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Best wishes,

Philippa Ross
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Professor Kevin Croft
Editor-in-Chief

1 Structure engineering of filled protein microbeads to tailor
2 release of oil droplets in gastric digestion

3

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15

16

17 **Abstract**

18

19 Oil-soluble components can be encapsulated in an O/W₁/W₂ microsystem, in which they are
20 dissolved in oil droplets dispersed in a gelled microbead (W₁), which forms a barrier between
21 the oil droplets and continuous, aqueous continuous phase (W₂). We investigated the rate and
22 mechanism of breakdown of protein microbeads in a simulated gastric system, and studied the

23 influence of microbead protein concentration, gelling method (cold-set, slow and fast heat-
24 set), and further processing (freeze-drying), on the breakdown process. Breakdown rate
25 decreased with increasing protein content of the beads, for the same method of production.
26 Due to the porosity of the slowly-heated heat-set beads, breakdown occurred evenly
27 throughout the entire bead. Cold-set microbeads of 10% protein broke down slightly slower
28 than the heat-set microbeads of 15%. The denser surface of the 10% beads slowed down the
29 diffusion of the enzymes into the bead's interior, causing the beads to be broken down from
30 the outside inward. All these beads broke down within one hour. Increasing the rate of
31 temperature increase during the heating step dramatically slowed breakdown. There was no
32 significant breakdown of rapidly heated beads within 138 minutes, even though no difference
33 in microstructure between rapidly and slowly heated beads was visible with electron
34 microscopy. Freeze-drying of the beads also slowed their breakdown. After 132 minutes more
35 than half the measured particle volume of were intact beads. Freeze-drying changed the
36 microstructure of the beads irreversibly: rehydrating the dried beads did not result in a
37 breakdown behaviour similar to that of unprocessed beads.

38 1. Introduction

39

40 For encapsulation systems to be effective, functional ingredients need to be protected from
41 external influences until they are delivered to specific locations where they need to be
42 released. The filled hydrogel bead is one of the many types of encapsulation systems that have
43 been developed and investigated in the past decades ¹. This is an O/W₁/W₂ system in which
44 sensitive oil soluble components are incorporated in an oil phase (O) dispersed within gelled
45 microbeads (W₁), which are dispersed in a liquid phase (W₂) ².

46

47 Models describing the breakdown and digestion of nutrients generally lack consideration for
48 the matrix in which the nutrients are entrapped ³. Parada & Aguilera concluded that the
49 bioavailability of a nutrient is more important than the actual amount of nutrient ingested, and
50 that this depends on the matrix in which the nutrient is located ⁴. The relatively large surface
51 area of small oil droplets promotes digestion by lipases ⁵. Li et al. have shown that
52 encapsulating lipids into filled hydrogel beads made of alginate retards lipid digestion ⁶. They
53 ascribe this retardation to presence of the matrix, which slows the diffusion of both lipase to
54 the interface and free fatty acids away from the interface of the oil droplets. Previously we
55 have investigated the strength of smaller, similar beads, made of WPI or alginate ⁷. However,
56 while alginate is not digested in the stomach, protein is extensively digested in the stomach.
57 The rate at which the protein matrix is digested will influence the rate of lipid digestion and
58 release of compounds that may be located in the oil phase. Digestive stability of whey protein
59 microcapsules has been investigated previously and have proven to protect certain strains of
60 probiotic microorganisms, such as *Lactobacillus rhamnosus* and *Bifidobacterium breve* ⁸⁻¹⁰.
61 These studies vary widely in how the protein matrixes are created (spray drying, cold-set Ca²⁺
62 gelation, cold-set acid gelation, heat-set gelation). Because both the production of the matrices

63 and the microorganism that is encapsulated vary, these studies do not allow comparison
64 between different encapsulation systems.

65 Recently we established that the method of production greatly affected the mechanical
66 strength of microbeads made with the same type and concentration of polymer. Both WPI or
67 alginate beads were stronger if the gelling agent was added externally, through the continuous
68 phase, instead of including it in the internal phase of the bead, and that this gelling method
69 was more important to bead strength than the matrix polymer. It was concluded that the
70 externally added gelling agent, which entered the system at the interface of the beads, created
71 a dense 'shell' which was very resistant to the high shear and extensional forces applied on
72 the beads during processing ⁷. Internally gelled microbeads had a more open and porous
73 structure and were severely damaged in conditions where externally gelled beads remained
74 intact.

