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Colon-specific Delivery of Bioactive Agents using Genipin-crosslinked Chitosan Coated Microcontainers

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Lactobacillus rhamnosus GG

Abstract

Oral administration of probiotics is beneficial for restoring the intestinal microbial balance and for the treatment of gastrointestinal (GI) tract-related disorders. In the current era characterized by the development of next-generation probiotic microorganisms, which are typically less robust towards

environmental challenges than the classically applied lactic acid producing probiotics, we anticipate a need for delivery of live organisms directly to the site where they need to colonize. Here, we thus present, for the first time, a proof of concept for using micrometer-sized polymeric containers, for the engineered delivery of probiotics, using spray dried *Lactobacillus rhamnosus* GG (LGG), as a model probiotic microorganism. To achieve colon-specific delivery, microcontainers are loaded with LGG and sealed with an enzyme-sensitive coating. A genipin-crosslinked chitosan coating is developed that: (i) is stable at gastric and intestinal pH; (ii) has tunable swelling; and (iii) is degradable by the colon-specific bacterial enzymes. The chitosan–genipin coated microcontainers are evaluated *in vitro*, *ex vivo* as well as *in vivo* in a rat model. Our results confirm that the genipin-crosslinked chitosan coating enables an effective local delivery in the cecum and colon without any premature release in the small intestine. Our findings suggest that the integration of modified polysaccharides with ingestible microdevices has great potential for controlled and site-specific delivery of live microorganisms.

1. Introduction

The colon of a healthy human contains hundreds of distinct species of bacteria, considerably more than other parts of the body^{1,2}. Disturbance of the healthy microbiota is associated with chronic illnesses such as inflammatory bowel diseases, in which case the administration of probiotics can be beneficial to eliminate the symptoms or even cure the patient^{3,4}. Therefore, encapsulation and targeted delivery of probiotic bacterial strains to the colon have been increasingly studied over the last few decades⁵. However, the delivery systems designed for this purpose face many challenges such as early release of bacteria while passing through the stomach

and the small intestine, which may reduce viability of bacteria that are sensitive to the conditions in the upper part of the digestive tract⁶. Research into next-generation probiotics is focused on putative applications on a number of alternative microorganisms, many of which are more vulnerable to the environment of the upper GI tract and can benefit from direct delivery to the colon⁷⁻¹⁰.

Microfabricated devices render a new approach for delivering active compounds to specific sites in the GI tract¹¹⁻¹³ and have great potential for colon-specific oral delivery of next-generation probiotic organisms¹⁴. Microcontainers, i.e. polymeric devices for oral drug delivery, are designed and fabricated as a reservoir with an opening on one side, to hold and carry a compound to the target site of release^{15,16}. Previous studies have shown that microcontainers can facilitate protection of loaded contents through the various local environments of the GI tract^{17,18}, controlled release of pharmaceuticals^{19,20} and their tunable properties can increase the intestinal retention of a drug formulation^{21,22}. After fabrication, the microcontainers are loaded with a compound followed by sealing with a polymeric lid²³. The polymeric lid coating enables further protection of the loaded cargo, modulates the controlled release of the loaded compound, as well as the bioadhesion of microcontainers *in vivo*^{24,25}.

Colon-specific delivery systems are often designed based on stimuli-responsive polymers, sensitive to the physiological characteristics of the colon²⁶. The distinguishable features of the colon in comparison with other sections of the gastrointestinal (GI) tract include: (i) pH (5.5-7.8 in the small intestine and 5-8 in the colon of healthy fasted humans)²⁷, (ii) transit time (3-4 h in the small intestine, whereas 24-48 h in the colon)²⁸ and (iii) colon-specific bacterial strains responsible for a high enzymatic activity²⁹. Reportedly, transit time and pH of the different sections of the GI tract are highly variable between individuals³⁰ and this variability is even more pronounced in

patients with GI disorders as e.g. ulcerative colitis³¹. Therefore, enzyme-sensitive delivery systems can be more reliable for the development of targeted delivery strategies compared to those triggered by time or pH.

