

Manuscript version: Author's Accepted Manuscript

The version presented in WRAP is the author's accepted manuscript and may differ from the published version or Version of Record.

Persistent WRAP URL:

<http://wrap.warwick.ac.uk/114211>

How to cite:

Please refer to published version for the most recent bibliographic citation information. If a published version is known of, the repository item page linked to above, will contain details on accessing it.

Copyright and reuse:

The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions.

© 2019 Elsevier. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International <http://creativecommons.org/licenses/by-nc-nd/4.0/>.



Publisher's statement:

Please refer to the repository item page, publisher's statement section, for further information.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk.

1 **Controlled bioactive compound delivery systems based on double**
2 **polysaccharide film-coated microparticles for liquid products and their**
3 **release behaviors**

4

5 Bo Zheng^a, Fengwei Xie^b, Wenbei Situ^a, Ling Chen^{a*}, Xiaoxi Li^{a*}

6 ^a *Ministry of Education Engineering Research Center of Starch & Protein Processing, Guangdong*
7 *Province Key Laboratory for Green Processing of Natural Products and Product Safety, School of*
8 *Food Science and Engineering, South China University of Technology, Guangzhou 510640, China*

9 ^b *School of Chemical Engineering, The University of Queensland, Brisbane, Qld 4072, Australia*

10

11 **Corresponding author:*

12 *Email addresses: felchen@scut.edu.cn, xxlee@scut.edu.cn; Tel: +86 20 8711 3252*

13

14

15

16

17

18

19 **Abstract:** A new carrier system for controlled release of immunologic peptides based on double
20 polysaccharide film-coated microparticles (PCMPs) used with liquid products was developed. The
21 release behavior of PCMPs was shown dependent on the thicknesses of the outer chitosan film and
22 the inner resistant starch acetate (RSA) film. The *in-vitro* release results indicated that, with
23 optimized polysaccharide coating thickness (RSA: 4–5%; chitosan: 6–7%), the release rate of
24 Thymopoietin (TP5) was less than 30% before the microparticles reached the colon, and was 50% in
25 the colon. Besides, the bioavailability of PCMPs was evaluated based on the cell proliferation and
26 protein expression. Compared with the intraperitoneal injection or oral administration, the
27 immunodeficient rats that were orally administrated with the yogurt containing TP5-loaded PCMPs
28 with different storage times possessed a good colon-targeting behavior, higher ratios of CD4/CD8
29 and IgG expression, indicating the improvement in the TP5 immunologic function.

30

31 **Keywords:** liquid products; resistant starch acetate; chitosan; colon-targeting; controlled release;
32 pH-responsiveness

33

34 Thymopoietin (PubChem CID: 50587); chitosan (PubChem CID: 71853); Fluorescein isothiocyanate
35 isomer (PubChem CID: 18730)

36

37 1. Introduction

38 Compared with traditional food, functional food provides significant health benefits by
39 regulating the physiological activity of the human body in addition to the nutritional and sensory
40 functions (such as color, smell, and taste) (Boer, Urlings, & Bast, 2016). Given that, bioactive
41 compounds are considered as the material basis of functional food (Izydorczyk et al., 2017).
42 However, bioactive compounds such as TP5 can be easily destroyed during food processing (Andrés,
43 Villanueva, & Tenorio, 2016; Buniowska, Carbonellcapella, Frigola, & Esteve, 2016) and storage
44 (Gonzálezolivares, Añorvemorga, Castañedaovando, Contreraslópez, & Jaimezordaz, 2014; Grace et
45 al., 2014), as well as in the human physiological environment (Lu, Zhang, Wang, & Chen, 2011;
46 Zanjani, Tarzi, Sharifan, & Mohammadi, 2013). For the improved effectiveness and bioavailability
47 of functional ingredients in food, it is significant to design a delivery system for bioactive
48 compounds with the enhanced stability of bioactive compounds in both the pre-consumption and the
49 human physiological environments.

50 Currently, liquid products systems such as yogurt play a major role in functional food (Tansey
51 & Worsley, 2014). However, technical difficulties, resulting from the pH variation, the digestion
52 enzymes, and the long transit time, are involved, which could negatively impact on the effective
53 storage and the oral delivery of these bioactive compounds to the specific parts of the digestive tract
54 as desired. To overcome these obstacles, it is important to design new controlled release delivery
55 system for bioactive compounds that can be used in liquid products. The recent progress on the
56 research of suitable carrier materials includes bacteria-degradable, pH-sensitive, pressure-sensitive,
57 and time-dependent polymer coating films for the enhanced stability and bioavailability of bioactive
58 compounds has provided renewed hope (Lin, Chen, & Luo, 2007; Maroni, Zema, Del Curto, Foppoli,

59 & Gazzaniga, 2012). Besides for effective storage, alginate (Champagne, 2006; Kailasapathy, 2006),
60 oligosaccharide (K. N. Chen, Chen, Liu, Lin, & Chiu, 2005), whey protein (Lambert, Weinbreck, &
61 Kleerebezem, 2008) and Arabia gum(A. Singh, Adak, Karmakar, & Banerjee, 2014) have been
62 reported to be used as carrier materials for coating bioactive compounds used in different foods such
63 as milk and fruit juice. Amylose and cacao oil have also been used as carrier materials in liquid
64 products like oat beverage (Lahtinen, Ouwehand, Salminen, Forssell, & Mylläriinen, 2007). Besides,
65 suitable materials have been developed for controlled-release delivery systems (Constantin,
66 Bucatariu, Doroftei, & Fundueanu, 2017; Deodhar, Adams, & Trewyn, 2016; Llopislorente,
67 Lozanotorres, Bernardos, Martinezmanez, & Sancenón, 2017). Given the enormous interest in recent
68 years towards maintaining biological activity, growing attention has been focused on many
69 polysaccharides, such as cellulose, pectin, hyaluronic acid and inulin, in developing controlled
70 release systems (Akhgari, Farahmand, Afrasiabi, Sadeghi, & Vandamme, 2006; Gurav, Kulkarni,
71 Khan, & Shinde, 2016; W. He, Du, Cao, Xiang, & Fan, 2008; Ribeiro et al., 2016; Zhou, Wang, Hu,
72 & Luo, 2016). However, in few studies so far, the development of release systems have addressed the
73 dual purposes of the controlled release of bioactive compounds and the improvement in the storage
74 stability of functional food. Thus, the paper reports our new efforts in developing controlled
75 bioactive compound delivery systems with these double advantages.

76 Starch and chitosan are two polysaccharides that are biocompatible and biodegradable and have
77 already been widely used in different foods (Z. He et al., 2017; J. Singh, Kaur, & McCarthy, 2007).
78 Starch can be modified easily to overcome its native hydrophilicity and limitations against the acid
79 and enzymes in the gastrointestinal tract (Bayat et al.; L. Chen, Li, Li, & Guo, 2007; Sharma, Yadav,
80 & Ritika, 2007). The modified starch may avoid being hydrolyzed in the small intestine but can still

81 be degraded by the microorganisms in the colon (Pu, Chen, & Li, 2011). It has been reported by our
82 group that resistant starch acetate (RSA) can be used as a potential carrier for oral colon-specific
83 delivery (Bie, Chen, Li, & Li, 2016; L. Chen et al., 2007; Li, Peng, Ling, & Long, 2011; Xiao, Liu,
84 & Sun, 2011). On the other hand, the dissolution and structure of chitosan are highly responsive to
85 pH in the upper GI tract (Bayat et al., 2008; Pan et al., 2016) and therefore can also be a promising
86 carrier material. Moreover, by adjusting the molecular structure and thus the film forming properties
87 of chitosan, the chitosan film can absorb water to form a gel in a weak-acid environment and
88 dissolve in a strong-acid environment. Therefore, by adjusting the digestion resistibility of starch and
89 the pH-responsiveness of chitosan based on molecular design, it is possible to develop a complex
90 polysaccharide material that can be used to construct a controlled-release delivery system for liquid
91 products.

92 In this study, colon-targeted controlled-delivery systems based on double polysaccharide
93 film-coated microparticles (PCMPs) for yogurt were designed using RSA (the degree of substitution:
94 1.9), which had digestion resistibility, and chitosan (M_w : 1.5×10^5 g/mol), which was pH-responsive,
95 as coating materials. TP5-loaded PCMPs were prepared, in which TP5 was used as a model bioactive
96 compound. Moreover, the release behavior during yogurt storage and *in-vitro* simulated GI
97 transportation were investigated, with the variation in the polysaccharide coating thicknesses.
98 Furthermore, the *in-vivo* effectiveness of TP5-loaded PCMPs was evaluated by tissue
99 immunocytochemistry, and the *in-vivo* TP5 bioactivity was studied in immune model rats.

