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A Double-Layer Polysaccharide Hydrogel (DPH) for the Enhanced Intestine-Targeted Oral Delivery of Probiotics

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ABSTRACT

Transplantation of probiotics to the intestine can positively regulate the gut microbiota, thereby promoting the immune system and treating various diseases. However, the harsh gastrointestinal environment and short retention time in the gastrointestinal tract significantly limit the bioavailability and intestinal colonization of probiotics. Herein, we present a double-layer polysaccharide hydrogel (DPH) in the form of a double-layer structure composed of a carboxymethyl cellulose (CMCL) supramolecular inner layer and a dialdehyde alginate (DAA) cross-linked carboxymethyl chitosan (CMCS) outer layer. This double-layer structure allows DPH to encapsulate and deliver probiotics in a targeted manner within the body. In the stomach, the cage structure of the DPH is closed, and the outer layer absorbs surrounding liquids to form a barrier to protect the probiotics from gastric fluids. In the intestine, the cage structure opens and disintegrates, releasing the probiotics. Thus, DPH endows probiotics with excellent intestine-targeted delivery, improved oral bioavailability, enhanced gastrointestinal tract tolerance, and robust mucoadhesion capacity. The encapsulated probiotics exhibit almost unchanged bioactivity in the gastrointestinal tract before release, as well as improved oral delivery. In particular, probiotics encapsulated by DPH exhibit 100.1 times higher bioavailability and 10.6 times higher mucoadhesion than free probiotics in an animal model 48 h post-treatment. In addition, with a remarkable ability to survive and be retained in the intestine, probiotics encapsulated by DPH show excellent *in vitro* and *in vivo* competition with pathogens. Notably, DAA-mediated dynamic crosslinking not only maintains the overall integrity of the hydrogels but also controls the release timing of the probiotics. Thus, it is expected that encapsulated substances (probiotics, proteins, etc.) can be delivered to specific sites of the intestinal tract by means of DPH, by controlling the dynamic covalent crosslinking.

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1. Introduction

The gut microbiota plays a critical role in health maintenance [1,2], acting as a barrier against pathogens by competing for nutrients, occupying ecological binding sites, and producing antimicrobial substances [3–5]. Disorder of the gut microbiota can result in numerous diseases through pathogen invasion or abnormal metabolism [6]. Probiotics supplementation is an efficient way to regulate the balance of the gut microbiota [7]. Thus, oral delivery of probiotics to the intestine is of great medical and scholarly interest, particularly as it provides a noninvasive alternative to invasive methods [8]. Nevertheless, gastric acid, digestive enzymes, and intestinal bile salt in the gastrointestinal tract result in limited bioavailability and intestinal colonization [9–11]. Thus, encapsulation is considered to be an effective method to protect probiotics from the harsh conditions of the gastrointestinal tract [12]. While many attempts have been made to enhance the survival rate of probiotics, the utilization of probiotics has been largely impeded due to their low bioavailability and poor retention in the intestine [10]. Hence, novel oral delivery systems with enhanced

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gastrointestinal fluid resistance, intestine-targeted release capability, and mucoadhesion properties are urgently needed.

Polysaccharides have been receiving extensive attention due to their ideal film-forming properties, biocompatibility, and sustainability [13,14]. However, delivery systems prepared directly from polysaccharides suffer from a lack of reactive functional groups for cross-linking and have difficulty maintaining structural integrity during mechanical digestion in the stomach. Unlike other polysaccharides, chitosan—a deacetylated derivative of chitin—contains amino groups [15,16]. This unique chemical composition makes it easier to achieve structural modification and enhanced mechanical properties through molecular modifications with chitosan than with other polysaccharides. However, chitosan has antibacterial activity [17], and direct encapsulation with chitosan-based materials can cause the death of probiotics. These properties lead to chitosan being widely used in the food industry but rarely applied in the field of probiotic delivery. Therefore, bypassing the antibacterial activity of chitosan against probiotics while maintaining its advantages is the key to breaking through the bottleneck of chitosan-based probiotic-delivery systems.

Herein, we develop a double-layer polysaccharide hydrogel (DPH) for the intestine-targeted oral delivery of probiotics. The DPH is in the form of a double-layer network, in which carboxymethyl cellulose (CMCL) molecules are cross-linked via hydrogen bonding to form the supramolecular inner layer, while carboxymethyl chitosan (CMCS) molecules are covalently cross-linked via dialdehyde alginate (DAA) to form the outer layer. Probiotics are separated from the chitosan-based outer layer by being encapsulated in the cellulose-based inner layer (Fig. 1). The structure, morphology, biocompatibility, controlled-release behavior, bioavailability, mucoadhesion property, and bacterial competition of the DPH are validated.

2. Methods

2.1. Preparation of DAA

Alginate (20 g) was added into 1 L of water, and $NaIO_4$ (17 g) was then added to the alginate solution. The resulting solution was incubated at 30 °C for 12 h. The reaction was quenched via the addition of ethylene glycol for 2 h.

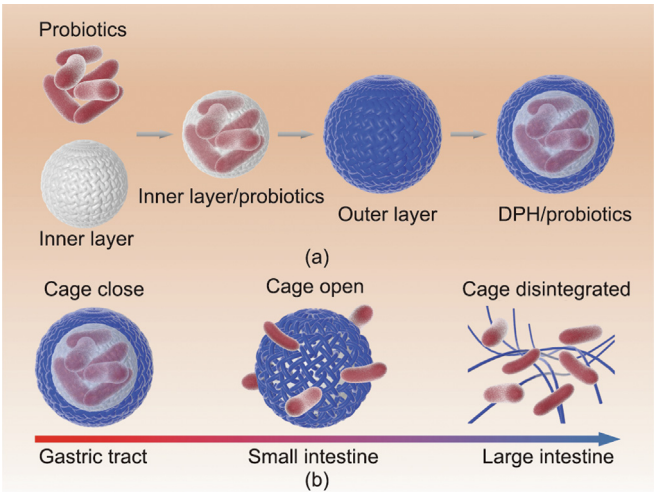

Fig. 1. Schematics of the DPH. (a) Design rationale of the DPH; (b) mechanism of intestine-targeted delivery.