Polysaccharides are mostly digested and fermented by glycosidase enzymes produced by the bacterial genera *Bifidobacterium* and *Bacteroides* residing in the colon³². Therefore, these biopolymers have great potential for the development of colon-specific delivery systems, that enable localized release of e.g. probiotics^{33–36}. However, as polysaccharides are mostly water-soluble and sensitive to pH variations throughout the GI tract, they need to be modified to prevent an early release before reaching the colon^{37,38}. Chitosan, has been studied for encapsulation and controlled release of probiotics due to its biodegradability in the colon and favorable mucoadhesive properties^{39–41}. However, chitosan is highly charged in the acidic environment of the stomach and semi-charged in the intestines, due to the amine functional groups on the backbone of the polymer chain⁴². Therefore, when incorporated into a delivery system, the transported cargo can be released in the fore-colon GI sections. In this regard, it has previously been shown that the release rate of encapsulated compounds can be controlled by crosslinking chitosan using genipin, a natural, biocompatible and non-toxic crosslinker^{43–45}. Although genipin has not been approved for commercial use in food or pharmaceutical industries by the U.S. Food and Drug Administration (FDA) or the European Food Safety Authority (EFSA), research studies have shown no significant signs of genotoxic effects e.g. DNA damage *in vivo*⁴⁶.

Figure 1. Illustrated concept of the study, comprising of (A) loading probiotics (pink) into microcontainers and deposition of a chitosan–genipin coating (green) and (B) in vivo evaluation of the colon-targeted delivery system in rats triggered by bacterial enzymes (blue).

In this study, we have developed and optimized a micro-sized delivery system for the inoculation of live microorganisms locally into the colon. The well-studied probiotic bacterial strain *Lactobacillus rhamnosus* GG (LGG)⁴⁷, chosen as a model microorganism due to its robustness and ease of handling, was spray dried and loaded inside microcontainers. Thereafter, the loaded microcontainers were sealed with genipin-crosslinked chitosan to ensure an enzyme-sensitive release in the lower GI tract (Figure 1-A). The delivery system was designed to sustain the release of spray dried LGG through the pH variations of the stomach and small intestine and facilitate

local delivery to the colon through biodegradation of the designed polymeric coating (Figure 1-B). The performance of the colon-targeted delivery system was evaluated *in vitro*, *ex vivo* and *in vivo*.

2. Materials and Methods

2.1. Materials

Chitosan (low molecular weight, degree of deacetylation of 76 % and viscosity (1 % concentration in 1 % acetic acid) of 87 CPS, specified by the supplier), skim milk powder, D-(+)-trehalose dihydrate (≥99.0%), phosphate buffer saline (PBS), acetic acid (≥99 %) and dibutyl sebacate were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Genipin (≥98 %) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and chitosanase derived from *Streptomyces* species was obtained from Merk Millipore, Calbiochem (Burlington, MA, USA). ø100 mm single-side (thickness=525 µm) polished silicon (Si) wafers were from Topsil Global Wafers A/S (Frederikssund, Denmark) and SU-8 2035, 2075 and SU-8 developer were purchased from micro resist technology GmbH (Berlin, Germany). Sylgard® 184 polydimethylsiloxane (PDMS) elastomer was obtained from Dow Corning (Midland, MI, USA) and Eudragit® L100 (EL-100) was supplied by Evonik (Essen, Germany). Ultrapure water was obtained using a water filtration unit from Millipore Corporation (Billerica, MA, USA), whereas Man/Rogosa/Sharp (MRS) agar plates and MRS bouillon were acquired from SSI Diagnostica (Hillerød, Denmark). Gelatin capsules, size 9 were purchased from Torpac® (Fairfield, NJ, USA).

2.2. Characterization of genipin-crosslinked chitosan

A µ-Diss profiler (pION INC, Woburn, MA, USA) was used to characterize the UV-visible spectra of chitosan (0.5 % w/v in 1 mM acetic acid), genipin (0.5 % w/v in PBS) and monitor

genipin-crosslinking of chitosan in real time for 24 h. Experiments were performed at room temperature in triplicates, using a UV probe with a 20 mm path length to monitor a wavelength range between 200 to 700 nm.

A QSense Analyzer (Biolin Scientific, Stockholm, Sweden) with a silica sensor was utilized to perform the quartz crystal microbalance with dissipation monitoring (QCM-D) experiments in flow. To prepare the chitosan–genipin single layer, 0.45 % w/v chitosan solution in 1 mM acetic acid containing 2 mM genipin was homogenized using a vortex mixer. The sensor was coated with chitosan–genipin using a WS-650-23B Spin Coater (Laurell Technologies, North Wales, PA, USA) with a speed of 3,000 rpm for 15 min. The coated sensor was kept in a chamber with 80 % relative humidity (RH) for 16-18 h for completion of the crosslinking process⁴⁸.