100

101

102

103 **2. Materials and methods**

104 **2.1 Chemicals and reagents**

105 RSA with the degree of acetyl substitution (DS) of 1.9 was synthesized from a high-amylose
106 starch (50% amylose content, from Penford, Australia) using the method as previously described
107 (Zhang, Chen, Zhao, & Li, 2013). Chitosan (M_w : 1.5×10^5 g/mol) was purchased from Kayon
108 Biological Technology Co., Ltd. (Shanghai, China). Lactic acid bacteria powder was supplied by
109 Chuanxiu Technology Co., Ltd. (Beijing, China). Sterilized pure whole milk was provided by Yili
110 Industrial Group Limited by Share Ltd. (Inner Mongolia, China). Microcrystalline cellulose (SH-102)
111 was purchased from Anhui Shanhe Medicinal Accessory Material Co., Ltd. (Huainan, China). TP5
112 was supplied by GL Biology and Chemistry Co., Ltd. (Shanghai, China). FITC, red blood cell lysis,
113 FITC anticat CD3, PE anticat CD8a, APC anticat CD4, and anticoagulation tubes were purchased
114 from BD Bioscience Co., Ltd. (USA). The ELISA Kit for Immunoglobulin G (IgG) was supplied by
115 Chenglin Biological Technology Co., Ltd. (Beijing, China) Cyclophosphamide was purchased from
116 Aladdin Co., Ltd. (Shanghai, China).

117 **2.2 Preparation of RSA films**

118 The RSA films were prepared by a flow-casting method. RSA was suspended in acetone and
119 stirred for 3 min to make it dissolve completely. An RSA solution was then prepared, with triacetin
120 as a plasticizer at a content of 25% (w/w) of RSA. The mixture was stirred for another 8 h, before
121 casting in a polypropylene plate with a diameter of 14 cm. The cast films were dried in an oven at
122 45 °C for 12 h, which could then be manually detached from the plate. Finally, 1 g of the RSA film

123 was added to 100 g of a fresh fermented yogurt, which was mixed evenly and then stored in a 4 °C
124 refrigerator for different days (1,7,13,19 days) based on the quality guarantee period of yogurt.

125 **2.3 X-ray diffraction(XRD)**

126 Crystalline structure was identified using an X-ray diffractometer (X'Pert Prox, Panalytial, The
127 Netherlands) operated at 40 kV and 40 mA with Cu-K α radiation (0.1542 nm). The diffractograms of
128 the samples were acquired at an angular angle (2θ) range of 4°to 40° with a step size of 0.033° and a
129 counting time of 4 s for each step. The ratio of the upper area (crystalline portion) to the total
130 diffraction area (based on a linear baseline) was taken as the relative crystallinity using the software
131 MDI Jade 6.0. The relative crystallinity of all these samples was calculated using the MDI Jade
132 software (Nara & Komiya, 1983).

133 **2.4 Dynamic mechanical properties of RSA films**

134 The dynamic mechanical properties of RSA films were investigated by a PerkinElmer Diamond
135 dynamic mechanical analyzer (DMA) (PerkinElmer, Inc., Waltham, MA, USA) using the tensile
136 mode. Rectangular specimens with a dimension of 40 (length) × 10 (width) mm were cut from the
137 central part of the films using a cutting mold. A frequency of 1.0 Hz was used. The storage modulus
138 (E'), loss modulus (E''), mechanical loss factor ($\tan \delta$) were recorded. The temperature scanning
139 proceeded from 30 °C up to 90 °C with a rate of 2 °C/min. Triple tests were carried out to each
140 sample to ensure data reliability.

141 **2.5 Preparation of polysaccharide-coated microparticles (PCMPs).**

142 TP5 was used as a model bioactive compound. TP5-loaded microparticles (containing
143 microcrystalline cellulose and starch in the ratio of 3:1) were obtained via extrusion-spheronization

144 (Pu et al., 2011). During the extrusion-spheronization, the temperature was kept at 5–10 °C to
145 maintain the activity of TP5.

146 The microparticle cores loaded with TP5 were then coated with RSA, and then with chitosan,
147 using a bottom spray fluid bed coater (Mini-XYT; Xinyite Technology Co., Shenzhen, China) until a
148 certain weight (thickness) of the coated film was achieved, which was representative of the dry
149 weight gain of the microparticles (Pu et al., 2011). In this way, seven samples of double
150 polysaccharide film-coated microparticles (PCMPs) were prepared: Type I: RSA 2.65%, chitosan
151 8.73%; Type II: RSA 4.15%, chitosan 9.56%; Type III: RSA 7.89%, chitosan 8.26%; Type IV: RSA
152 4.45%, chitosan 1.47%; Type V: RSA 4.45%, chitosan 3.87%; Type VI: RSA 5.14%, chitosan 7.07%;
153 Type VII: RSA 4.15%, chitosan 0%) The process parameters were: the inlet temperature at 44±1 °C;
154 temperature of TP5-loaded microparticles at 30±2 °C; spray rate of coating dispersion at 0.7–0.8
155 mL/min; atomization pressure of 0.15 MPa; and fluidization pressure at 0.15 MPa. PCMPs were
156 finally dried in an oven at 45 °C for 24 h. 1 g of PCMPs were added into 100 g of a fresh fermented
157 yogurt, mixed evenly and then stored in a 4°C refrigerator for 1 to 19 days for the establishment of
158 liquid products delivery systems.

159 **2.6 Release tests during yogurt storage**

160 After stored in the yogurt for different times, PCMPs were taken out and washed with distilled
161 water. Furthermore, PCMPs were soaked and fully dissolved in a hydrochloric acid solution of pH
162 1.2, then ground and filtered. The filtrate was diluted with water to 100 mL. The amount of TP5
163 released from the PCMPs was determined using a UV spectrophotometer at a wavelength of 275 nm.

164 **2.7 *In-vitro* release tests**

165 The *in-vitro* release behavior of PCMPs was studied according to the China Pharmacopoeia
166 (2015) dissolution method using a dissolution rate test apparatus (J. Chen et al., 2016). 1 g of PCMPs
167 stored in yogurt for different times was taken out and washed with distilled water twice, then
168 immersed in the simulated gastric fluid (SGF) for the first 2 h, in the simulated intestinal fluid (SIF)
169 for another 6 h, and afterward in the simulated colonic fluid (SCF) for an additional 40 h, in
170 sequence, all at 37 ± 0.5 °C with agitation using a paddle at a rotation speed of 100 rpm by an
171 intelligent medicine dissolving instrument (RCZ-8B, Tianjin Tianda Tianfa Technology Co., Ltd.,
172 Tianjin, China) (Pu et al., 2011; Situ, Chen, Wang, & Li, 2014). When the simulated digestive fluid
173 changed, PCMPs were filtered by filter paper under vacuum, washed with distilled water twice, and
174 then put into the following simulated digestive fluid. At appropriated time intervals, 5 mL of the
175 sample was collected from the simulated digestive tract fluid for analysis every one hour, and the
176 amount of TP5 released from the PCMPs was determined using a UV spectrophotometer at a
177 wavelength of 275 nm.

178 **2.8 *In-vitro* fluorescent imaging**

179 Five SPF-grade nude mice (7 to 8 weeks old, all females, Vital River Laboratory Animal
180 Technology Co., Ltd., Beijing, China) weighing approximately 12–14 g were fasted for 12 h before
181 the study. The yogurt containing FITC-labeled PCMPs after storage for different times (1 and 13
182 days) were orally administrated to the stomach via polyethylene tubing under light ether anesthesia.
183 Meanwhile, three blank groups containing PBS buffer, the yogurt, and FITC-labeled PCMPs were
184 imaged for comparison. The PCMPs were dosed at 0.2 mg per gram of body weight. At 10, 15, 30,

185 45, 60, 90, 120, 150 and 180 min after oral administration, the fluorescence intensity and
186 transmission of the PCMPs in the nude mice were observed by an small-animal whole-body *in-vivo*
187 imaging system (IVIS 200, Xenogen Corp., Alameda, CA, USA) at 445–490 nm of exciting light
188 and 515–575 nm of emitted light with an exposure time of 5 s. The nude mice were anesthetized by
189 isoflurane before being photographed.

190 **2.9 TP5 bioactivity**

191 Thirty female rats were randomly divided into six groups for the pharmacodynamics study. The
192 ratio of CD4/CD8 in the peripheral blood of each rat was determined as an “internal control” before
193 implantation. The first group of rats were orally administrated with PBS buffer, 2 mL/kg/day, and the
194 second to the sixth group were immunosuppressed by intraperitoneal injection of cyclophosphamide
195 (CTX) at a dosage of 35 mg/kg/day. After CTX treatment for 3 days, if the ratio of CD4/CD8 was
196 beyond the range of 1.5–2.0, the immune deficiency model was considered to be established
197 successfully. After the establishment of the model of the immune deficiency rats, the second group of
198 rats was orally administrated with PBS buffer, 2 mL/kg/day, as an immune suppression control group.
199 In the third to fifth group, each rat was respectively orally administrated with the yogurt without TP5
200 microparticles, with the TP5 solution (12 mg/kg/day), and with the yogurt containing TP5-loaded
201 PCMPs after storing 13 days (the dosage of TP5 was 12 mg/kg/day). Meanwhile, in the sixth group,
202 each rat was given TP5 solution by intraperitoneal injection, 12mg/kg/day. A 200 μ L aliquot of blood
203 was collected into a heparinized tube via the caudal vein 2, 5, 8, 12 and 16 days after implantation
204 and stored at 4 °C. All the samples were analyzed by flow cytometry within 15 min.