2.2. Preparation of DPH

Lactobacillus plantarum (*L. plantarum*) was used as a model probiotic in this study. *L. plantarum* was mixed with CMCL and then added to the mixture of CMCS and DAA.

2.3. Characterization

The molecular structures of CMCS and DAA were confirmed by Fourier-transform infrared (FT-IR) spectroscopy (Nicolet iS10; Thermo Fisher Scientific, USA) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Avance III; Bruker, USA). A scanning electron microscope (SEM; Regulus8100; Hitachi, Japan) and a transmission electron microscope (TEM; HT7700; Hitachi, Japan) were used to visualize the morphology of the samples.

2.4. Experimental groups

In this work, probiotics encapsulated by DPH are referred to as the DPH group, unencapsulated probiotics (UEP) are denoted as the UEP group, samples treated with *Salmonella* are referred as the *Salmonella* group, and samples without treatment comprise the control group.

2.5. Cell viability assay

A cell counting kit (CCK)-8 assay was used to determine cell viability. To prepare the sample-conditioned medium, 1 g of the sample was diluted in 10 mL of Dulbecco's modified Eagle's medium. Then, 20 μL of conditioned medium along with L929 fibroblast cells were inoculated into a 96-well plate and cultured for 24 h at 37 °C. Next, CCK-8 solution was added to each well, and the optical density (OD)/absorbance value was measured at 450 nm. Cell compatibility was further tested using a live/dead assay. L929 fibroblast cells were stained using a live/dead BacLight bacterial viability kit, followed by fluorescent imaging using a confocal microscope (A1R HD25; Nikon, Japan).

2.6. Evaluation of hematological parameters

C57BL/6JNifdc was used as the animal model for the hematological test. Blood was collected in tubes with ethylenediaminetetraacetic acid (EDTA), and red blood cell (RBC), white blood cell (WBC), and platelet (PLT) counts and hemoglobin (HGB) levels were assessed using a hematology analyzer.

2.7. Probiotic distribution in the gastrointestinal tract

The gastrointestinal distribution of the probiotics was examined by an *in vivo* imaging system (IVIS Lumina XRMS; PerkinElmer, USA). In brief, the model animals were fed with samples labeled with SYTO 64. C57BL/6JNifdc from each group were euthanized by CO_2 inhalation at 1, 2, 4, 24, and 48 h post-administration, and the gastrointestinal tract was collected for *in vivo* imaging.

2.8. In vitro *resistance assessment*

Samples (1×10^7 colony-forming units per milliliter ($CFUs{\cdot}mL^{-1}$), 1 mL) were suspended in 10 mL of simulated gastric fluid (SGF) for 2 h. Then, the SGF-treated bacteria were collected and resuspended in 10 mL of simulated intestinal fluid (SIF) for 2 h. Next, the probiotics were rinsed with phosphate-buffered saline (PBS), spread onto De Man, Rogosa, and Sharpe (MRS) agar plates, and incubated at 37 °C for 48 h, after which the number of colonies was counted. The bacterial suspension was withdrawn for SEM and TEM observation at each time point.

2.9. Controlled-release behavior of DPH

The controlled-release behavior of the probiotics from the DPH was tested in SGF and SIF. The samples (1×10^7 CFUs·mL^{-1}, 1 mL) were incubated in SGF (9 mL) at 37 °C for 2 h with stirring at 200 r·min^{-1}. Next, the samples were transferred from SGF to SIF. Then, the survival and release of the probiotics were measured.

2.10. Flow cytometry

Encapsulated probiotics labeled with SYTO 8 and propidium iodide were added to 1 mL of SGF (10 g·L^{-1} of pepsin, HCl, pH 1.2) and SIF (10 g·L^{-1} of trypsin in KH_2PO_4 solution, NaOH, pH 7.4), and incubated at 37 °C. Then, the samples were taken and evaluated by means of flow cytometry at predetermined time points.

2.11. In vitro *adhesion tests*

Caco-2 cells were seeded in 12-well plates, followed by the addition of probiotics from various groups to the 12-well plates. The cells were then allowed to adhere in the presence of 5% CO_2 at 37 °C for 4 h. Next, the monolayers were washed with PBS to remove nonadherent probiotics. The Caco-2 monolayers were then treated with trypsin. The resulting samples were centrifuged, and the pellet was resuspended in PBS. The binding rate was calculated as follows:

Adhesion rate (%) = [bound probiotics/(bound probiotics + unbound probiotics)] × 100%

2.12. Animal studies

All animal care and experiments were approved by the Animal Investigation Ethics Committee of the Ocean University of China (SPXY2022041803).

2.13. Oral bioavailability and mucoadhesion properties of probiotics

C57BL/6JNifdc were fed probiotics (2×10^7 CFUs) by means of oral gavage. Animals fed probiotics without encapsulation were used as a control. The animals were euthanized by CO_2 inhalation, and the intestinal tracts were collected at 12, 48, and 120 h. To assess the bioavailability and mucoadhesion of the probiotics, the intestinal tissues, intestinal contents, and feces were collected. The collected tissues were ground and diluted with normal saline, while the intestinal contents and feces were soaked in normal saline. Suspensions from the tissues, intestinal contents, and feces were withdrawn and spread onto MRS agar plates for counting.