75
76 These differences in microstructure of the beads may not only affect mechanical stability of
77 the beads, but also the rate of breakdown of the beads in the gastrointestinal tract. Guo et al.
78 showed that protein gels with different hardness or different protein concentrations broke
79 down differently in a simulated gastrointestinal system ¹¹. No exact description of the
80 microstructure of the 'hard' or 'soft' macroscopic gel was given, however it was clear that
81 pepsin had more effect on the soft gel. Luo et al. also found that heat-set WPI gels of 15 and
82 20% protein had different breakdown rates, with respectively 50% vs 11% of the gels broken
83 down after 6 hours ¹². They also found that, at pH 3, the breakdown was entirely caused by
84 the presence of pepsin and not a result of the low pH. Barbé et al. concluded that the rate of
85 digestion of protein can be changed by applying different processes on the same mix of
86 proteins, in their case heating and/or gelation of the protein mixes ¹³. Microbeads produced
87 with different methods and different gel strengths are therefore also likely to break down

88 differently. The difference in gelling method and resulting microstructure would thus give
89 opportunities for the development of different types of microencapsulation systems for release
90 at different locations in the digestive tract.

91

92 For the rate of diffusion of a component into or out of a microbead, the porosity, size, and
93 charge of the bead, and size and charge of the component are of great significance^{1, 14}. For
94 filled hydrogel beads, the encapsulated components are oil soluble and are therefore generally
95 slow or unable to diffuse out of the microbead. Lipase itself may diffuse through the bead,
96 digesting and releasing small amounts of fatty acids, but for complete and rapid digestion to
97 occur the matrix of the bead should be broken down. Current knowledge on how such beads
98 break down, and which variables determine the rate of the process is still lacking.

99

100 In the gastrointestinal tract food encounters a wide range of pH's and enzymes, which digest
101 food. The structure and material of the beads will determine how well and how long the
102 sensitive component inside is protected. A larger size, a higher polymer concentration and
103 higher degree of cross-linking will slow down the release of the component. The porosity may
104 also play a role, because a finer gel with smaller pores may slow down the diffusion of the
105 enzyme into the bead. When the rate of the enzymatic reaction is much higher than the
106 enzyme can diffuse into the interior of the bead we call the system diffusion limited, and the
107 system will break down from the outside in. This would be visible in a size distribution of the
108 beads as a gradual shift to smaller bead sizes. When the enzyme diffuses into the beads much
109 faster than the enzyme needs to break down the substrate, we call the system reaction limited.
110 In this case the bead will be digested evenly over its entire volume, which would be visible in
111 the size distribution as a gradual decrease of the intensity of the peak representing the average
112 bead size, and an increase in intensity for the peak representing free oil droplets.

113

114 The charge of WPI is dependent on the pH, which in turn has an effect on the microstructure
115 of the gelled beads. At the isoelectric point (WPI: 5.1)¹⁵ the molecules will exhibit a net
116 attraction between each other. This will cause the beads to shrink, which might reduce access
117 of proteolytic enzymes. In addition, beads may aggregate into clusters, possibly reducing
118 enzyme access even further. Exposing beads to the low pH of the stomach however, proteins
119 become more positively charged, causing repulsion between the molecules and swelling of the
120 bead³. This leads to bigger pores and easier access for enzymes.

121

122 Recent research has shown that digestion of proteins is affected by changing the processing of
123 the proteins^{11-13, 16}. For the creation and development of new, protein gel-based, delivery
124 systems, we need a better understanding of the relation between microstructure and the rate of
125 breakdown of the delivery systems, and also the factors that are important to the rate of
126 breakdown. The objective of this study was to investigate the impact of variations of the
127 microstructure, created by changing the interior protein concentration and the production and
128 processing method, on the gastric digestion rates and mechanisms of oil filled protein beads.