The investigation of pH-sensitivity of genipin-crosslinked chitosan was conducted in two different media. Initially, the chambers were filled with 1 M acetic acid (pH=2.5) for 30 min, followed by PBS (pH=7.4) for 100-120 min. Lastly, the chambers were filled again with 1 M acetic acid. Frequency shifts (Δf) and shifts in dissipation (ΔD) were monitored and recorded for all overtones and plotted for the 3rd, 5th and 7th overtones as a function of time. All measurements were performed with a 100 μ L/min flow rate at room temperature.

2.3. Culture and spray drying of LGG

Lactobacillus rhamnosus GG (ATCC 53103) was cultivated on MRS agar plates and incubated at 37°C under anaerobic conditions. In order to identify the introduced LGG strain in the non-sterilized contents of the GI tract in the performed *ex vivo* and *in vivo* experiments, a rifampicin-resistant LGG mutant was used. The spontaneous rifampicin mutation was selected by streaking the wild type LGG, in high concentrations, onto MRS agar supplemented with rifampicin (50

µg/mL). Resistant colonies were checked by a rifampicin gradient, ranging from 50 to 100 µg/mL. The selected rifampicin mutant grew on MRS with the same growth rate as the wild type LGG.

A BÜCHI mini spray dryer B-290 (BÜCHI Labortechnik AG, Flawil, Switzerland) was used for spray drying the LGG cultures using a 0.7 mm nozzle. The LGG cultures were centrifuged at 5,000 rpm for 10 min. Prior to spray drying, the obtained bacterial pellets were re-suspended in an aqueous solution containing 10 % v/v of D-(+)-trehalose dihydrate and 10 % v/v of skim milk powder. The prepared suspension was then spray dried at 4.5 mL/min using an inlet temperature of 100°C and an outlet temperature of 60°C, with 100 % aspirator and a 40 % air flow.

The survival of LGG during the spray drying process was calculated based on the colony-forming units (CFU)/mL of the bacteria before and after spray drying. 100 µL of samples were serially diluted and plated on MRS agar plates (for wild type LGG) and MRS agar plates supplemented with rifampicin (50 µg/mL, for rifampicin-resistant LGG) and incubated at 37°C for 48 h under anaerobic conditions.

2.4. Fabrication of microcontainers

Microcontainers were produced from the epoxy-based negative photoresist, SU-8, similarly to the previously established UV-lithography based fabrication schemes^{18,49}. Briefly, a 35 µm thick bottom layer (SU-8 2035) was spin coated (RCD8 manual spin coater, Süss MicroTec, Garching, Germany) on Si substrates, which was then subjected to a soft bake at 50°C (ramping=2°C/min) for 2 h. Next, the layer was UV-exposed using a Karl Süss Mask Aligner MA6 (Süss MicroTec, Garching, Germany) in soft contact mode (250 mJ/cm², 30 sec dwell time) and a post exposure bake at 50°C for 6 h. In order to define the sidewalls, a layer of SU-8 2075 was spin coated to obtain a thickness of approximately 220 µm and soft baked at 50 °C for 10 h. The layer was then exposed in proximity mode (2x250 mJ/cm², 30 sec dwell time). Subsequently, the substrates were

subjected to a post exposure bake at 50 °C for 10 h before developing the SU-8 in two mr-Dev 600 baths with magnetic stirring. The development was done using 2x20 min immersion followed by flushing with isopropanol and drying. The wafers containing microcontainers were cut into 1.2×1.2 cm chips using an automatic dicing saw (DAD 321, DISCO, Tokyo, Japan). For the fabrication of the microcontainers used for *in vivo* experiments, a release layer of 5 nm Ti and 20 nm Au was deposited by E-beam evaporation (Temescal, Ferrotec GmbH, Unterensingen, Germany) on the Si wafers. The release layer facilitated easy detachment of the single microcontainers, which were later filled in gelatin capsules.