New Zealand white rabbits were fed probiotics (1×10^9 CFUs) from different groups by oral gavage. Rabbits fed with free probiotics were used as a control. The feces were collected at 12, 24, 48, 72, 96, and 120 h to determine the bacteria retention *in vivo*. Next, the bacteria were further cultured for 12 h at 37 °C for plate counting. Then, the intestines—including the duodenum, jejunum, ileum, cecum, colon, and rectum—were collected 2 days after administration. The collected tissues were ground and diluted using normal saline, and the suspensions were spread on MRS agar plates for counting. Then, the major organs and intestines were processed for histological analyses.

2.14. Treatment of Salmonella typhimurium (S. typhimurium) colonization

C57BL/6JNifdc were administered 1×10^7 CFUs of *S. typhimurium*. Probiotics (1×10^7 CFUs) of various groups were administered for 7 days. Free probiotics were used as a control. At days 1, 3, and 5 after treatment, feces were collected and diluted in normal saline. The suspensions were spread on *Salmonella* chromogenic medium plates and then incubated at 37 °C for counting. Next, the animals were sacrificed at day 7 to collect the intestinal tract tissues for counting probiotics, which were processed for hematoxylin and eosin (H&E) staining, Alcian blue periodic acid-Schiff (AB-PAS) staining, immunohistochemical analysis, and cytokine assay.

3. Results

3.1. Preparation of DPH

The preparation of CMCS and DAA is presented in Fig. S1 in Appendix A. The chemical structures of the prepared molecules were confirmed by FT-IR and ^{1}H NMR spectroscopy (Figs. S2 and S3 in Appendix A).

3.2. Biocompatibility tests

Cell viability was assessed by live/dead assay. As exhibited in Fig. 2(a), there was no observable difference in viability for L929 fibroblast cells incubated in the pristine culture medium and those incubated in the culture medium exposed to DPH for 1–3 days. The cytocompatibility of the samples was assessed by means of a CCK-8 assay. No observable cytotoxicity was detected in response to the DPH-conditioned solution (Fig. 2(b)).

In vivo biocompatibility was tested by performing a histological analysis of the major organs and colon (Fig. 2(c)). No detectable histological damage was found in the H&E staining images, and no morphological changes or inflammatory responses associated with the oral administration had emerged in the intestinal tissues by 5 days post-administration, suggesting that DPH is a safe system for the oral delivery of probiotics. In addition, the hematological parameters—including RBC, WBC, and PLT counts and HGB levels—were in the normal range (Fig. 2(d)). These experimental results suggest that DPH has excellent cytocompatibility without long-term adverse effects.

3.3. In vitro *resistance of DPH against simulated gastrointestinal fluids*

The greatest challenge in the oral administration of probiotics is to ensure that the probiotics survive in the environment of the upper gastrointestinal tract, which has a low pH and multiple digestive enzymes [11,18]. To assess the protective effect of DPH, encapsulated probiotics were immersed in an SGF and then consecutively transferred to an SIF (Fig. 3(a)). Quantitative survival rates after incubation were measured at predetermined time points. In the presence of DPH, the probiotics exhibited outstanding resistance to SGF, and the survival rates decreased by only 14.2% when the exposure time was extended from 1 to 4 h. In contrast, complete death of the probiotics was observed in the UEP group after exposure to SGF for only 2 h, indicating the outstanding protective effects of DPH against acidic and enzymatic stomach conditions.

The intestine-targeted release and protective effects of the DPH on the probiotics were tested *in vitro* using SGF and SIF (Fig. 3(b)). The samples were incubated in SGF (2 h) and were then transferred to SIF (4 h) to simulate the release of the probiotics. After 2 h of exposure to SGF, no release was observed, and all the probiotics remained in the DPH. Probiotics began to be released after the transferal to SIF. The probiotics release was not significant at the beginning of the transfer; after 3 h of transfer, the proportion of released probiotics increased significantly as the exposure time increased, reaching 90.0% after 6 h of treatment.

To confirm that the enhanced tolerance could be ascribed to the presence of DPH, SEM and TEM were used to examine the morphological integrity of the probiotics after incubation in SGF and SIF. As shown in Figs. 3(c) and (d), the structural integrity of the probiotics without encapsulation was destroyed, and the cellular surface