129

130 2. Materials and methods

131

132 2.1 Materials

133 Whey protein isolate (WPI) purity 98% (Davisco Foods international, France), Alginate
134 (Texturas, Barcelona, Spain), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (purity $\geq 99\%$, Merck, Germany), ethanol absolute
135 (purity $\geq 99.2\%$, Merck, Germany), polyglycerol polyricinoleate (PGPR) 90 kosher (Danisco,
136 Denmark), Medium Chain Triglycerides (MCT) (Miglyol 812 N, Sasol, Germany), Dextran-
137 FITC (40 000 Da), (Sigma Aldrich, Germany) were all used as received. For the simulated
138 gastric system, KCl, NaCl, MgCl_2 , $(\text{NH}_4)_2\text{CO}_3$ (Sigma Aldrich, USA), KH_2PO_4 , NaHCO_3
139 (Merck, Germany) were used. Pepsin from porcine gastric mucosa (EC.3.4.23.1, Sigma
140 Aldrich, USA) had an activity of 3802 U/mg. Solutions were made in demineralized water.

141

142 2.2 Production of WPI heat-set beads (15%, 20%, 25%, and rapidly heated 25%)

143 A primary emulsion, which later formed the inner oil phase, was made by mixing 5% (w/w)
144 MCT in a 15, 20 or 25% (w/w) protein in water solution (pH 6.8) with the Ultra-Turrax (T 25,
145 Germany) at 8000 rpm for 2 minutes. The emulsion was then further homogenized (Delta
146 instruments, Drachten, The Netherlands) for 4 passes at 100 bar. The primary emulsion itself
147 was then emulsified by slowly adding 10% (v/v) to an MCT solution containing 2.5% (w/w)
148 PGPR, while mixing with the Ultra-Turrax at 4200 rpm, thereby creating an O/W/O emulsion.
149 After full addition, the double emulsion was mixed for another 4 minutes. The double
150 emulsion was then put in a water bath of 80 °C for 20 minutes, while continuously stirring to
151 allow the protein in the water phase to gel.

152 The rapidly heated 25% beads were made by slowly adding (in about 10 minutes) the double
153 emulsion to MCT containing 2.5% PGPR which was preheated to 80 °C. The temperature of
154 the mixture was kept above 79°C during the addition of the double emulsion, after which the

155 emulsion was stirred for 20 more minutes at 80 °C. Since the continuous phase is already at
156 about 80 °C upon addition of the double emulsion droplets, these beads are produced at a
157 much higher temperature gradient ($\sim 10^6$ K/m), then beads produced from double emulsions
158 prepared at room temperature, followed by heating of the entire double emulsion ($\sim 10^3$ K/m).
159 For both methods the beads were cooled to room temperature using tap water and stored at 4
160 °C for at least a night and until use.

161

162 **2.3 Production of CaCl₂ nanocrystals**

163 Calcium nanocrystals were made according to the method of Paques et al.¹⁷. A volume of 5%
164 of a solution of 0.1 molal CaCl₂·2H₂O in ethanol was emulsified in MCT containing 6%
165 (w/w) PGPR, with a sonicator (Sonicator S-250A, Branson Ultrasonics, USA) for 1 minute.
166 The emulsion was stirred and heated overnight at 60 °C without a cover, which allowed the
167 ethanol to evaporate, giving a 5% CaCl₂ nanocrystals dispersion.

168

169 **2.4 Production of WPI externally gelled beads**

170 Protein aggregates were made by heating a 10% (w/w) protein solution of pH 8 for 30
171 minutes at 80 °C while stirring. After heating, the solutions were cooled with tap water and
172 stored at 4 °C. The double emulsion was prepared as for the heat-set protein beads, however
173 instead of using a 15, 20 or 25% WPI solution the 10% WPI aggregate solution was used.
174 After emulsification the double emulsion was gelled with calcium by adding the CaCl₂
175 nanocrystal dispersion in a ratio of 7.23 mL per 10 mL of double emulsion and stirring it
176 overnight. The beads were stored at 4 °C until use.

177

178 **2.5 Removal of beads from the outer oil phase**

179 Protein microbeads were removed from the oil phase by centrifuging the dispersion at 2500 g
180 for 2 hours. To remove residual oil sticking to the surface of the beads, the beads were
181 washed. The pellet was redispersed in a 2% (w/w) WPI solution, mixed with the Ultra-Turrax
182 at 6000 rpm for 2 minutes and then homogenized at 100 bar for 2 passes. The dispersion was
183 then again centrifuged, now at 400 g for 45 minutes. The redispersion, mixing, and
184 centrifugation steps were repeated twice, after which the pellet was redispersed in water.