205 **2.10 Flow cytometric analysis of peripheral blood**

206 The lymphocyte populations in the peripheral blood were analyzed by dual-color flow
207 cytometry. An antibody solution (1 mL) containing 1% serum was transferred to a new centrifuge
208 tube coated with aluminum foil and then mixed with anti-rat CD4 (25 µL) and anti-rat CD8a (25 µL).
209 Blood samples (200 µL) were washed with PBS by centrifugation at 1500 rpm for 10 min and then
210 mixed with the antibody solution (22 µL). After incubation in the dark at room temperature for 30
211 min, red blood cell lysis buffer (200 µL) was added to the blood sample, incubated in the dark at
212 room temperature for 10 min, and washed twice with PBS by centrifugation at 1500rpm for 10 min.
213 Data was analyzed by the CELL Quest software and represented as dual-parameter density plots.

214 **2.11 Enzyme-linked immunosorbent assay (ELISA) analysis of serum antibodies**

215 The concentrations of serum antigen-specific IgG in individual animals were analyzed by
216 ELISA, according to the manufacturer's instructions (Bethyl, USA). Individual serum samples at
217 1:1–1:5 dilutions were tested in triplicate and incubated at 37 °C for 30 min. Subsequently, the bound
218 antibodies were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (1:100)
219 (Bethyl, USA) at 37 °C for 30min. After washing, the bound HRP-conjugated secondary antibodies
220 were detected with a tetramethylbenzidine substrate. The reaction was stopped by adding 50 µL/well
221 of 1 M H₂SO₄, and the optical density was measured at 450 nm.

222 **2.12 Statistical Analysis**

223 All data were subjected to statistical analysis using the SPSS 16.0 statistical package and were
224 presented as the mean ± standard deviation (±SD). Differences between groups were estimated by

225 analysis of *t*-test, and $P < 0.01$ was considered to indicate a statistically significant difference
226 between two groups.

227 **3. Results and Discussion**

228 **3.1 Changes in crystalline structure of RSA film during Yogurt storage**

229 **Figure 1a** shows the wide-angle XRD spectra of the native RSA film and four samples with the
230 RSA film in the yogurt after storage for different times (1, 7, 13, 19 days). It can be seen that the
231 crystalline structure (related to the V-type diffraction pattern) of the RSA film stored in the yogurt
232 for different days was the same as the native RSA film. For these 5 samples, the relative crystallinity
233 values were calculated to be 9.2, 10.6, 11.1, 11.6, and 12.0, respectively. The relative crystallinity
234 was slightly increased with a longer storage time. This was due to the influence of acid and water
235 molecules in the liquid products, which promoted the degradation and rearrangement of RSA chains.
236 As a result, the aggregation structure of RSA films was changed, and the ordering of RSA chains was
237 increased.

238 **3.2 Changes in dynamic thermal-mechanical properties of RSA film during
239 storage in yogurt**

240 The dynamic thermal-mechanical properties of RSA film as affected by the yogurt were
241 investigated by DMA, and the results are shown in **Fig. 1 b-d**. It could be seen that when all samples
242 were at a low temperature, the E' values were in the range of $4.0 \times 10^8 - 7.0 \times 10^8$ Pa, indicating that
243 the films had strong rigidity. With the increased temperature, E' decreased all along, but $\tan \delta$ firstly
244 increased and then decreased. The peak of a $\tan \delta$ curve describes the glass transition temperature (T_g)

245 of materials (Zhu, Li, Huang, Chen, & Li, 2013). Here, the storage in the yogurt influenced T_g of the
246 films significantly, with T_g increased with a longer storage time.

247 During storage in the yogurt, with the presence of acid and water, the ordering of RSA chains
248 was improved by rearrangements, leading to the increased crystallinity of the RSA film. Moreover,
249 the infiltration of water into the film allowed the interactions between RSA chains and water
250 molecules through hydrogen bonding. These interactions could restrict the movement of starch
251 molecules and the rigidity of the film, as reflected by increased E' and T_g . The longer the storage
252 time, the higher were E' and T_g . A longer storage time could also result in embrittlement of the film.

253 **3.3 Effect of coating thicknesses on the release behavior of PCMPs during storage
254 in yogurt**

255 The PCMPs delivery systems were obtained using TP5 as the model bioactive compound, and
256 the effect of film coating thicknesses on the TP5 release behavior of PCMPs in the yogurt and in the
257 simulated human GI tract was investigated. Firstly, PCMPs were coated with three different
258 thicknesses of RSA but with a similar thickness of chitosan (as shown in **Method 2.5 Types I-III**).

259 The release behavior of these three PCMP samples in the yogurt was illustrated in **figure 2a**. It
260 can be seen that, for each sample, the release rate of PCMPs was increased with the storage time.
261 Comparing the cumulative release percentages of TP5 from the three PCMP samples, it was
262 suggested that under the same storage time and with the same coating thickness of chitosan, the
263 release rate, which reflected the amount of TP5 in yogurt, was decreased with the increased thickness
264 of the inner RSA coating. Besides, after storage for 1 and 22 days in the yogurt, PCMP Sample 1
265 released 16.41% and 29.69% of TP5, respectively. This was mainly due to the loose film of RSA.

266 Based on the results of XRD and DMA analysis, the rigidity of RSA film was increased during
267 storage in the yogurt, which made the RSA film brittle. In this way, TP5 in PCMPs was released
268 through the gel formed by the outer chitosan layer after storage, which prevented the intrusion by the
269 surrounding liquid products. Thus, the coating thickness of the inner RSA film was a determinant
270 factor influencing the release behavior of the bioactive compounds during storage in liquid products.

271 **3.4 Effect of polysaccharide coating thicknesses on the release behavior of**
272 **PCMPs in the simulated human GI tract**

273 The release behaviors of three PCMP samples in the simulated human GI tract after storage in
274 the yogurt for 1, 7, 13, and 19 days was shown in **Figure 2b-e**. It can be seen that the release rate for
275 TP5 in every PCMP sample was increased with the increased storage time in the yogurt. Besides, the
276 release rate for TP5 was decreased with a greater thickness of the RSA coating. It can be proposed
277 that when PCMPs entered the simulated GI tract environment, the time for the dissolution of chitosan
278 in gastric acid delayed the release of TP5 in the upper GI tract. After the dissolution of the outer
279 chitosan layer, the inner RSA film still resisted the erosion by digestive enzymes and gastric acid in
280 the small intestine, which allowed the targeting delivery of TP5 to the colon. These dual functions
281 could maximize the biological activity of TP5.

282 To investigate the effect of chitosan coating thickness on the release rate of the bioactive
283 compound, PCMPs were coated with three different thickness of chitosan (as shown in **Method 2.5**
284 Types IV–VI). The release behaviors of these three PCMP samples in the simulated human GI tract
285 were shown in **Figure 2f-i**. The release of TP5 in the colon could be adjusted by the coating
286 thickness of the outer chitosan layer. When the coating thickness of the inner RSA layer and the outer

287 chitosan layer were between 4–5% and 6–7%, respectively, the cumulative TP5 release rates in the
288 upper GI tract could be controlled to be about 30% and 80% in the colon after storage in the yogurt
289 for 19 days. This meant that the colon-targeted delivery of TP5 was achieved.

290 **Table 1** shows the release rates of different PCMP samples after storage in the yogurt for
291 various days. After storage, part of TP5 had already been released at release time 0 h. Moreover, the
292 release rate of PCMPs in the simulated GI tract was significantly increased. It can be seen that with a
293 similar coating thickness of RSA, PCMP samples had similar release rate when stored in the yogurt,
294 while they had different release rate in the GI tract. For RSA-coated microparticles, 39.08% and
295 50.74% of TP5 were released in the upper GI tract after storage in the yogurt for 1 and 19 days,
296 respectively, while PCMPs released 16.26–29.78% and 24.08–31.94% after storage for the same
297 time periods, respectively. These results further suggested that the outer chitosan layer avoided the
298 release of TP5 before reaching the colon. Furthermore, a certain thickness of chitosan helped restrict
299 the release of TP5 before the colon, which improved the bioavailability of bioactive compounds.

300 **3.5 Release mechanism of liquid products delivery system with pH responsiveness
301 and colon-targeted release**

302 Based on the data discussed above, a release mechanism of the delivery systems based on liquid
303 products with pH responsiveness and colon-targeted release is proposed here as shown in **Figure 3**.

304 In the beginning, the bioactive compounds were evenly distributed throughout PCMPs with
305 RSA as the inner layer and chitosan as the outer layer (**Figure 3 I**).

306 When PCMPs were stored in the yogurt, the outer chitosan layer absorbed water. With the
307 increased storage time, the swelling of the chitosan layer led to the formation of a gel structure

308 around the particle (**Figure 3 II**). The disintegration of the chitosan layer allowed the water and other
309 substances in the yogurt to be gradually in direct contact with the RSA layer. However, RSA was
310 capable of resisting water intrusion from the yogurt because of its certain hydrophobicity (**Figure 3**
311 **III**).