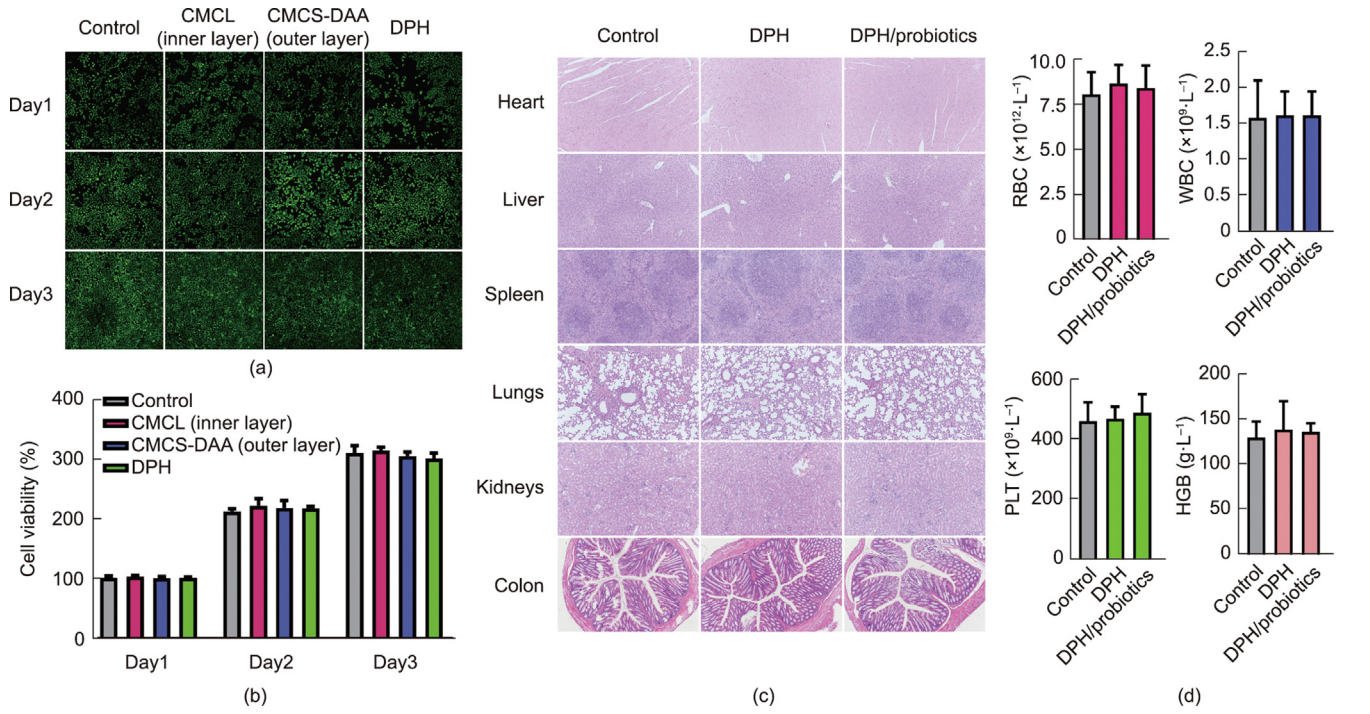

Fig. 2. Biocompatibility tests. (a) Live/dead staining fluorescence images; (b) CCK-8 assay; (c) micrographs of H&E-stained major organ and colon tissue slices; (d) levels of hematological parameters.

became fuzzy. This occurs because stomach pH and digestive enzymes rapidly increase the permeability of the probiotic cell wall and consequently deactivate the probiotics via diffusion. In contrast, DPH effectively prevented the penetration of the simulated gastrointestinal fluids and protected the probiotics from acidic, enzymatic, and salt infiltration, as the DPH remained intact even after 4 h of consecutive incubation in SGF and SIF. These results indicate that DPH was the key factor in the probiotics' resistance to environmental assault.

Flow cytometry with double staining with SYTO 8 and propidium iodide was used to determine the cell state caused by treatment with simulated gastrointestinal fluids. As shown in Fig. 3(e), almost all the probiotics encapsulated with DPH remained alive after 4 h of consecutive incubation in SGF and SIF, whereas most of the probiotics without encapsulation lost their activity upon exposure to SGF.

3.4. In vitro *mucoadhesion test*

Caco-2 cells are commonly used as an *in vitro* model for evaluating intestinal absorption [19]. To assess the intestinal retention of DPH, a mucoadhesion test was performed with Caco-2 cells. As shown in Fig. S4 in Appendix A, the Caco-2 cell binding levels of the control, the UEP, and the DPH groups were 12.4%, 7.1%, and 11.1%, respectively. The binding ability of the DPH group was significantly higher than that of the UEP group and the control group.

3.5. *Oral bioavailability and mucoadhesion in animal models*

We assessed the oral bioavailability and intestinal colonization in a mouse model. The number of probiotics in the intestines and feces was counted at predetermined time points. As presented in Fig. 4(a), the number of probiotics in the DPH group far exceeded those of the control and UEP groups in all these locations. Representatively, the probiotic numbers of the DPH group in the intestine, intestinal contents, and feces of the DPH-treated group were 252.2, 13157.9, and 7549.0 times higher than those of the control group and 10.6, 190.8, and 157.2 times higher than those of the UEP-treated group 12 h after oral gavage. Even with the time increased to 48 h, the bioavailability and colonization of the DPH group were considerably increased in comparison with those of the control and UEP groups. The total probiotic numbers in the intestine, intestinal content, and feces were respectively 239.5, 4982.5, and 3817.3 times higher than those of the control group and 10.2, 147.9, and 124.1 times higher than those of the UEP group for a prolonged time of up to 48 h after gavage. These results suggest that encapsulating probiotics with DPH can markedly improve their oral bioavailability and mucoadhesion.

The enhanced bioavailability and mucoadhesion properties were further evaluated in a rabbit model (Fig. 4(b)). The probiotic numbers of the DPH group located in the intestines and feces were found to be considerably higher than those in the control and UEP groups. The overall probiotic colonization of the DPH group reached 33 641.7 CFUs·mL^{-1}, whereas almost negligible colonization of probiotics (175.8 CFUs·mL^{-1}) appeared in the control group. To assess the survival of the probiotics, the probiotics in the intestinal contents and feces were quantified after oral gavage. The probiotic numbers in the intestinal contents and feces of the DPH-treated group were respectively 2707.4 times and 3387.9 times higher than those of the control group and 188.4 times and 134.3 times higher than those of the UEP-treated group. These experimental results in the rabbit model further confirmed that DPH can endow orally delivered probiotics with excellent bioavailability and mucoadhesion properties.

Gram staining further confirmed that DPH encapsulation enhanced the probiotic colonization in the intestinal mucosae (Figs. 4(c) and (d)). In contrast, negligible colonization was observed from the control group and UEP group.