185

186 **2.6 Freeze-dried protein beads**

187 The redispersed beads were frozen with liquid nitrogen and Freeze-dried (Christ Alpha 2-4
188 LD plus, Martin Christ, Germany). The powder was stored in a desiccator until use.

189

190 **2.7 Surface morphology**

191 Surface morphology was examined by scanning electron microscopy (SEM; Magellan 400,
192 FEI, Eindhoven, the Netherlands). The samples were adhered to coverslips, critical point
193 dried with CO₂, and then sputter coated (Leica EM SCD 500, Leica, Vienna, Austria) with a
194 10nm iridium layer.

195

196 **2.8 Protein and oil content**

197 To measure the encapsulation efficiency the beads were freeze-dried to remove all water. The
198 protein content of the dried beads was measured by DUMAS (Flash EA 1112 Analyzer,
199 Thermo Fisher Scientific, USA). The rest of the weight was assumed to be oil.

200

201 **2.9 Dry matter content**

202 Addition of the amount of beads to the simulated gastrointestinal system was based on equal
203 amounts of beads, which was based on dry matter content. A measured volume of dispersion

204 was put in an oven of 105 °C overnight. Based on the dry matter content a calculation was
205 made (with the assumption that no oil was added or lost during the second emulsification
206 step) to find the volume of beads. The bead dispersion was diluted to a volume fraction of
207 0.20. MCT is not volatile and did not noticeably evaporate during 20 hours at 105 °C.

208

209 **2.10 Simulated gastric system**

210 The gastrointestinal system was made as described by Minekus et al. ¹⁸. We assumed the
211 beads to be present in a liquid phase, thereby bypassing the oral phase. Simulated gastric fluid
212 (SGF) electrolyte stock solutions were made as described. A 20% (v/v) bead dispersions and
213 the CaCl₂ solution were added to SGF after which the pH was adjusted to 3. The solution was
214 put on an automatic stirrer at 37 °C. Then pepsin was added in the concentrations mentioned
215 (2000 U/mL). Every 6 minutes, before and during enzyme treatment, samples were taken and
216 bead size was measured.

217

218 **2.11 Size distribution**

219 The size distribution of the beads, before and during the treatment in the simulated
220 gastrointestinal system, was measured with the Mastersizer (Malvern Mastersizer 2000,
221 Malvern Instruments, UK) using laser diffraction. The volume percent of the beads, oil
222 droplets, and aggregates was determined by calculating the surface area of the respective
223 peaks in the particle size distribution curve. The results shown are an average of two
224 measurements, the error bars show the standard deviation.

225

226 **2.12 Light microscopy**

227 Pictures of the rehydrating freeze-dried 25% beads were made with a light microscope
228 (Axioskop 2 plus, Carl Zeiss, Germany)

229

230 **2.13 Diffusion of probe molecules**

231 To further investigate whether the breakdown of the protein beads was diffusion or reaction
232 limited, the diffusion through the matrix was investigated using a tracer particle of similar
233 molecular weight as pepsin. Dextran-FITC (40 000 Da) was added in 20mmol (similar as to
234 the pepsin concentration in the stomach) to 1 % samples of the 25% heat-set, 25% rapidly
235 heated, 25% hydrated freeze-dried and 10% cold-set beads in simulated gastric fluid (SGF) at
236 pH 3. The diffusion of the Dextran-FITC over time was examined by confocal laser scanning
237 microscopy (CSLM, Leica TCS-SP5, Leica Microsystems B.V., Rijswijk, Netherlands).