2.5. Loading of LGG into microcontainers and lid deposition

Microcontainers were loaded with the spray dried LGG using a previously established masking technique⁵⁰. In short, Sylgard[®] 184 two-part (resin and curing agent) PDMS elastomer were mixed with a weight ratio of 10:1 and poured in 25 well chip trays containing the microcontainer chips. The chip trays were kept at 70°C for 30 min until the PDMS was fully crosslinked. The masked microcontainers were then loaded using a spatula to press the LGG powder on top of the chips. The excess powder was removed using a soft brush and an air gun. To calculate the amount of powder inside the microcontainers, the masked chips were weighed before and after loading. After loading, the PDMS mask was manually peeled off of the chip using tweezers.

LGG-loaded microcontainers were coated using an ultrasonic spray coater (Sono-Tek, Milton, NY, USA) with an AccuMist[™] nozzle and a heating plate. The spray coating parameters for chitosan (control), chitosan–genipin and EL-100 (control) coatings are provided in Table 2.

Chitosan lids were spray coated using chitosan 0.5 % w/v in 1 mM acetic acid, while for the chitosan–genipin lids chitosan 0.45 % w/v in 1 mM acetic acid solution containing 2 mM genipin was used. After the chitosan–genipin lid was deposited, the coated microcontainers were kept in

80 % RH for 16-18 h for completion of the crosslinking process. EL-100 lids were spray coated using an EL-100 1 % w/v in isopropanol solution containing 5 % w/w (in relation to the polymer) dibutyl sebacate as a plasticizer.

Microcontainers were characterized post-fabrication, during and after the loading and coating processes using a TM3030Plus tabletop scanning electron microscope (SEM, Hitachi High Technologies Europe GmbH, Krefeld, Germany) with a voltage of 15 keV.

Table 1. Parameters used for spray coating polymeric lids onto the loaded microcontainers.

Polymers	Chitosan/chitosan–genipin	EL-100
Atomizer pressure (kPa)	0.01	0.02
Nozzle speed (mm.s ⁻¹)	25	10
Total No. of loops	200	40
Temperature (°C)	50	40
Generator power (W)	1.3	2.2

2.6. In vitro and ex vivo release of LGG from microcontainers

2.6.1. In vitro release studies

The release of spray dried LGG from uncoated and coated (chitosan and chitosan–genipin) microcontainers was measured using a μ -Diss profiler (pION INC, Woburn, MA, USA) in 1 M acetic acid for 2 h and PBS for 12-16 h (pH=2.5 and 7.4, respectively). The chips containing the loaded and coated microcontainers were attached to magnetic stirrers in glass vials, filled with 10 mL of the respective release media. Cumulative release profiles were obtained through monitoring the UV spectrum of skim milk powder present in the spray dried formulation, therefore indirectly measuring the release of the bacteria. The UV spectra of pure skim milk powder and the spray

dried LGG powder were matched and linear calibration curves were obtained for the second derivative of the UV spectrum at 300 nm (supporting information S1).

The stability of the chitosan–genipin coatings was studied in conditions simulating the pH, transit time^{51,52} and the colonic bacterial enzymes⁵³ in the GI tract of rats. The release studies were carried out in 1 M acetic acid (pH=2.5) for 30 min, followed by changing the release medium to PBS (pH=7.4) for 3 h, then, the samples were transferred to 10 mL PBS (pH=7.4) containing 0.08 U/mL chitosanase for 12-16 h^{54,55}.

All experiments were carried out in triplicates at 37°C with a stirring rate of 100 rpm and using uncoated microcontainers loaded with LGG as control. The release profiles were monitored real time through *in situ* UV probes with a path length of 20 mm.

2.6.2. Ex-vivo release studies in rat GI contents

Non-sterile cecal and intestinal contents were obtained from the cecum, proximal (pro) colon and distal (dis) colon of male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA). The cecal and intestinal contents were diluted in ultrapure water to obtain the release media with dry content of 10 mg/mL⁵⁶. The prepared media was added to vials containing the chips holding the microcontainers (loaded with LGG and coated with chitosan–genipin) and was kept under anaerobic conditions at 37°C with continuous shaking at 90 rpm. To examine the release and survival of LGG, 100 µL samples were taken at 90, 180 and 270 min. CFU of LGG for each sample was determined and standardized by the weight of the spray dried powder loaded into the microcontainers. All experiments were carried out in triplicates and the release of LGG from uncoated microcontainers in PBS was used as control.