3.6. In vivo *resistance of DPH against the gastrointestinal tract environment*

To further determine the resistance of DPH against the gastrointestinal tract environment *in vivo*, the retention of delivered probiotics was observed using an intestinal transit assay via an animal

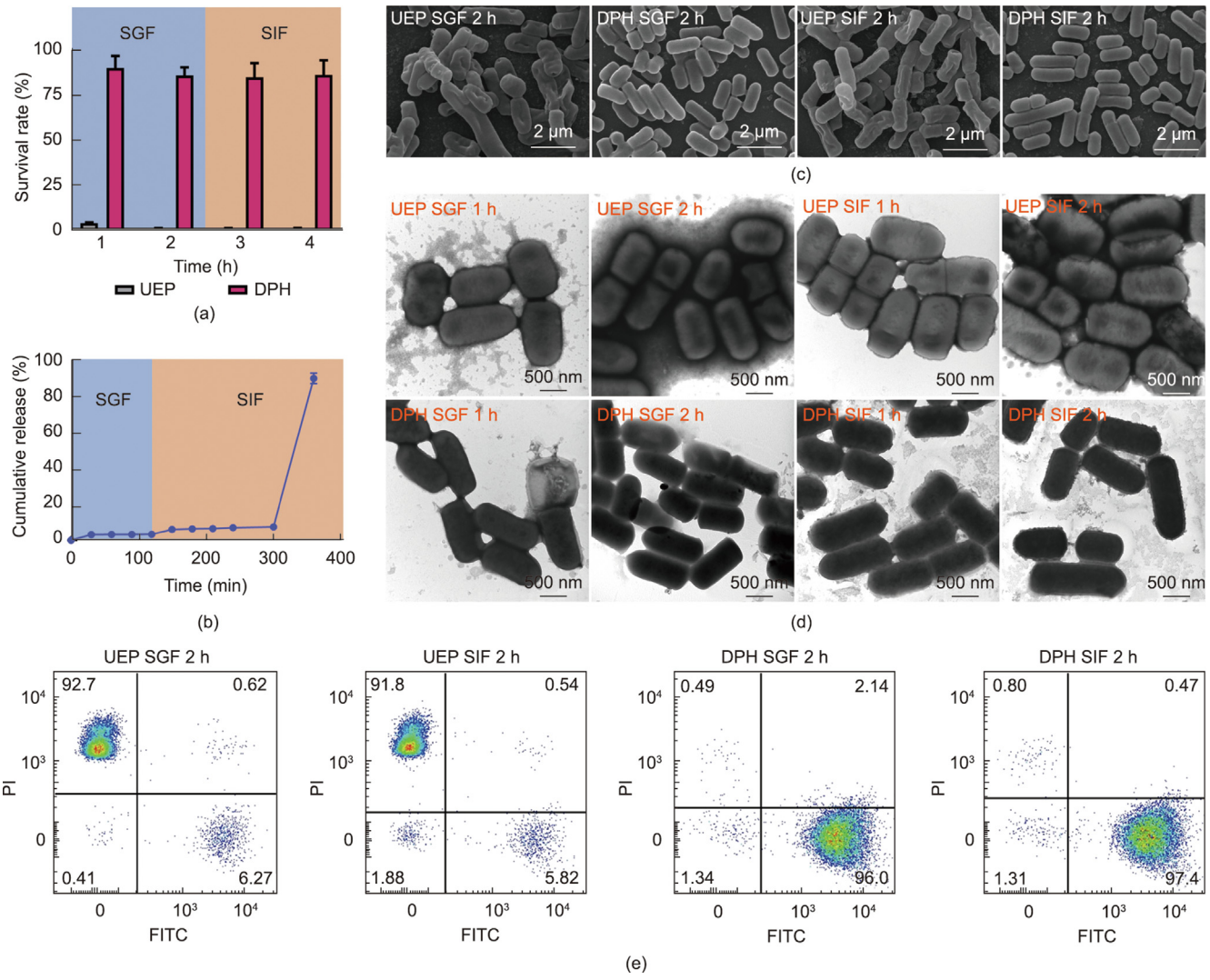

Fig. 3. *In vitro* resistance of DPH against simulated gastrointestinal fluids. (a) Survival rate of probiotics encapsulated with DPH and probiotics without encapsulation; (b) controlled-release behavior of the DPH; (c) SEM and (d) TEM images of simulated gastrointestinal fluids-treated probiotics; (e) flow cytometry analysis of probiotics encapsulated with DPH and probiotics without encapsulation.

imaging system 4, 24, and 48 h post-administration. As shown in Fig. 4(e), free probiotics exhibited intestinal retention for 4 h and completely disappeared after 24 h. Notably, the fluorescence of the DPH group could be observed even after 48 h, indicating the improved retention and mucoadhesion of DPH-encapsulated probiotics in the intestine. This result suggests that DPH is stable in gastrointestinal fluids and can be retained in the intestine for a prolonged period of time.

3.7. *Treatment of* Salmonella-*induced colitis*

Salmonella, a common and serious pathogen, can colonize the intestine, resulting in a large number of infectious diseases [20]. Probiotics can treat *Salmonella*-associated diseases by competing for nutrients, occupying ecological binding sites, and producing antibacterial substances. An *in vivo* competitive experiment was performed to assess the therapeutic effects of DPH-encapsulated probiotics on a *Salmonella*-infected animal model. We tested the efficacy of DPH-encapsulated probiotics on the inhibition of *Salmonella* growth and treatment of *Salmonella*-induced colitis (Fig. 5(a)). The excluded *Salmonella* in the intestines and feces were counted at predetermined time points. As expected, the DPH group presented potent inhibition capability against *Salmonella*, and the number of *Salmonella* was significantly reduced after DPH treatment, with a 22.5 times higher reduction in the intestines in the DPH-treated group than in the UEP-treated group 2 days after treatment. In addition, the counts of *Salmonella* in feces returned to normal levels for the DPH-treated group within 1 day after administration. Consequently, the superior inhibition achieved by dosing with DPH alleviated colitis-related symptoms, as indicated by the colon length, thymus index, and spleen index (Fig. 5(b)).