238

239 3. Results and discussion

240

241 **3.1 Surface morphology (SEM)**

242 Protein beads were created by the various preparation methods and imaged with SEM, the
243 results of which can be found in Figure 1. The 15, 20, 25 and rapidly heated 25% heat-set
244 beads have an open and porous structure. The pores appear to be large enough for a pepsin
245 molecule (34620 Da, about 10nm) to enter, which suggests that these beads should display a
246 reaction-limited breakdown. The 10% cold-set beads have a much denser surface structure.
247 There are still pores visible, however they are much smaller than in the heat-set beads. This
248 might slow or completely stop the diffusion of pepsin into the beads, therefore the cold-set
249 beads are more likely to give a diffusion limited breakdown. The actual concentration of
250 protein in the 10% cold-set bead is however lower than for the heat-set beads, which are made
251 from protein concentrations of 15% and above, which means that less protein needs to be
252 broken down before the inner oil droplets are released. The freeze-dried 25% beads show a
253 very dense surface with several large surface defects. The surface of the 25% heat-set beads is
254 thus changed by the process of freezing and freeze-drying. The denser the surface, the more
255 likely it is to hinder diffusion and access of pepsin. The cracks in the surface are however
256 likely to promote bead breakdown.

257

258 **3.2 Protein content**

259 Table 1 shows the measured vs. the calculated protein content. The calculated protein content
260 is the amount of protein that was put into the system. The measured protein content is the
261 amount of protein actually measured by DUMAS. Since the sample was completely dried, we
262 have assumed for the calculated value that the beads consist primarily of protein and oil, and
263 have neglected any contributions from salts to the total weight. Based on the DUMAS results,

264 it can be seen that for all samples the measured protein content is lower than the expected
265 protein content. This reduction of protein content means that either extra oil was emulsified
266 into the protein phase during the double emulsification step, or that protein was lost from the
267 matrix during washing. It is probable that during the washing steps, protein that was not
268 tightly bound in the matrix was washed out. The 10% beads which were gelled with the cold-
269 set method lost the most protein, about 9% while the other samples lost on average about 2%.
270 The amount of aggregation that occurs between proteins is dependent on the protein
271 concentration¹⁹. The cold-set gel of only 10% protein is therefore expected to have the
272 highest concentration of non-aggregated protein present. These non-aggregated proteins can
273 diffuse out of the bead during washing. Other authors also saw loss of protein from their
274 system^{8, 20, 21}. Though it is impossible to say if oil was lost during emulsification and
275 gelation, based on the DUMAS results it can be concluded that a large amount of the oil
276 originally added to the interior of the beads is still present.

277

278 **3.3 Bead breakdown**

279 The graphs of Figure 2 show the size distribution of components present during the
280 breakdown of the beads in SGF. Enzyme was added separately after 6 minutes, to see if the
281 beads react to the SGF itself.

282

283 During the breakdown of the beads we determined the size distribution of the beads as a
284 function of time. Figure 3 shows an example of the size distribution graphs over time. The
285 solid line is the size distribution of the oil droplets in the 25% WPI solution, after the first
286 emulsification, but before the second emulsification and gelation step. At t=0 (min) a sample
287 was taken just before the addition of pepsin. The size distribution shows only the beads which
288 are about 10 μm in size. During the breakdown of the beads, sometimes particles were

289 detected with a size of around 100 μm . These large particles were few in number and were
290 most likely aggregates of beads and/or oil droplets. These peaks always disappeared when the
291 beads were completely broken down which suggests that flocculation was induced by the
292 partially broken down protein. The actual content of these peaks are discussed per sample. At
293 $t=42$ (min) the breakdown is in progress, oil droplets, beads and aggregates are present. At
294 $t=54$ (min) no further breakdown occurs and only oil droplets, which are not affected by the
295 presence of pepsin, are present.

296

297 For the 25% beads, after the enzyme was added, the average diameter of the beads increased
298 over 18 minutes, from about 8 μm to 9 μm . This suggests that the enzymes diffused into the
299 beads where they have broken bonds. Together with the low pH of the system, this led to
300 swelling of the beads. After 18 minutes the bead diameter decreased, which suggests a
301 breakdown of the beads. Single oil droplets were first detected 30 minutes after the addition
302 of pepsin. When about 20% of the volume represented single oil droplets, and thus a
303 significant amount of beads had broken down, the diameter of the beads (8.9 μm) was still
304 larger than the diameter of the beads before pepsin was added (8.2 μm). These observations
305 suggest a mechanism where the pepsin was able to diffuse into the bead relatively fast.
306 Subsequently the enzyme started to break down the protein matrix throughout the entire bead,
307 and when enough bonds had been broken the bead disintegrated into smaller pieces, releasing
308 the oil droplets. The smaller pieces initially (at least partially) aggregated into larger clusters,
309 and are subsequently broken down even further. Therefore for the 25% heat-set beads the
310 breakdown mechanism is reaction limited. Within 48 minutes of the beads entering the
311 simulated gastric system, all the internal oil droplets were released.