2.7. *In vivo* evaluation of colon-specific delivery of LGG

Animal experiments were performed according to the Danish and European guidelines for animal housing and care and the euthanasia procedures followed the humane endpoints stated in welfare norms at the National Food Institute at the Technical University of Denmark. The experiments were carried out under the approval of the local institutional Animal Welfare Committee, license number 2015-15-0201-00553 and in compliance with Danish laws regulating experiments on animals and EC Directive 2010/ 63/EU.

Microcontainers were loaded with spray dried LGG and spray coated with EL-100 (sample 1) and chitosan–genipin (sample 2) (Figure 2-A). The coated microcontainers were detached from the chips and loaded in the gelatin capsules, each capsule containing 580 ± 19 microcontainers hence, 5.41 ± 0.18 mg of spray dried LGG.

Localization of the released LGG from microcontainers coated with chitosan–genipin in the GI tract and the visualization of the coated microcontainers in the intestines of rats were investigated during two *in vivo* experiments as described in the following (Figure 2-B,C).

2.7.1. Localization of the delivered LGG in the GI tract of rats

14 male 8-week-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were housed in pairs and had access to water and standard feed ad libitum. The animals were weighed before the start of the experiment (434 ± 21.7 g) and dosed with one capsule/animal by oral gavage. Group 1 (n=6) was dosed with sample 1 (EL-100 coated microcontainers), while the animals in group 2 (n=8) were dosed with sample 2 (chitosan–genipin coated microcontainers). Animals from both groups were sacrificed at 6 or 24 h (n=3 for group 1 and n=4 for group 2) after dosing and dissected (Figure 2-B). The intestinal and cecal contents were sampled from the ileum, cecum, pro and dis colon and serial diluted. The diluted samples were plated and incubated at 37°C

for 48 h under anaerobic conditions, followed by CFU counting. The obtained CFU values were standardized by the weight of the spray dried powder loaded in the capsules.

Figure 2. Schematic representation of the *in vivo* studies, showing (A) the two tested samples, namely EL-100 (sample 1) and chitosan–genipin (samples 2) coated microcontainers containing spray dried LGG, (B) the experimental plan for the evaluation of the location and quantity of LGG released from samples 1 and 2 at 0, 6 and 24h and (C) the strategy for the visualization of the GI tract of rats dosed with sample 2 at 0, 2, 4 and 8h.. For both experiments, the rats were sacrificed and dissected at the defined time points during 24 h.

2.7.2. Visualization of microcontainers in the intestines of rats

As indicated in Figure 2-C, the microcontainers were visualized *in vivo* using 6 male 8-week-old Sprague-Dawley rats, housed in pairs with access to water and standard feed and weighed before the start of the experiment (366.4 ± 18 g). In order to visualize the chitosan–genipin coated microcontainers in the GI tract, animals were orally dosed with sample 2 and sacrificed at 2, 4 and 8 h after dosing ($n=2$ at each time point, Figure 2-C). At each time point, the rats were dissected and their GI tract from the duodenum to the dis colon was removed, sectioned into 5 cm fragment, placed on glass slides and opened using a scalpel. The visualization of the microcontainers in the

intestines was performed using an optical microscope (U-RFL-T mercury/xenon burner with a U-LH100HG microscope (Olympus®, Tokyo, Japan) and CellSens Entry software (version 1.12, Olympus®, Tokyo, Japan)).

2.8. Data analysis

All data is presented as mean $\pm$ standard deviation (SD). Statistical analysis and plotting of data were conducted using OriginLab software (OriginLab Corporation, Northampton, MA, USA). p -values were calculated using an un-paired t-test ($p \leq 0.05$ was considered significant).

3. Results and Discussion

3.1. Characterization of genipin-crosslinked chitosan

3.1.1. Characterization of the crosslinking process

Covalent crosslinking of chitosan with genipin was investigated using UV-visible spectroscopy (Figure 3). The complex mechanism of the crosslinking process consists of multiple reactions, leading to a green-blue coloration in the reaction mixture⁵⁷. The progress of the crosslinking reaction was monitored in real time for 24 h as shown in Figure 3-A, which reveals a distinct change in the peaks at 210, 280, 360 and 600 nm during the measurement. Figure 3-B shows the increase in the absorbance of the reaction mixture at 600 nm, corresponding to the formation of blue pigment coloration of the chitosan–genipin mixture over time. The coloration of the chitosan and genipin mixture can be observed in the inset of Figure 3-B.