Moreover, an enzyme-linked immunosorbent assay (ELISA) was carried out to evaluate the inflammation caused by *Salmonella*. Compared with treatment with the UEP group, treatment with the DPH group remarkably reduced the inflammatory reaction, as reflected in the lower levels of proinflammatory cytokines (interleukin (IL)1β, IL-6, and IL-17) (Fig. S5 in Appendix A). A histological assessment with H&E and AB-PAS staining showed that significant damage was observed in the *Salmonella* group and UEP-treated group (Figs. 5(c) and (d)). In contrast, the DPH-encapsulated probiotics restored the damaged mucosa, and no observable histological damage was detected in the DPH-treated group. Furthermore, an immunohistochemical analysis showed that a large amount of F4/80 and CD68—the major macrophage

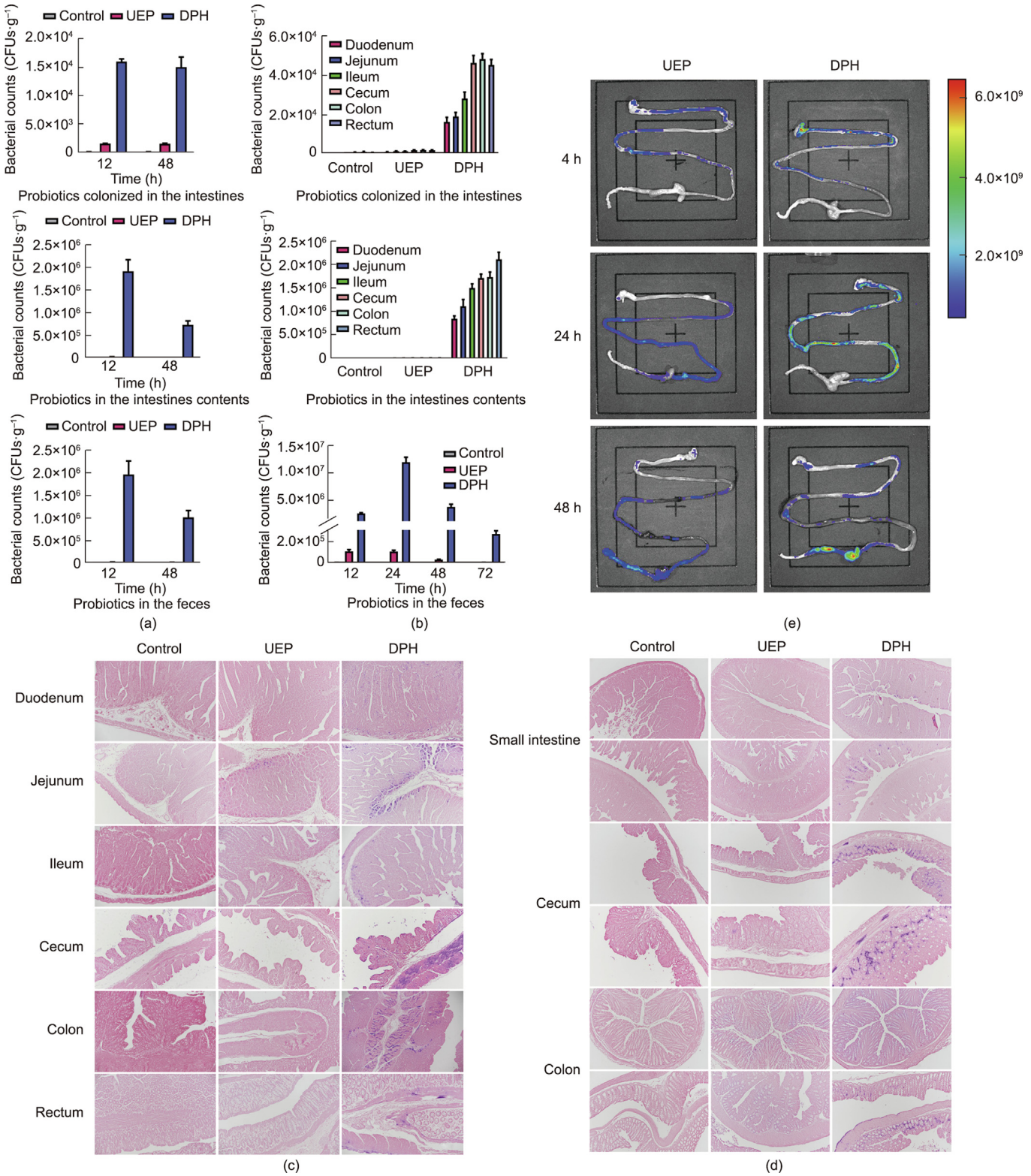

Fig. 4. Oral bioavailability and colonization in animal models. Probiotic counts in (a) a mouse model and (b) a rabbit model; microscopic images of intestinal tissues from (c) a mouse model and (d) a rabbit model; (e) representative fluorescence images of probiotic distribution in the intestines.

markers—were observed in the *Salmonella* group and UEP-treated group (Fig. 5(e)), whereas a significant decrease in F4/80 and CD68 was observed after treatment with the DPH group. The observed effective yet long-term suppression against *Salmonella* can be attributed to the ample reservation of the probiotics encapsulated by DPH in the intestines.