312

313 The 20% WPI beads started releasing oil droplets 12 minutes after addition of the enzyme. No
314 significant increase in diameter was seen as for the 25% beads. However, because of their
315 microstructure, which is similar to that of the 25% beads (as seen in the SEM images) and the
316 lower amount of protein present, we assume that here the enzymes were also able to diffuse
317 relatively fast throughout the bead, and the breakdown mechanism is reaction limited. After
318 the first 12 minutes, where oil droplets were first observed, most of the beads broke down
319 within the next 12 minutes. For the 20% beads, both the lag time before release of oil droplets
320 and the time within which the oil droplets were completely released were significantly shorter
321 than for the 25% heat-set beads.

322

323 The 15% heat-set beads started releasing their oil immediately after addition of pepsin which
324 is too fast to allow any conclusions about the mechanism of breakdown. From the similarities
325 in microstructure observed in the SEM images between the 15% beads and the 25 and 20%
326 heat-set beads, it is reasonable to assume that the breakdown was again reaction limited.
327 Where the 25% beads were stable for 24 minutes, and the 20% beads were stable for 12
328 minutes, the 15% beads were stable for less than 6 minutes. Extrapolating these observations,
329 the 10% beads were expected to break down more quickly than the 15% beads.

330

331 However the 10% cold-set beads showed a release of individual oil droplets 12 minutes after
332 pepsin addition. In that same time span the peak height in the volume distribution of the beads
333 reduced by about 20% and the bead diameter was reduced from 12 to about 8 μm . This more
334 gradual decrease (compared to that observed in the 15 to 25% beads) in size suggests that the
335 beads were broken down from the outside in, and the breakdown is therefore predominantly
336 diffusion limited, which is different from the heat-set beads. A decrease in diameter from 12
337 to 8 μm corresponds to a decrease in volume of the beads of 70%. With such an extent of

338 breakdown and the fact that oil droplets were present throughout the bead, it is unlikely that
339 oil droplets were not released during the first 12 minutes. The oil droplets may have initially
340 been in a flocculated state and hence represented by the aggregate peak.

341
342 When comparing the extent of breakdown of the beads after 12 minutes it is visible that for
343 the 15% heat-beads the amount of beads has decreased more than for the 10% cold-set beads,
344 about 75% decrease versus 15%. The 15% heat-set beads thus broke down faster than the
345 10% cold-set beads, despite the lower protein content of the 10% beads. The SEM results
346 show that the 10% beads have a much denser surface and this denser surface may slow down
347 the diffusion of pepsin into the interior of the beads. As a result the breakdown mechanism
348 has shifted from reaction limited to diffusion limited.

349
350 The rapidly-heated 25% heat-set beads broke down much more slowly than the standard 25%
351 heat-set beads. The rapidly-heated beads showed a much more significant degree of swelling
352 of the beads in this initial phase of the breakdown (from 7.5 to 13.6 μm). After 90 minutes,
353 the radius did not increase further, and remained stable. No significant release of oil droplets
354 was registered in a time span of 138 minutes, which is longer than the average residence time
355 of 2 hours in the stomach, according to Minekus et al. ¹⁸. Beads which are put in SGF without
356 pepsin did not show a significant change in radius (results not shown) so the pepsin clearly
357 plays a role in the increase in radius. The fast temperature increase from room temperature to
358 80°C, apparently resulted in a significantly different protein matrix which was much more
359 resistant to enzymatic breakdown. This difference in matrix is however not visible in SEM
360 analysis and there was also no significant difference in protein content of the beads.