312 During the storage in the yogurt, the ordering of RSA chains was increased through molecular
313 rearrangements. Moreover, because of the infiltration of water molecules from the surrounding liquid
314 products for the interaction and hydrogen-bonding formation with starch chains, the movement of
315 starch chains was restricted, leading to the increased rigidity and brittleness of the film. If the
316 RSA layer was not thick enough, with a longer storage time, a small amount of TP5 would be
317 released from the PCMPs into the surrounding yogurt through the chitosan gel layer. Therefore, the
318 swelling capacity of the chitosan film, together with the hydrophobicity of the RSA layer, maintained
319 the stability of the bioactive compound in the liquid products during storage.

320 After transported from the yogurt to the simulated human GI tract, PCMPs were firstly in
321 contact with low pH gastric juice. The low pH allowed the outer chitosan gel layer to be dissolved
322 gradually due to a pair of non-shared electrons on the nitrogen atom of the amino group of chitosan,
323 which contributed to the combination with a hydrogen ion from the gastric juice (**Figure 3 IV**).
324 Meanwhile, gastric juice also began to reach the inner RSA layer. Nevertheless, owing to the high
325 DS of RSA and thus the resistant starch content, the inner RSA layer was intact, and the release of
326 bioactive compounds was prevented. After PCMPs had been transported to the small intestine, the
327 chitosan gel layer was mostly eliminated. The integrity of the RSA layer was mostly kept but could
328 contain minor damages, which allowed the release of the bioactive compound (**Figure 3 V**).

329 After the PDMCs had been transported to the colon, the colonic microbial fermentation resulted
330 in holes in the RSA coating layer, which allowed the release of the bioactive compound to the
331 surrounding colon environment (**Figure 3 VI**).

332 In this scheme, RSA had a significant number of acetyl groups, which formed steric hindrance
333 and resist to digestion. Besides, a certain degree of hydrophobicity of RSA was helpful to resist the
334 degradation by the acid and various digestive enzymes initially, whereas RSA could still be
335 fermented by colonic microflora.

336 **3.6 *In-vivo* colonic targeting and bioadhesion of PCMPs**

337 Using *in-vivo* fluorescent imaging observed by a small-animal whole-body *in-vivo* imaging
338 system, the oral colonic targeting capability of FITC-labeled PCMPs was studied. **Figure 4** shows
339 the distribution of PCMPs in different parts of the GI tract of nude mice at different times after oral
340 administration. Before administration, no fluorescence was shown in the nude mice (**Figure 4a**).
341 With the increased transit time after the oral administration of PCMPs (from 10 to 45 min), the
342 fluorescence spots moved from the stomach to the small intestine and then to the colon. At 45 min
343 after oral administration, all the fluorescence spots concentrated in the colon, indicating that PCMPs
344 had reached the colon (**Figure 4d–i**). As the transit time was prolonged further, the fluorescence
345 intensity at the colon was gradually increased, and the fluorescence spots were enlarged, which could
346 be due to the release of FITC from PCMPs. The results here indicated good colonic targeting of
347 PCMPs for liquid products.

348 From **Figure 4**, it was seen that after oral administration, the FITC-labeled PCMPs that were
349 stored in the yogurt for 1 and 13 days showed some differences in the time and intensity of

350 fluorescent spots in the body. At 15 min after oral administration of the FTIC-labeled PCMPs that
351 were stored in the yogurt for 13 days, the fluorescent spots appeared in the small intestine, and the
352 intensity of spot increased gradually for the growing time. At 45 min, the PCMPs that were stored in
353 the yogurt for 13 days had been transported to the upper colon, and the intensity of spots was
354 brighter than those without storage or with only one-day storage in the yogurt. This result indicated
355 that the storage time for PCMPs in liquid products had little influence on colon-targeted delivery.

356 **3.7 Bioavailability of thymus peptide five (TP5)**

357 **3.7.1 Effect of TP5 on CD4⁺ and CD8⁺ cells**

358 TP5 is a natural polypeptide that promotes the growth of thymus(Janway, 1992). However, if
359 directly injected or orally administered, TP5 could be degraded easily in the animal body, which
360 limited its function on T-lymphocytes (Amin et al., 2016). As **Figure 5a-d** shows, the populations of
361 CD4⁺ and CD8⁺ cells in rats that were orally administrated with the yogurt containing TP5-loaded
362 PCMPs with different storage time for 7 days were higher than those for the other groups.

363 From **Figure 5e-h** it can be seen that as the time increased for oral administration, the
364 proportions of CD4⁺ or CD8⁺ cells to the total lymphocytes in immune deficiency rats were
365 increased gradually. Also, the proportions reached the maxima on the first day after oral
366 administration. Subsequently, with the end of the oral administration, the proportions of CD4⁺ and
367 CD8⁺ cells to the total lymphocytes were decreased gradually.

368 **Table 2** presents the ratio of CD4/CD8 in immune model rats after different days of oral
369 administration or injection of TP5 solution with the same dosage of TP5. The ratio of CD4/CD8 in
370 the model control group, the TP5 oral administration group, and the TP5 injection group were all less

371 than 1.5. In the TP5 PCMPs yogurt group, the ratio of CD4/CD8 was significantly increased with the
372 increased time ($P < 0.01$). At Day 8, the ratio reached the maximum of 1.52, but still within the
373 normal range. When the time was further extended, the ratio was decreased gradually, which was
374 similar to the model control group. Moreover, there was no significant difference between the model
375 control group, the TP5 oral administration group, and the TP5 injection group. These results showed
376 the pH-responsiveness and the colon-targeted controlled-release performance of PCMPs. After stored
377 in the yogurt and transported into the rat GI tract, PCMPs released TP5 mainly in the colon. Also,
378 there were plenty of lymphoid tissue in the colon, which could directly absorb and utilize proteins
379 such as TP5. This mechanism could prevent the adverse effect of the liver on the bioactive
380 compound, and allowed TP5 to be absorbed in the colon to make it act on the targeted cells, which
381 improved the immunological activity of the cells.

382 **Figure 6a-b** shows the results regarding the effect of the dosage of oral administration of the
383 TP5-loaded PDMCs received by immune model rats on the CD4/CD8 value. After 7 days of oral
384 administration, the CD4/CD8 ratio of the TP5 high-dosage group was significantly increased, which
385 was even close to the normal range, and better than that of the TP5 injection group. Besides, the
386 CD4/CD8 ratio was slightly higher in the rats that were orally administrated with the TP5-loaded
387 PCMPs that were stored in the yogurt for 19 days than that with the TP5-loaded PCMPs that were
388 stored in the yogurt for 7 days.

389 **3.7.2 Effect of TP5 on rat immunoglobulin (IgG) expression**

390 **Table 2** also shows the results regarding the effect of PCMPs on the change in the TP5
391 concentration in immune deficiency model rats. After modeling, the content of IgG in the blood of

392 rats in each group was similar (9.50–12.33 mg/mL) without significant difference. Furthermore, with
393 a prolonged time after oral administration of the yogurt containing PCMPs, the content of IgG in the
394 blood of rats was increased gradually, and reached the maximum at Day 12. This was because the
395 TP5 that was released in the colon was absorbed by the body of the rats, which improved the
396 immunity of T cells and provided the relevant auxiliary stimulation signal and cytokines, thus
397 promoting the production of antibodies for B cells. Compared with the TP5 PCMPs yogurt group,
398 the TP5 oral administration group and the TP5 injection group had a lower content of IgG in the
399 blood, though there was an improvement within their individual groups with time.

400 **Figure 6c-d** shows the effect of dosage of oral administration of TP5-loaded PCMCs on the
401 change in the IgG expression in immune model rats. It could be seen that the IgG expression of
402 immune model rats with yogurt treatment contained the different dosage of TP5-loaded PCMPs was
403 higher (18.6–29.7 mmol/L) than those of the normal group, the control group, and TP5 injection
404 group. As mentioned earlier, B cell activation requires costimulatory signals and cytokines provided
405 by T cells. Thus, it was reasonable to see that, regardless of the dosage of TP5, TP5 in PCMPs could
406 be absorbed by rats and then acted on T cells, which led to the recovery of the cellular immune
407 activity and improved the immunity of rats eventually.

408 **4. Conclusion**

409 This research is focused on the development of a colon-targeted controlled release system based
410 on PCMPs for liquid products. The results indicated that both the storage stability and the
411 colon-targeted controlled release performance of the bioactive compound, TP5, could be achieved
412 with PCMPs with 4–5% and 6–7% of the inner RSA film and the outer chitosan film, respectively.

413 The release rate of TP5 was 50% in the colon, which improved the bioavailability of immune peptide.

414 Thus, this work has provided a new approach for enhancing the bioavailability of functional foods.

415 **Potential conflict of interest statement**

416 The authors declare no competing financial interest.

417 **Acknowledgement**

418 This research has been financially supported under the National Natural Science Foundation of

419 China (NSFC)-Guangdong Joint Foundation Key Project (U1501214), YangFan Innovative and

420 Entrepreneurial Research Team Project (2014YT02S029), the R&D Projects of Guangdong Province

421 (2014B090904047), the Science and Technology Program of Guangzhou (201607010109), the Key

422 R&D Projects of Zhongshan (2014A2FC217), the innovative projects for Universities in Guangdong

423 Province (2015KTSCX006), and the Fundamental Research Funds for the Central Universities

424 (2015ZZ106).

