of Application of Acknowledge of Mathematics of Senior High Schools

(4) The fifth Peking Competition of Application of Acknowledge of Mathematics of Senior High Schools
The first grade award for thesis on the application of mathematic acknowledge, awarded by Organizing Commission of Peking Competition of Application of Acknowledge of Mathematics of Senior High Schools