361

362 The difference in protein matrix that was found between the 25% heat- set and rapidly-heated
363 25% heat-set beads might be found in the mechanism of protein gelation. Protein gelation
364 occurs roughly in two steps: the unfolding of the protein and the progressive aggregation of
365 the unfolded protein until a network is formed. The rapid heating most likely did not allow for
366 complete unfolding of the protein before aggregation occurred, resulting in a network with
367 proteins with more native folds than a network induced by slow heating. Barbé et al. found
368 that unheated β -lactoglobulin shows more resistance to hydrolysis in the stomach than heated
369 β -lactoglobulin, which breaks down more easily¹³. Dalgalarondo et al. similarly found that β -
370 lactoglobulin was only cleaved in ethanol concentrations higher than 20%, where the presence
371 of ethanol caused changes in the structure of β -lactoglobulin²².

372

373 Figure 4 shows the breakdown of freeze-dried, 25% heat-set beads. The freeze-dried 25%
374 beads broke down slower than the standard 25% heat-set beads. At the start of the
375 measurement we observed large aggregates, which were most likely flocs of beads, which
376 were slowly broken up by gentle stirring, both in water and after addition of SGF. The
377 addition of pepsin increased the speed in which the flocs fell apart into separate beads. After
378 30 minutes most beads appeared to be separated. Their size and number was then stable for
379 another 66 minutes before oil droplets were released. The data shows more variation here.
380 However, on average, after an enzyme treatment of 132 minutes, longer than the average
381 residence time of 2 hours in the stomach, about 60% of the volume still consisted of intact
382 beads. These beads hence broke down much more slowly than the standard 25% heat-set
383 beads.

384

385 Upon microscopic observation of the freeze-dried 25% heat-set beads during enzymatic
386 breakdown in the simulated gastric system, a distinct line within the beads was observed,

387 which moved further inward over time (Figure 5, left). This is most likely a hydration front
388 which slowly moved into the bead. The breakdown of the beads might be slowed down by the
389 time the bead needs to fully hydrate. In regions that are not fully hydrated, the enzyme cannot
390 diffuse easily and thus cannot break down the matrix. To see if the removal of this hydration
391 time would give a similar breakdown pattern as the standard 25% heat-set beads, a sample of
392 the freeze-dried beads was left in water overnight and given the SGF and pepsin treatment the
393 next day. Microscopic observation showed that this hydration line was not present anymore
394 and full hydration was assumed (Figure 5, right).

395
396 The hydrated, freeze-dried beads also showed flocs at the start of the measurement, but these
397 were smaller than those observed in the sample which was not rehydrated. After about 18
398 minutes (compared to 30 minutes for the freeze-dried sample) the flocs were broken down to
399 single beads by the presence of pepsin. After 36 more minutes, 6 minutes more than for the
400 standard 25% heat-set beads but 36 minutes less than for the freeze-dried beads, individual oil
401 droplets started being released. Again the data shows significant variation here, but substantial
402 release occurs within the average time of 2 hours that food stays in the stomach.

403
404 In the detailed graph of the freeze-dried and rehydrated, freeze-dried beads, it can be seen that
405 when oil droplets were released, the bead diameter was still as large as it was before oil
406 droplets were released, as was also with the standard 25% beads. This means that, though the
407 breakdown was markedly slower than the standard 25% beads, it was still a reaction limited
408 breakdown.

409
410 Compared to the normal 25% heat-set beads, it took more time before oil droplets were
411 released from the hydrated beads, and it took more time before all beads were broken down.

412 Compared to the freeze-dried beads, it took less time before oil droplets were released from
413 the hydrated beads and it took less time for the beads to break down. This was partly caused
414 by the flocculation and dehydration, as hydrated freeze-dried beads broke down more quickly,
415 but removal of the flocculation and dehydration did not revert the freeze-dried sample to the
416 behaviour of the non- freeze-dried sample. The breakdown was still markedly slower. In the
417 SEM results it can be seen that the surface morphology of the freeze-dried and non-freeze-
418 dried samples was very different. Strambini and Gebellieri found that changes in secondary
419 and tertiary structure occur in single tryptophan molecules when freezing²³. Li et al. found
420 that freeze-drying increased aggregate size of soy proteins²⁴. The freezing and freeze-drying
421 process thus changed the structure markedly, as also seen in the SEM images. This change
422 slowed down the breakdown of the beads. Because both systems are reaction limited, we
423 expect that the process of freeze-drying created protein structures within the bead, which were
424 harder for pepsin to cut.