425 **References**

426 Akhgari, A., Farahmand, F., Afrasiabi, G. H., Sadeghi, F., & Vandamme, T. F. (2006). Permeability
427 and swelling studies on free films containing inulin in combination with different
428 polymethacrylates aimed for colonic drug delivery. *European Journal of Pharmaceutical
429 Sciences*, 28(4), 307-314.

430 Amin, N. D., Zheng, Y., Bk, B., Shukla, V., Skuntz, S., Grant, P., Pant, H. C. (2016). The interaction
431 of Munc 18 (p67) with the p10 domain of p35 protects in vivo Cdk5/p35 activity from
432 inhibition by TFP5, a peptide derived from p35. *Molecular Biology of the Cell*, 27(21).

433 Andrés, V., Villanueva, M. J., & Tenorio, M. D. (2016). The effect of high-pressure processing on
434 colour, bioactive compounds, and antioxidant activity in smoothies during refrigerated
435 storage. *Food Chemistry*, 196, 328-335.

- 436 Bayat, A., Dorkoosh, F. A., Dehpour, A. R., Moezi, L., Larijani, B., Junginger, H. E., & Rafieetehrani,
437 M. (2008). Nanoparticles of quaternized chitosan derivatives as a carrier for colon delivery of
438 insulin: ex vivo and in vivo studies. *International Journal of Pharmaceutics*, 356(356),
439 259-266.
- 440 Bie, P., Chen, L., Li, X., & Li, L. (2016). Characterization of concanavalin A-conjugated resistant
441 starch acetate bioadhesive film for oral colon-targeting microcapsule delivery system.
442 *Industrial Crops & Products*, 84, 320-329.
- 443 Boer, A. D., Urlings, M. J. E., & Bast, A. (2016). Active ingredients leading in health claims on
444 functional foods. *Journal of Functional Foods*, 20, 587-593.
- 445 Buniowska, M., Carbonellcapella, J. M., Frigola, A., & Esteve, M. J. (2016). Bioaccessibility of
446 bioactive compounds after non-thermal processing of an exotic fruit juice blend sweetened
447 with Stevia rebaudiana. *Food Chemistry*.
- 448 Champagne, C. P. (2006). Starter Cultures Biotechnology: The Production of Concentrated Lactic
449 Cultures in Alginate Beads and Their Applications in the Nutraceutical and Food Industries
450 (Review paper). *Chemical Industry & Chemical Engineering Quarterly*, 12(1), 11-17.
- 451 Chen, J., Zhang, K., Yang, Z. Q., Wang, X. L., Pan, F., School, G., & University, T. M. (2016).
452 Research of dissolution determination method of dexlansoprazole sustained-release capsules.
453 *Chinese Journal of New Drugs*.
- 454 Chen, K. N., Chen, M. J., Liu, J. R., Lin, C. W., & Chiu, H. Y. (2005). Optimization of incorporated
455 prebiotics as coating materials for probiotic microencapsulation. *Journal of Food Science*,
456 70(5), M260-M266.
- 457 Chen, L., Li, X., Li, L., & Guo, S. (2007). Acetylated starch-based biodegradable materials with
458 potential biomedical applications as drug delivery systems. *Current Applied Physics*, 7,
459 e90-e93.
- 460 Constantin, M., Bucatariu, S. M., Doroftei, F., & Fundueanu, G. (2017). Smart composite materials
461 based on chitosan microspheres embedded in thermosensitive hydrogel for controlled
462 delivery of drugs. *Carbohydrate Polymers*, 157, 493-502.
- 463 Deodhar, G. V., Adams, M. L., & Trewyn, B. G. (2016). Controlled release and intracellular protein
464 delivery from mesoporous silica nanoparticles. *Biotechnology Journal*.
- 465 Gonzálezolivares, L. G., Añorvemorga, J., Castañedaovando, A., Contreraslópez, E., & Jaimezordaz,

- 466 J. (2014). Peptide separation of commercial fermented milk during refrigerated storage.
467 *Ciência E Tecnologia De Alimentos*, 34(4), 674-679.
- 468 Grace, M. H., Yousef, G. G., Gustafson, S. J., Truong, V. D., Yencho, G. C., & Lila, M. A. (2014).
469 Phytochemical changes in phenolics, anthocyanins, ascorbic acid, and carotenoids associated
470 with sweetpotato storage and impacts on bioactive properties. *Food Chemistry*, 145(4),
471 717-724.
- 472 Gurav, D. D., Kulkarni, A. S., Khan, A., & Shinde, V. S. (2016). pH-responsive targeted and
473 controlled doxorubicin delivery using hyaluronic acid nanocarriers. *Colloids & Surfaces B
474 Biointerfaces*, 143, 352.
- 475 He, W., Du, Q., Cao, D. Y., Xiang, B., & Fan, L. F. (2008). Study on colon-specific
476 pectin/ethylcellulose film-coated 5-fluorouracil pellets in rats. *Int J Pharm.*, 348(1-2), 35-45.
- 477 He, Z., Santos, J. L., Tian, H., Huang, H., Hu, Y., Liu, L., Mao, H.-Q. (2017). Scalable fabrication of
478 size-controlled chitosan nanoparticles for oral delivery of insulin. *Biomaterials*, 28–41.
- 479 Izydorczyk, M. S., McMillan, T., Bazin, S., Kletke, J., Dushnicky, L., Dexter, J., Rossnagel, B.
480 (2017). Milling of Canadian oats and barley for functional food ingredients: Oat bran and
481 barley fibre-rich fractions. *Canadian Journal of Plant Science*, 94(3), 573-586.
- 482 Janway, C. A. (1992). The T cell receptor as a multicomponent signalling machine: CD4/CD8
483 coreceptors and CD45 in T cell activation. *Annual Review of Immunology*, 10(10), 645-674.
- 484 Kailasapathy, K. (2006). Survival of free and encapsulated probiotic bacteria and their effect on the
485 sensory properties of yoghurt. *LWT - Food Science and Technology*, 39(10), 1221-1227.
- 486 Lahtinen, S. J., Ouwehand, A. C., Salminen, S. J., Forssell, P., & Mylläriinen, P. (2007). Effect of
487 starch- and lipid-based encapsulation on the culturability of two *Bifidobacterium longum*
488 strains. *Letters in Applied Microbiology*, 44(5), 500-505.
- 489 Lambert, J. M., Weinbreck, F., & Kleerebezem, M. (2008). In vitro analysis of protection of the
490 enzyme bile salt hydrolase against enteric conditions by whey protein-gum arabic
491 microencapsulation. *Journal of Agricultural & Food Chemistry*, 56(18), 8360.
- 492 Li, X., Peng, L., Ling, C., & Long, Y. (2011). Effect of resistant starch film properties on the
493 colon-targeting release of drug from coated pellets. *Journal of Controlled Release*, 152 Suppl
494 1(1), e5.
- 495 Lin, Y., Chen, Q., & Luo, H. (2007). Preparation and characterization of

-
- 496 N-(2-carboxybenzyl)chitosan as a potential pH-sensitive hydrogel for drug delivery.
497 *Carbohydrate Research*, 342(1), 87-95.
- 498 Llopislorente, A., Lozanotorres, B., Bernardos, A., Martinezmanez, R., & Sancenón, F. (2017).
499 Mesoporous Silica Materials for Controlled Delivery based on Enzymes. *Journal of*
500 *Materials Chemistry B*.
- 501 Lu, H. W., Zhang, L. M., Wang, C., & Chen, R. F. (2011). Preparation and properties of new micellar
502 drug carriers based on hydrophobically modified amylopectin. *Carbohydrate Polymers*, 83(4),
503 1499-1506.
- 504 Maroni, A., Zema, L., Del Curto, M. D., Foppoli, A., & Gazzaniga, A. (2012). Oral colon delivery of
505 insulin with the aid of functional adjuvants. *Advanced Drug Delivery Reviews*, 64(6),
506 540-556.
- 507 Nara, S., & Komiya, T. (1983). Studies on the Relationship Between Water-saturated State and
508 Crystallinity by the Diffraction Method for Moistened Potato Starch. *Starch - Stärke*, 35(12),
509 407-410.
- 510 Pan, Q., Lv, Y., Williams, G. R., Tao, L., Yang, H., Li, H., & Zhu, L. (2016). Lactobionic acid and
511 carboxymethyl chitosan functionalized graphene oxide nanocomposites as targeted anticancer
512 drug delivery systems. *Carbohydrate Polymers*, 151, 812.
- 513 Pu, H., Chen, L., & Li, X. (2011). An Oral Colon-Targeting Controlled Release System Based on
514 Resistant Starch Acetate: Synthetization, Characterization, and Preparation of Film-Coating
515 Pellets. *Journal of Agricultural & Food Chemistry*, 59(10), 5738-5745.
- 516 Ribeiro, S. D., Guimes, R. F., Meneguin, A. B., Prezotti, F. G., Boni, F. I., Cury, B. S. F., & Gremião,
517 M. P. D. (2016). Cellulose triacetate films obtained from sugarcane bagasse: Evaluation as
518 coating and mucoadhesive material for drug delivery systems. *Carbohydrate Polymers*, 152,
519 764.
- 520 Sharma, A., Yadav, B. S., & Ritika. (2007). Resistant Starch: Physiological Roles and Food
521 Applications. *Food Reviews International*, 24(2), 193-234.
- 522 Singh, A., Adak, S., Karmakar, S., & Banerjee, R. (2014). Impact of Processing Condition on
523 Nutraceutical Potency of Soy Whey Hydrolysate. *Journal of Food Quality*, 37(37), 403–414.
- 524 Singh, J., Kaur, L., & McCarthy, O. J. (2007). Factors influencing the physico-chemical,
525 morphological, thermal and rheological properties of some chemically modified starches for