Figure 3. (A) UV-visible spectra of chitosan and genipin and genipin-crosslinked chitosan over time. (B) Increase in the absorbance of chitosan–genipin mixture at 600 nm, over 24 h (correlating to the spectra presented in (A)). (B-inset) coloration of the chitosan–genipin reaction mixture over time.

3.1.2. pH-stability and swelling

pH-responsiveness of genipin-crosslinked chitosan was investigated using QCM-D. For this purpose, a silica sensor was coated with a thin layer of chitosan–genipin (Figure 4-A) and exposed to pH of 2.5 and 7.5 to monitor frequency and dissipation shifts over time (Figure 4-B). It can be observed that a small shift occurs in frequency and dissipation of the 3rd overtone ($\Delta f=6$ Hz, $\Delta D=-2E-6$), when changing the medium from pH=2.5 to pH=7.4 and the same shift can be seen when changing back to pH=2.5. A similar trend is visible for the 5th and 7th overtones. This trend is explained by the slightly swollen film at pH=2.5 due to the highly charged status of chitosan at this pH ($pK_a = 6-6.5^{58}$), and a decrease in the amount of swelling after changing to pH=7.4 due to the semi-charged nature of chitosan in neutral pH⁵⁹. These results also suggest that covalent crosslinking of chitosan can lead to a higher pH-stability, compared to the conventional approach of using the electrostatic interactions in a polyelectrolyte complex^{60,61}.

Figure 4. QCM-D analysis of genipin-crosslinked chitosan, showing (A) chitosan–genipin coated sensor and (B) pH responsiveness of the deposited layer. A-inset schematically illustrates the genipin-crosslinked chitosan and arrow points to the edge of the coated layer on the sensor.

3.2. LGG-loaded microcontainers coated with chitosan–genipin

Figure 5-A shows the viability of LGG before and after the spray drying process, which reveals a survival rate of 118.87 ± 29.59 %. Figure 5-(B-E) represents empty microcontainers loaded with spray dried LGG and coated with chitosan–genipin. It is evident from the SEM images that the coating successfully seals the reservoir of the microcontainers (elaborated further in supporting information S2).

Figure 5. (A) Bacterial count of LGG before and after spray drying. SEM images of (B) a chip containing empty microcontainers, (C) single microcontainer, (D) loaded with spray dried LGG and (E) coated with chitosan–genipin. scale bar=200 μ m. Data is presented as mean (n=3) $\pm$ SD.

To test the potential of chitosan–genipin as a coating for colon targeted delivery of probiotics, the release of LGG from the coated microcontainers was investigated. Figure 6-A,B shows the cumulative release profiles from uncoated and coated (chitosan and chitosan–genipin) microcontainers in media with pH=2.5 and pH=7.4. A cumulative release of $103.9 \pm 2.1\%$ in pH=2.5 and $88.8 \pm 6.5\%$ in pH=7.4 was observed from chitosan coated microcontainers, similar to the release recorded from uncoated ones ($p=0.234$ and $p=0.103$, respectively). As hypothesized earlier, the rapid release from the chitosan-coated microcontainers at pH=2.5 (Figure 6-A) can be explained by the highly charged polymeric chains at this pH⁴². On the other hand, the slower release from this coating at pH=7.4 (Figure 6-B) can be attributed to hydration and partial swelling of chitosan. This observation is in accordance with previous studies on the release of drugs from chitosan matrixes⁶² and matches with previous reports on chitosan-coated microcontainers^{24,25}.

Figure 6. Cumulative release of spray dried LGG from microcontainers with no coating, coated with chitosan and chitosan–genipin in media with (A) pH=2.5 and (B) pH=7.4. Data is presented as mean (n=3) $\pm$ SD.

The release profiles from microcontainers coated with chitosan–genipin in gastric pH (Figure 6-A) indicates that only a small amount of LGG was released (16.2 ± 4.4 %) over a period of 2 h. On the other hand, the cumulative release is slightly higher (32.4 ± 6.0 %) at intestinal pH (Figure 6-B) during 12 h. The higher release at pH=7.4 compared to that of pH=2.5 can be explained by the faster dissolution of the spray dried LGG at this pH, through comparison of the cumulative release of the powder from uncoated microcontainers at pH=2.5 (55.4 ± 33.0 after 10 min) compared to that of pH=7.4 (93.4 ± 12.4 after 10 min). These results show a statistically significant difference between the capabilities of chitosan and chitosan–genipin coatings to sustain the release of LGG in pH=2.5 and 7.5 ($p=2.32 \times 10^{-4}$ and 8.1×10^{-3} , respectively). These findings are in accordance with previous studies showing controlled release of encapsulated drugs by genipin-crosslinking of chitosan microspheres⁶³. As the potential of the chitosan–genipin coating for colon-specific delivery of LGG was established, the coating was further investigated in *in vitro* and *ex vivo* assays.