3.8. Short-chain fatty acid contents

Short-chain fatty acids (SCFAs) are important metabolites produced by probiotics via fermentation [21]. As signaling molecules, SCFAs play a critical role in modulating metabolism [22]. To evaluate the bioavailability of the delivered probiotics, the

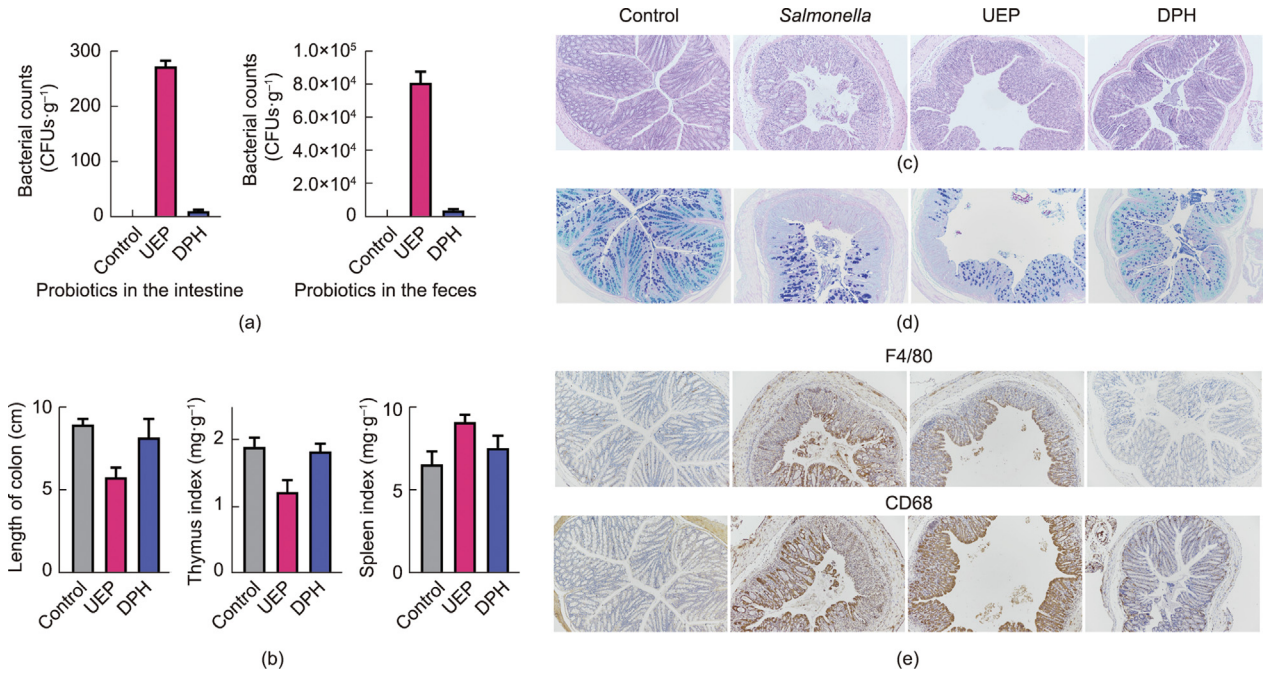

Fig. 5. Treatment of *Salmonella*-induced colitis. (a) Counts of *Salmonella* in intestines and feces after treatment; (b) colitis-related symptoms of different groups; (c) H&E staining, (d) AB-PAS staining, and (e) immunohistochemical analysis of different groups.

concentrations of the total SCFAs—including acetic acids, propionic acids, butyric acids, and pentanoic acids—in the intestine were measured (Fig. S6 in Appendix A). It was found that both the unencapsulated and encapsulated probiotics increased the concentration of SCFAs; however, treatment with the encapsulated probiotics resulted in higher concentrations of SCFAs than that with the UEP, suggesting that DPH improves the bioavailability and activity of probiotics by protecting them from the adverse environment of the gastrointestinal tract.

4. Discussion

To exert beneficial effects, probiotics should be taken orally and pass through the early gastrointestinal tract, with its low pH and multiple digestive enzymes, to reach the small intestine and then colonize the intestinal mucosa [11]. Thus, survival in the upper gastrointestinal tract and the improvement of mucoadhesion present the greatest challenges in the oral delivery of probiotics [7]. DPH tends to close in gastric acid because of the increased hydrogen bonding between polymer chains that results from deprotonation of the carboxymethyl groups at an acidic pH [23,24]. Meanwhile, the covalently cross-linked CMCS that comprises the outer layer of the DPH rapidly absorbs the surrounding liquid to form a polymer–water barrier at the surface of the DPH, further preventing diffusion of gastric fluids across the DPH and protecting the probiotics inside. The mildly basic conditions in the intestinal tract lead to deprotonation of the carboxymethyl groups [23], which decreases the intermolecular hydrogen bonding between the CMCSs of the outer layer and leads to electrostatic repulsion between the negatively charged polymer chains [23]. At this time, the encapsulated probiotics begin to be released in the small intestine. Then, the dynamic imine bonds break as the incubation time increases further, resulting in complete destruction of the cage shape of the hydrogel and the rapid release of the encapsulated probiotics in the large intestine (Fig. 1(b)). Thus, the release timing of the probiotics can be controlled by controlling the formation of the imine bonds. Overall, DPH protects probiotics from gastric fluids during oral delivery, resulting in significantly improved probiotic bioavailability. Moreover, it is expected that substances encapsulated in DPH, including probiotics, proteins, and so forth, can be protected and delivered to specific sites of the intestine by controlling the dynamic covalent cross-linking.

The binding of probiotics to host tissues is mediated by macromolecules on the cell surface, including proteins such as mucin-binding proteins and pili and nonprotein components such as peptidoglycans, exopolysaccharides, and lipoteichoic acid [25]. These components have been shown to be related to binding to the intestine, leading to prolonged transit times and enhanced barrier integrity [25]. These macromolecules are sensitive to gastric acid and easily become inactivated in it. DPH enhances the adhesion of probiotics by protecting these colonization-associated macromolecules from gastrointestinal fluids, leading to prolonged retention of probiotics in the intestine.