-
- 526 food applications—A review. *Food Hydrocolloids*, 21(1), 1-22.
- 527 Situ, W., Chen, L., Wang, X., & Li, X. (2014). Resistant Starch Film-Coated Microparticles for an
528 Oral Colon-Specific Polypeptide Delivery System and Its Release Behaviors. *Journal of
529 Agricultural & Food Chemistry*, 62(16), 3599.
- 530 Tansey, G., & Worsley, A. (2014). *The Food System*: Routledge.
- 531 Xiao, L., Liu, C., & Sun, Y. (2011). A novel oral colon-targeting drug delivery system based on
532 resistant starch acetate. *Journal of Controlled Release*, 152 Suppl 1(1), e51-52.
- 533 Zanjani, M. A. K., Tarzi, B. G., Sharifan, A., & Mohammadi, N. (2013). Microencapsulation of
534 Probiotics by Calcium Alginate-gelatinized Starch with Chitosan Coating and Evaluation of
535 Survival in Simulated Human Gastro-intestinal Condition. *Iranian Journal of Pharmaceutical
536 Research*, 13(13), 843-852.
- 537 Zhang, B., Chen, L., Zhao, Y., & Li, X. (2013). Structure and enzymatic resistivity of debranched
538 high temperature-pressure treated high-amylose corn starch. *Journal of Cereal Science*, 57(3),
539 348–355.
- 540 Zhou, M., Wang, T., Hu, Q., & Luo, Y. (2016). Low density lipoprotein/pectin complex nanogels as
541 potential oral delivery vehicles for curcumin. *Food Hydrocolloids*, 57, 20-29.
- 542 Zhu, J., Li, X., Huang, C., Chen, L., & Li, L. (2013). Plasticization effect of triacetin on structure and
543 properties of starch ester film. *Carbohydrate Polymers*, 94(2), 874-881.
- 544
- 545
- 546
- 547
- 548
- 549
- 550
- 551

Figure captions

552
553 **Figure 1.** XRD pattern and DMA results of the RSA film in yogurt for different storage times (a:
554 XRD patterns; b: storage modulus (E'); c: loss modulus (E''); d: loss angle tangent ($\tan \delta$)).

555 **Figure 2.** a: Release behaviors of PCMPs during storage in yogurt; b-i: Release behaviors of PCMPs
556 in the GI tract after storage in yogurt for different days (b and f: 1 day; c and g: 7 days; d and h: 13
557 days; e and i: 19 days).

558 **Figure 3.** Release mechanism of PCMPs stored in yogurt (upper) and that of PCMPs after being
559 transported to the GI tract (lower).

560 **Figure 4.** Transition after oral administration of FITC-labeled PCMPs in the GI tract of nude mice at
561 different time intervals (from left to right are: the normal group, the control group, the TP5 oral
562 administration group, the TP5 PCMPs yogurt stored 1 day group, and the TP5 PCMPs yogurt stored
563 in 13 days group, respectively).

564 **Figure 5.** a-d: Flow cytometry graph of rats in different groups after 7 days of administration (a:
565 control group; b: TP5 oral administration group; c: TP5 injection group; and d: TP5 PCMPs yogurt
566 group); e-h: Flow cytometry graph of immune model rats at different days after administration with
567 the yogurt containing TP5-loaded PCMPs (e: 0 day; f: 2 days; g: 8 days; and h: 16 days).

568 **Figure 6.** a-b: CD4/CD8 ratios of rats after continuous administration of TP5-loaded PCMPs with
569 different TP5 dosages for 7 days, with TP5-loaded PCMPs stored in yogurt for 1 day (a) and 19 days
570 (b). And c-d: IgG concentrations in rats after continuous administration of TP5-loaded PCMPs with
571 different TP5 dosages for 7 days, with TP5 PCMPs stored in yogurt for 1 day (c) and 19 days (d).
572 (Low dosage group: 4 mg/kg/d oral administration; medium dosage group: 8 mg/kg/d oral
573 administration; high dosage group: 12 mg/kg/d oral administration).