3.3. *In vitro* and *ex vivo* evaluation of chitosan–genipin coated microcontainers

3.3.1. Release of LGG in simulated GI conditions

The release of LGG from chitosan–genipin coated microcontainers was further tested in media simulating the sequential pH variations and transit times of the GI tract and the presence of colonic enzymes of rats^{51,53}. Figure 7-A shows the cumulative release from chitosan–genipin coated microcontainers with uncoated microcontainers as a control, in the simulated phases of stomach, small intestine and colon. It can be observed that in accordance with the previous results (Figure 6-A,B), there was a 9.7 ± 1.8 % release in the gastric phase (pH=2.5), followed by a release of up to 37.3 ± 6.7 % in the small intestinal phase (pH=7.4).

Figure 7. (A) Cumulative release profiles from microcontainers loaded with LGG with no coating and coated with chitosan–genipin in simulated GI conditions of rats. (B) Bacterial counts of LGG released from chitosan–genipin coated microcontainers in contents isolated from cecum, pro colon and dis colon of rats with uncoated microcontainers in PBS as control. Data is presented as mean ($n=3$) $\pm$ SD.

This premature release, which was also observed in Figure 6-B, can be explained by the infusion of the spray dried powder with the coating during the lid deposition process and subsequent release through partial swelling of the lid. Such observations have also been reported in previous studies on enteric coated microcontainers^{20,64}. In the final step, in the presence of chitosanase enzymes, there was a gradual release of up to 91.6 ± 5.6 %. Based on these findings, it can be concluded that the chitosan–genipin coating is degraded slowly by the enzymes, which resulted in a release of 78.16 ± 3.6 % after 2 h.

These results were confirmed by microscopy images of the coated microcontainers after the full release of LGG (Figure S3). Although, these results confirm the enzyme-sensitive mechanism of release through degradation of the coating, they do not provide sufficient information on the performance of the delivery system under *in vivo* conditions³⁰. Therefore, the performance of the coating was investigated in an *ex vivo* assay in the cecal and intestinal contents of rats.

3.3.2. Release of LGG in rat GI contents

Figure 7-B shows CFU of LGG released from the chitosan–genipin coated microcontainers after 90, 180 and 270 min in rat cecal and intestinal contents. We could observe that LGG was released after 90 min in the cecum, pro colon and dis colon, which is in accordance with previous reports on biodegradation of chitosan in the GI contents of rats^{65,66}. The release mechanism was also investigated by SEM imaging, which suggested a similar trend in the disintegration of the coating in the rat GI contents and in the presence of chitosanase (supporting information S3). These observations indicate an enzyme-triggered release, and furthermore confirm that LGG is detectable among other bacteria present in the contents of the lower GI tract of rats. Therefore, these findings were proceeded by *in vivo* studies in rats.

3.4. *In vivo* examination of chitosan–genipin coated microcontainers

3.4.1. Localization of LGG in the GI tract of rats

Figure 8-A,B presents chitosan–genipin coated microcontainers loaded with LGG after detachment from the carrier chip and loaded into a gelatin capsule. SEM and optical microscopy images of the detached microcontainers (Figure 8-A, B-inset) show that the coating was intact and undamaged after detachment from the chips. Figure 8-C,D shows CFU counts of LGG in samples collected from the GI tract of rats at 6 and 24 h after dosing. Based on the measured CFU counts, and the assumption that LGG does not proliferate rapidly in the crowded colonic environment, we assume that the enumerated LGG are ‘primary’ released bacterial cells, and not bacteria proliferated after release. We observed (Figure 8-C,D) that there were no detectable LGG in the ileum of the animals dosed with the chitosan–genipin coated microcontainers at 6 or 24 h; however LGG was present in the cecum and pro colon after 6 h and reached the dis colon at 24 h. Therefore, these results confirm that LGG is contained inside the chitosan–genipin coated microcontainers while passing through the ileum.