5. Conclusions

In summary, we have reported a double-layer DPH for the intestine-targeted oral delivery of probiotics. DPH-encapsulated probiotics exhibit significantly enhanced survival in the extreme conditions of the gastrointestinal tract with almost unchanged viability and activity. Moreover, DPH exhibits excellent mucoadhesive properties and enhanced colonization in the intestinal tract. Overall, we anticipate that DPH can be a good alternative to existing carriers for intestine-targeted delivery.

Acknowledgments

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Compliance with ethics guidelines

Wen-Can Huang, Wenjie Wang, Wei Wang, Yanan Hao, Changhu Xue, and Xiangzhao Mao declare that they have no conflicts of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2023.05.024.

References

[1] Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol 2021;19(1):55–71.
[2] Pérez-Burillo S, Molino S, Navajas-Porras B, Valverde-Moya ÁJ, Hinojosa-Nogueira D, López-Maldonado A, et al. An *in vitro* batch fermentation protocol for studying the contribution of food to gut microbiota composition and functionality. Nat Protoc 2021;16(7):3186–209.
[3] Kamada N, Seo SU, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol 2013;13(5):321–35.
[4] Gareau MG, Sherman PM, Walker WA. Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol 2010;7(9):503–14.
[5] Piewngam P, Zheng Y, Nguyen TH, Dickey SW, Joo HS, Villaruz AE, et al. Pathogen elimination by probiotic *Bacillus* via signalling interference. Nature 2018;562(7728):532–7.
[6] Lin W, Conway LP, Vujasinovic M, Löhr JM, Globisch D. Chemoselective and highly sensitive quantification of gut microbiome and human metabolites. Angew Chem Int Ed Engl 2021;60(43):23232–40.
[7] Wang X, Cao Z, Zhang M, Meng L, Ming Z, Liu J. Bioinspired oral delivery of gut microbiota by self-coating with biofilms. Sci Adv 2020;6(26):eabb1952.
[8] Feng P, Cao Z, Wang X, Li J, Liu J. On-demand bacterial reactivation by restraining within a triggerable nanocoating. Adv Mater 2020;32(34):e2002406.
[9] Centurion F, Merhebi S, Baharfar M, Abbasi R, Zhang C, Mousavi M, et al. Cell-mediated biointerfacial phenolic assembly for probiotic nano encapsulation. Adv Funct Mater 2022;32(26):2200775.
[10] Yao M, Xie J, Du H, McClements DJ, Xiao H, Li L. Progress in microencapsulation of probiotics: a review. Compr Rev Food Sci Food Saf 2020;19(2):857–74.
[11] Centurion F, Basit AW, Liu J, Gaisford S, Rahim MA, Kalantar-Zadeh K. Nanoencapsulation for probiotic delivery. ACS Nano 2021;15(12):18653–60.
[12] Stamatopoulos K, Kafourou V, Batchelor HK, Konteles SJ. Sporopollenin exine microcapsules as potential intestinal delivery system of probiotics. Small 2021;17(7):e2004573.
[13] Deng J, Zhu E, Xu G, Naik N, Murugadoss V, Ma M, et al. Overview of renewable polysaccharide-based composites for biodegradable food packaging applications. Green Chem 2022;24(2):480–92.
[14] Zhang Y, Dong L, Liu L, Wu Z, Pan D, Liu L. Recent advances of stimuli-responsive polysaccharide hydrogels in delivery systems: a review. J Agric Food Chem 2022;70(21):6300–16.
[15] Nam S, Mooney D. Polymeric tissue adhesives. Chem Rev 2021;121(18):11336–84.
[16] Peng X, Dong K, Zhang Y, Wang L, Wei C, Lv T, et al. Sweat-permeable, biodegradable, transparent and self-powered chitosan-based electronic skin with ultrathin elastic gold nanofibers. Adv Funct Mater 2022;32(20):2112241.
[17] Bai L, Liu L, Esquivel M, Tardy BL, Huan S, Niu X, et al. Nanochitin: chemistry, structure, assembly, and applications. Chem Rev 2022;122(13):11604–74.
[18] Luan Q, Zhang H, Chen C, Jiang F, Yao Y, Deng Q, et al. Controlled nutrient delivery through a pH-responsive wood vehicle. ACS Nano 2022;16(2):2198–208.
[19] Liu L, Zhang Y, Yu S, Zhang Z, He C, Chen X. pH- and amylase-responsive carboxymethyl starch/poly(2-isobutyl-acrylic acid) hybrid microgels as effective enteric carriers for oral insulin delivery. Biomacromolecules 2018;19(6):2123–36.
[20] Xue L, Jin N, Guo R, Wang S, Qi W, Liu Y, et al. Microfluidic colorimetric biosensors based on MnO_2 nanozymes and convergence–divergence spiral micromixers for rapid and sensitive detection of salmonella. ACS Sens 2021;6(8):2883–92.
[21] Frampton J, Murphy KG, Frost G, Chambers ES. Short-chain fatty acids as potential regulators of skeletal muscle metabolism and function. Nat Metab 2020;2(9):840–8.
[22] Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol 2015;11(10):577–91.
[23] Huang W, Ying R, Wang W, Guo Y, He Y, Mo X, et al. A macroporous hydrogel dressing with enhanced antibacterial and anti-inflammatory capabilities for accelerated wound healing. Adv Funct Mater 2020;30(21):2000644.
[24] Shi Q, Liu H, Tang D, Li Y, Li X, Xu F. Bioactuators based on stimulus-responsive hydrogels and their emerging biomedical applications. NPG Asia Mater 2019;11:64.
[25] Sanders ME, Benson A, Lebeer S, Merenstein DJ, Klaenhammer TR. Shared mechanisms among probiotic taxa: implications for general probiotic claims. Curr Opin Biotechnol 2018;49:207–16.