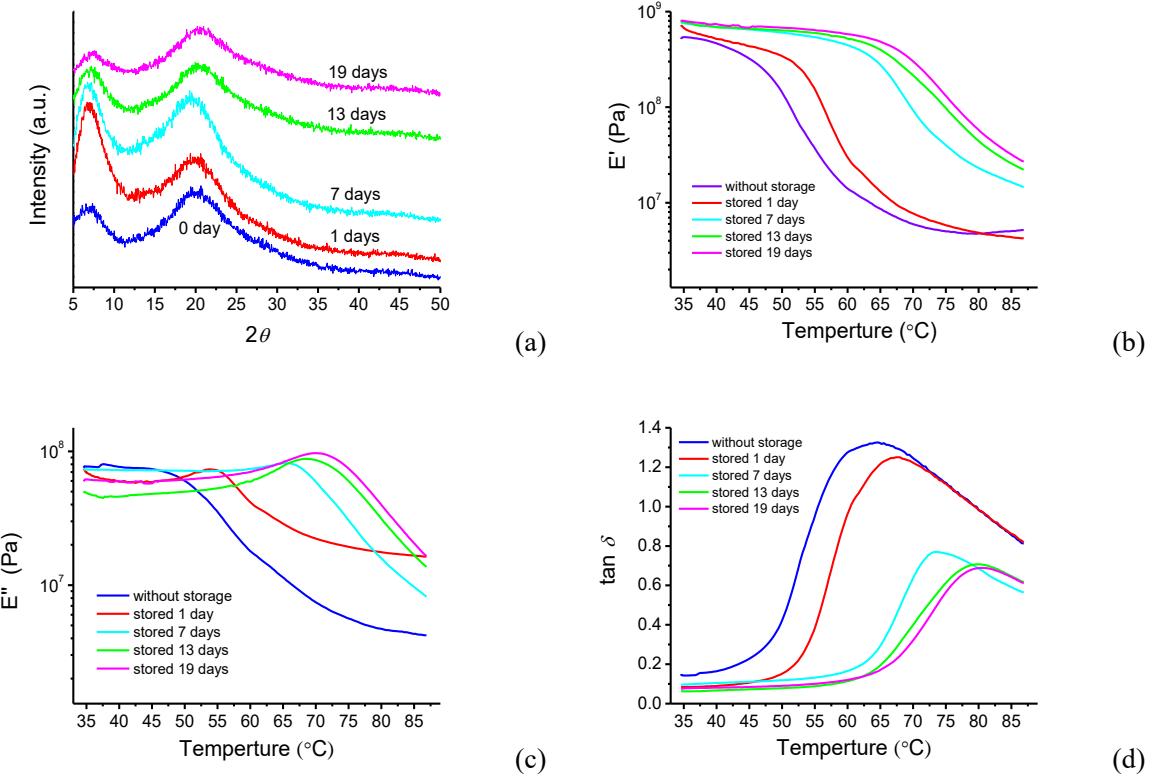


Figure 1

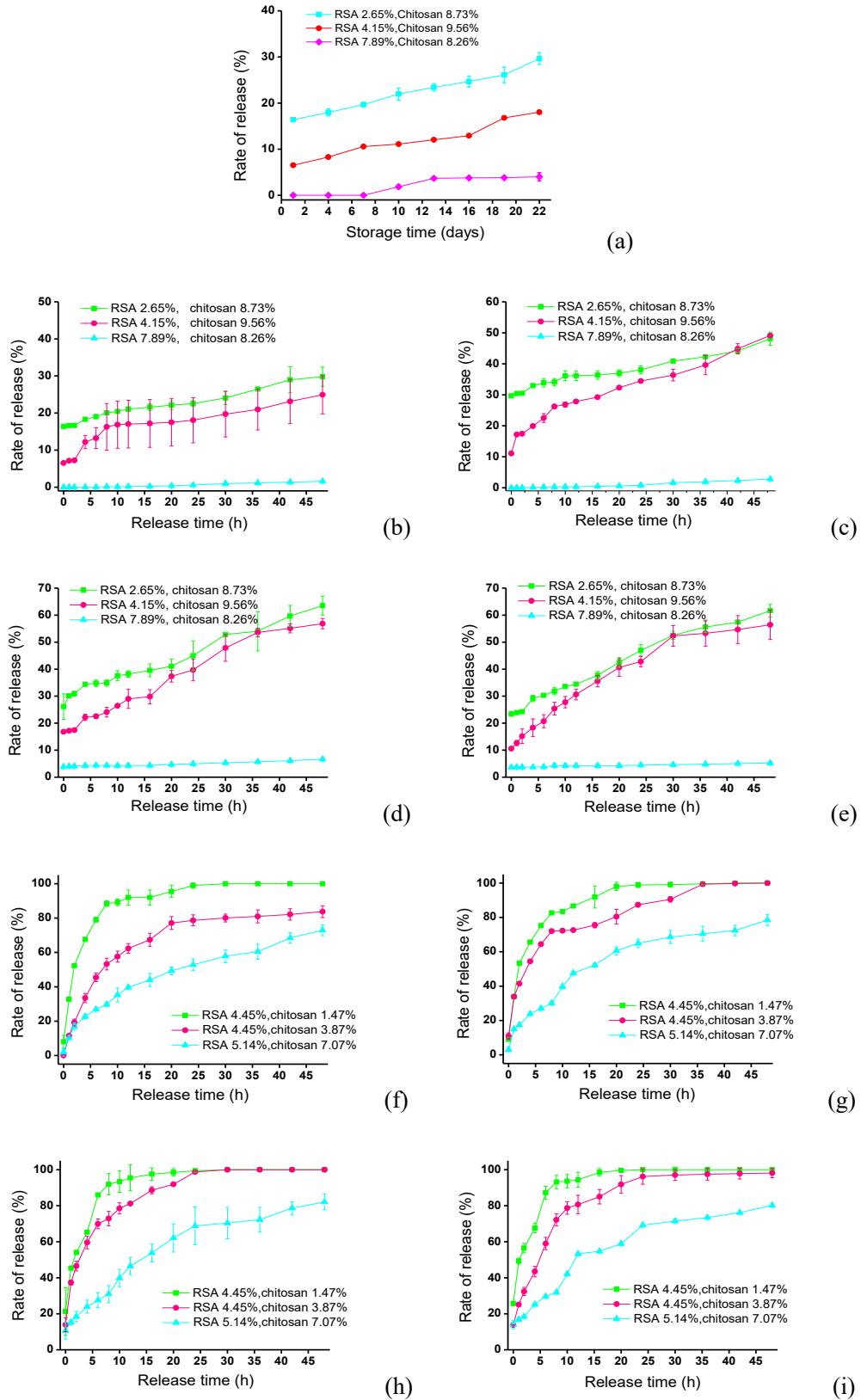


Figure 2

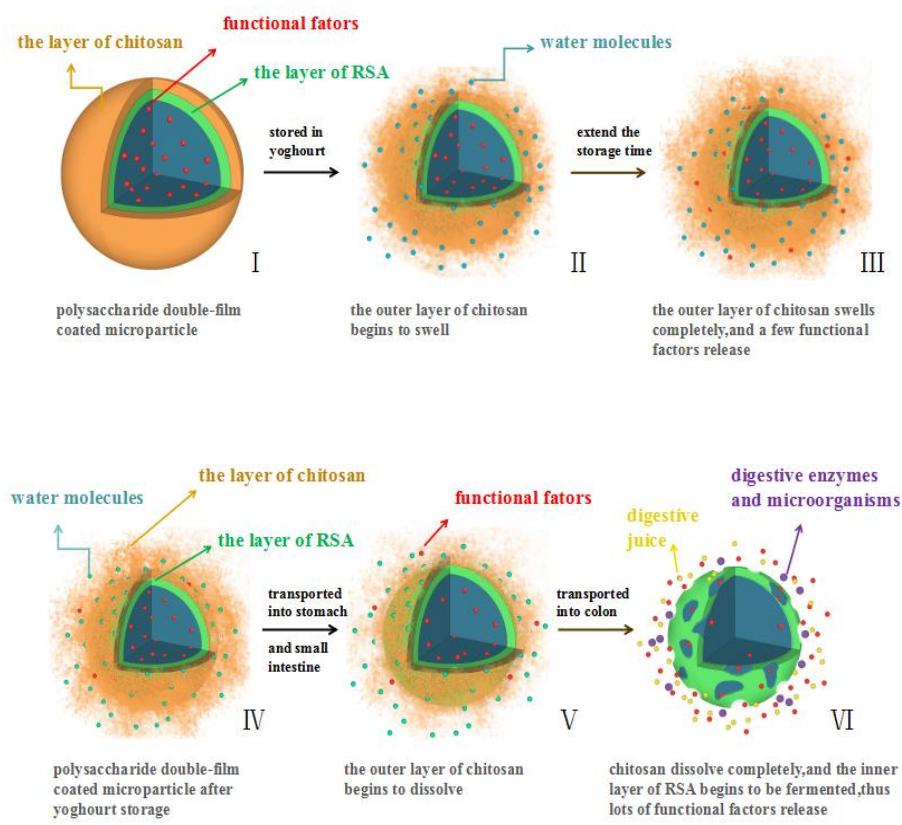


Figure 3

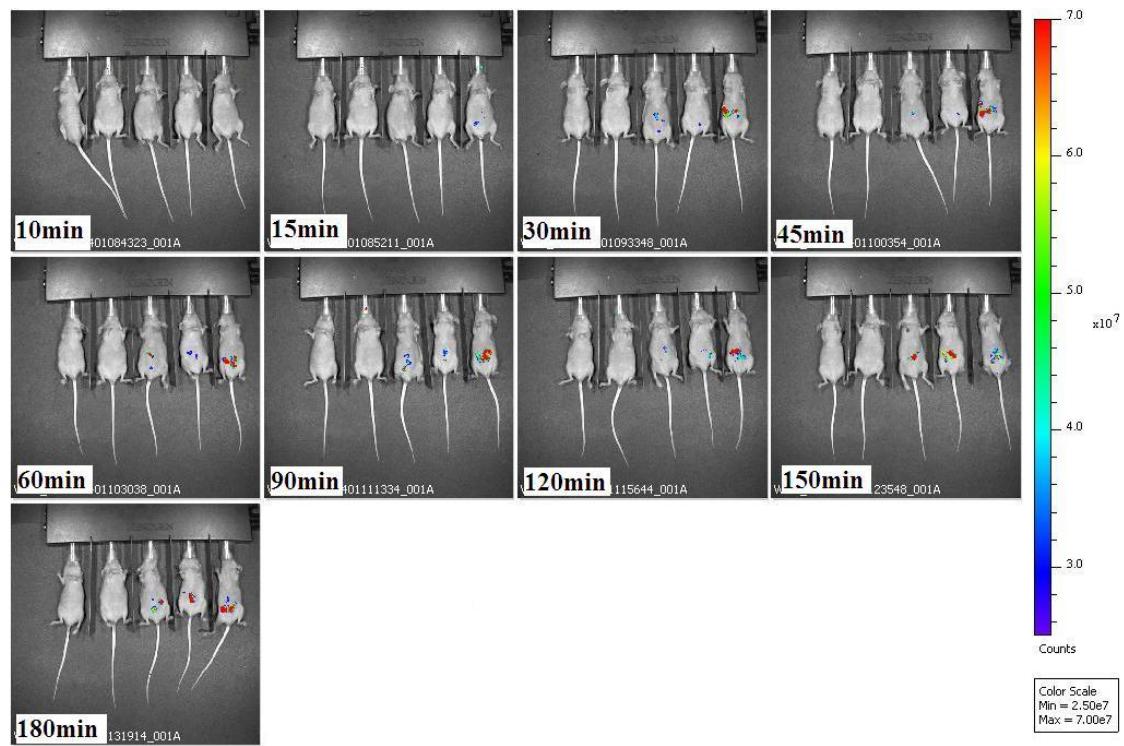


Figure 4

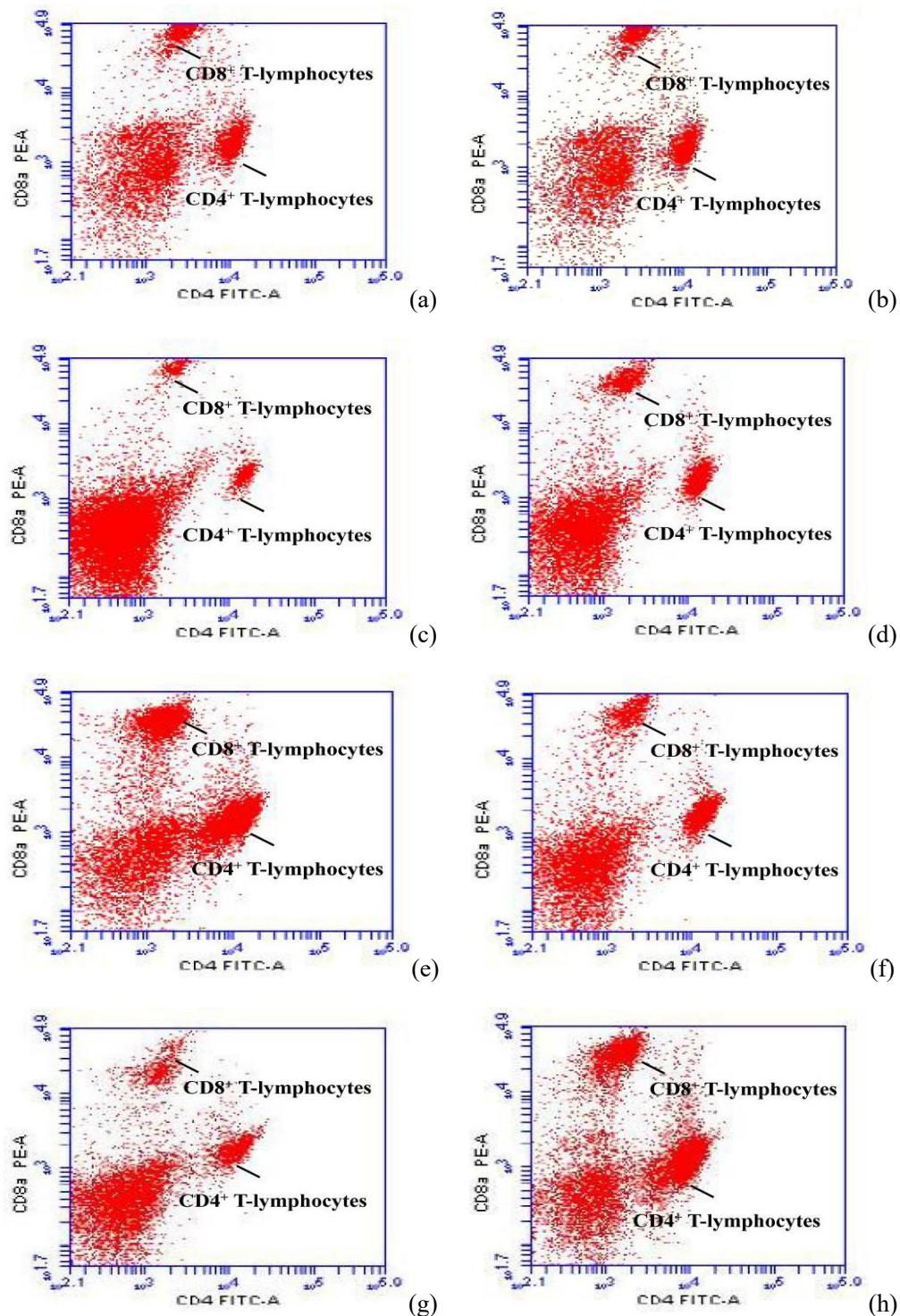


Figure 5

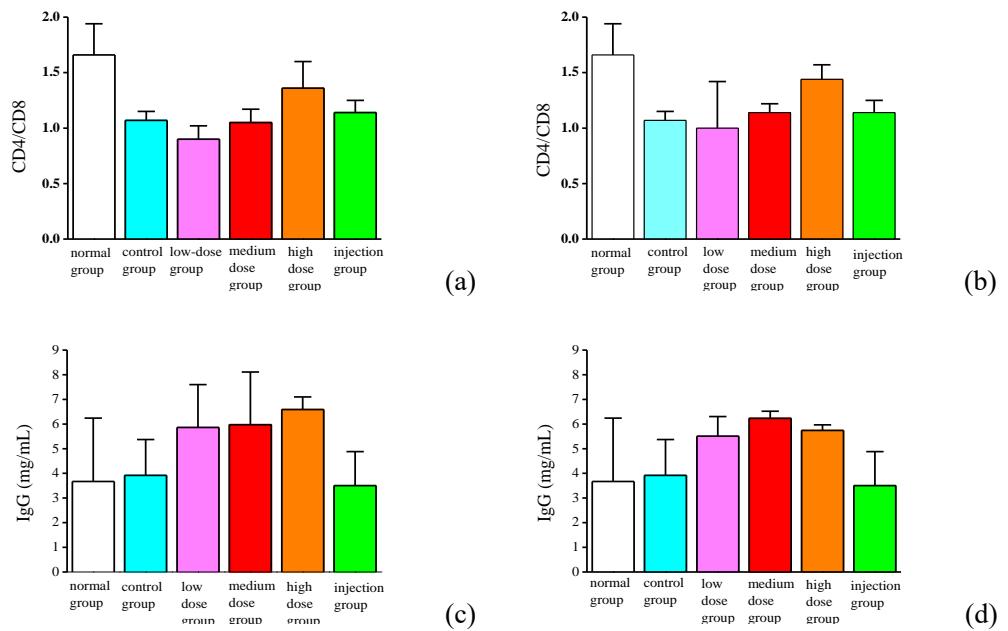


Figure 6

Table 1 Release behaviors of RSA film-coated microparticles and PCMPs (mean±SD)

	Release time	0 h	2 h	8 h	24 h	48 h
Stored for 1 day	RSA 4.15%	1.50±2.13	22.78±4.65	39.08±4.32	67.43±2.54	91.54±0.29
	RSA 4.15% + chitosan 9.56%	6.52±0.04	7.22±0.03	16.26±6.29	18.06±6.11	24.92±5.16
	RSA 5.14% + chitosan 7.07%	2.09±2.40	16.94±2.13	29.78±1.01	52.82±3.45	72.90±3.15
Stored for 7 days	RSA 4.15%	5.73±3.44	22.85±0.35	39.80±0.23	64.56±2.40	86.98±4.96
	RSA 4.15% + chitosan 9.56%	11.10±0.01	17.43±0.13	26.23±0.65	34.44±0.05	49.13±0.26
	RSA 5.14% + chitosan 7.07%	3.06±0.40	17.41±0.13	30.14±1.01	64.90±2.45	78.47±3.15
Stored for 13 days	RSA 4.15%	16.39±2.24	36.97±0.24	54.32±0.77	80.14±2.16	100.00±0.00
	RSA 4.15% + chitosan 9.56%	10.58±0.01	15.19±2.67	25.39±2.38	42.76±1.96	56.42±5.45
	RSA 5.14% + chitosan 7.07%	10.66±4.72	18.43±2.93	30.91±4.75	68.82±10.47	82.11±4.29
Stored for 19 days	RSA 4.15%	15.48±5.90	34.22±0.63	50.74±0.25	75.50±0.44	96.12±5.49
	RSA 4.15% + chitosan 9.56%	16.80±0.01	17.43±0.13	24.08±1.79	39.71±1.04	56.85±1.90
	RSA 5.14% + chitosan 7.07%	13.99±2.36	18.47±0.39	31.94±0.42	69.21±0.12	80.23±0.77