In the case of EL-100-coated microcontainers (Figure 8-C,D), LGG was present in the small intestine at 6 and 24. These findings are in accordance with previous studies on EL-100 coated microcontainers, which showed the release of a loaded compound in the beginning of the small intestine^{20,51,64}. Based on these observations, we infer that it is indeed the chitosan–genipin coating which facilitates the protection of LGG in the upper GI tract.

Figure 8. (A) SEM image of chitosan–genipin coated microcontainers after detachment from the chip and (B) photo of coated microcontainers loaded into a rat size gelatin capsule. (B)-inset shows Optical microscopy image of chitosan–genipin coated microcontainers. CFU of detected LGG released from microcontainers coated with chitosan–genipin (n=4) and microcontainers coated with EL-100 (n=3) in the ileum, cecum, pro colon and dis colon of rats, (C) 6h and (D) 24h after dosage. Scale bar=100μm. Data is presented as mean ± SD. Detection limit=10³ CFU/g.

The presented results show a decrease in bacterial cell viability in the samples coated with chitosan–genipin compared to those coated with EL-100, suggesting that the sample preparation steps i.e. spray coating temperature and humidity during crosslinking may need to be further optimized.

The findings in Figure 8-C additionally suggest that the chitosan–genipin coated microcontainers had traveled slower through the gut compared to the EL-100-coated, as LGG from the chitosan–genipin samples was not detectable in the distal colon after 6 h. This observation could be related to the stronger adhesion of the chitosan–genipin coated microcontainers to the intestinal mucus layer as a result of the mucoadhesive properties of chitosan^{67,68}. Overall, our findings confirm that the developed carrier system can deliver the probiotics locally in the colon without any premature release in the small intestine.

3.4.2. Visualization of microcontainers in the intestines

Figure 9 shows microscopy images of individual microcontainers located in the distinct segments of the GI tract at different time points (Elaborated further in supporting information S4). 2 h after dosing, microcontainers were found mainly in the jejunum and few were located in the ileum, where the microcontainers were found sealed and the chitosan–genipin lids were intact. After 4 h, the delivery devices were found mostly in the distal part of the ileum and some were located in the cecum and the pro colon. We could observe that the microcontainers found in the small intestine were still sealed. However, those located in the cecum and the pro colon, were found open and the lids had degraded. After 8 h, the microcontainers were located in the middle and the distal part of the colon, where the chitosan–genipin coatings had degraded, as can be seen in Figure 9. Our findings further confirm the ability of the developed coating to control the release of probiotics through an enzyme-sensitive mechanism and deliver LGG locally to the colon.

Figure 9. Visualization by optical microscopy of chitosan–genipin coated microcontainers loaded with LGG in the GI tract of rats at, 2, 4 and 8 h after oral dosage. Arrows point at the microcontainers embedded in the colonic contents.

4. Conclusions

In this study, we have developed an enzymatically triggered delivery system based on microcontainers sealed with crosslinked chitosan coatings for the widely used bacterial strain, *Lactobacillus rhamnosus* GG, as a model microorganism in order to provide proof-of-concept for colon-specific delivery of bioactive agents. Chitosan was covalently crosslinked using genipin as a strong, yet nontoxic and biocompatible crosslinker, to design a coating that is stable at gastric and intestinal pH with controllable swelling.

The results of *in vitro*, *ex vivo* and *in vivo* studies confirm that the coated microcontainers can delay the release of the loaded microorganisms throughout the pH variations of the upper GI tract and deliver the probiotics locally in the cecum and colon of rats. Based on the presented results,

the modification of chitosan through chemical crosslinking as a coating for orally ingestible microdevices, shows great promise for colon-targeted delivery of bioactive agents. The obtained results of this study shall be investigated further to understand the effect of colon-specific delivery on the growth and colonization of probiotic microorganisms delivered locally by chitosan–genipin coated microcontainers.

Supporting Information

UV-vis spectra of skim milk and spray dried LGG; Optical microscopy and SEM images of microcontainers before, during and after the loading and coating processes; SEM images of chitosan–genipin coated microcontainers after release of LGG; Additional optical microscopy images of chitosan–genipin coated microcontainers inside the GI tract of rats.

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Notes

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