Table 2 The ratio of CD4/CD8 and IgG concentrations of rats in different days after administration

Time	Normal group		Control group		TP5 oral administration group		TP5 injection group		TP5 PCMPs yogurt group	
	CD4/CD8	IgG	CD4/CD8	IgG	CD4/CD8	IgG	CD4/CD8	IgG	CD4/CD8	IgG
0d	1.60±0.06 ^{Aa}	9.82±1.42 ^{Ab}	1.09±0.25 ^{Ba}	12.00±0.98 ^{Ab}	0.90±0.09 ^{Ba}	12.33±2.32 ^{Ac}	1.06±0.25 ^{Ba}	11.81±2.39 ^{Ab}	0.90±0.17 ^{Bb}	9.50±1.07 ^{Ac}
2d	1.53±0.14 ^{Aa}	11.92±2.77 ^{Aab}	1.18±0.13 ^{ABA}	11.90±0.25 ^{Ab}	0.96±0.11 ^{Ba}	12.39±1.60 ^{Ac}	1.19±0.22 ^{Ab}	12.21±0.60 ^{Ab}	1.19±0.23 ^{ABa}	12.15±2.14 ^{Ab}
5d	1.61±0.29 ^{Aa}	14.23±0.70 ^{Aa}	1.14±0.01 ^{ABA}	14.72±0.51 ^{Aa}	0.98±0.20 ^{Ba}	14.09±0.81 ^{Abc}	1.16±0.14 ^{Ab}	14.76±1.98 ^{Aab}	1.33±0.29 ^{ABab}	15.64±1.16 ^{Aab}
8d	1.52±0.12 ^{Aa}	11.22±1.21 ^{Bab}	1.15±0.11 ^{Ba}	11.60±1.07 ^{Bb}	1.11±0.17 ^{Ba}	16.55±0.94 ^{Aa}	1.07±0.13 ^{Ba}	12.95±1.36 ^{Bb}	1.52±0.16 ^{Aa}	16.48±1.16 ^{Aa}
12d	1.52±0.09 ^{Aa}	14.43±0.31 ^{Ca}	0.98±0.12 ^{Ba}	16.44±0.14 ^{Ba}	0.99±0.06 ^{Ba}	16.23±1.19 ^{ABab}	1.03±0.15 ^{Ba}	13.38±0.52 ^{Ba}	1.14±0.04 ^{Bab}	18.71±0.20 ^{Aa}
16d	1.59±0.15 ^{Aa}	11.26±0.19 ^{Aab}	0.82±0.21 ^{Ba}	15.18±1.23 ^{Aa}	0.66±0.37 ^{Ba}	10.92±2.26 ^{Ac}	0.46±0.08 ^{Bb}	11.98±1.19 ^{Ab}	0.89±0.04 ^{Bb}	10.89±1.92 ^{Ac}

Values followed by different upper case letters within a column differ significantly ($P < 0.01$); capital letters

differed from each group at the same time; lower-case letters differed from the time at the same group.