425

426 **3.4 Diffusion of probe molecules**

427 Images of the diffusion of Dextran-FITC through the beads can be found in Figure 6. The
428 green areas in the image indicate where Dextran-FITC is present. Images A, B and C show
429 that Dextran-FITC diffuses into and throughout the 25% heat-set, 25% rapidly heated and
430 25% hydrated freeze-dried beads within 1.5 minutes. For the 25% heat-set and 25% hydrated
431 freeze-dried, and thereby by extension also 20% and 15% heat-set beads, the diffusion is
432 therefore confirmed to be very fast and the breakdown of the beads is predominantly reaction
433 limited. Images D show that the Dextran-FITC does not diffuse easily into the 10% cold-set
434 bead. After 1.5 minutes, there is clearly more Dextran-FITC at the outer perimeter than in the
435 centre of the bead. Only after 12 minutes is the Dextran-FITC homogenously distributed

436 throughout the bead. The mechanism of breakdown for the 10% beads is therefore more likely
437 to be diffusion than reaction limited.

438

439 The results of this study have been summarised in Table 2

440

441 In this study we showed that the structure of filled hydrogel beads is important for the rate and
442 way these beads break down and release their load. The variations of release found in this
443 study, while using the same building blocks, gives opportunities in the creation of novel
444 targeted release systems. When a quick release of the encapsulated compound is required, a
445 low protein concentration and a reduced heating gradient during gelation are recommended.
446 When a delayed release in the intestinal tract is required, a high protein concentration,
447 external gelation, an increased heating gradient during gelation and application of freeze-
448 drying should be used. In the case of slow release systems, and thus slow digestion of the
449 proteins, one must however be aware that the release and digestion can be so slow as to
450 reduce the nutritional availability and also that not fully digested proteins can cause allergic
451 reactions in the intestine ²⁵.

452

453 4. Conclusions

454

455 In this study we investigated the influence of protein concentration, method of gelation, and
456 freeze-drying on the speed and way of breakdown of oil filled protein microbeads.

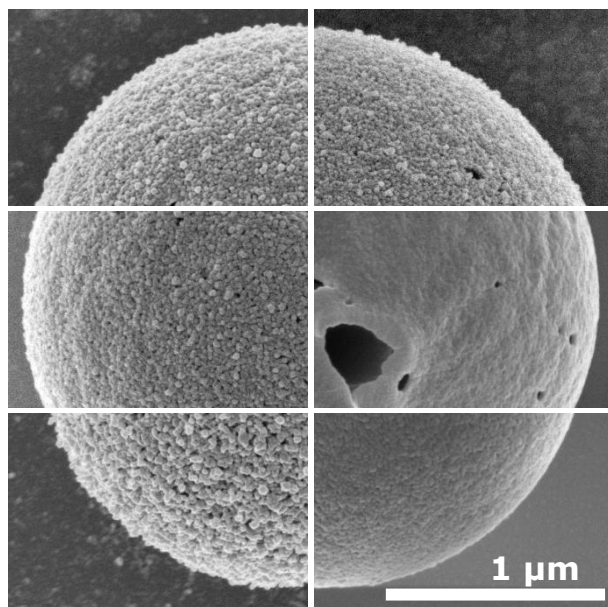
457 Similarly produced microbeads with a higher protein concentration break down slower. The
458 porous nature of the heat-set beads assured that the pepsin could diffuse throughout the bead
459 and break the entire bead down evenly, resulting in a reaction limited breakdown process. The
460 cold-set 10% protein microbead was broken down slightly slower than the 15% heat-set
461 beads. Because of the method of gelation, the surface of the 10% beads was much denser,
462 which resulted in a diffusion barrier for the pepsin and resulted in a diffusion-limited
463 breakdown of the beads. Increasing the rate of temperature increase of the heating step
464 changed the breakdown kinetics of the 25% heat-set beads, and these beads needed more than
465 2 hours to break down. Both the standard 25% and rapidly heated 25% heat-set beads allowed
466 diffusion of enzyme throughout the bead and no difference in surface morphology or probe
467 diffusion was seen. When the 25% beads are freeze-dried, the rate of breakdown is greatly
468 slowed. Flocculation and dehydration of the beads were important factors but rehydrating did
469 not revert the breakdown kinetics of the freeze-dried beads back to that of the standard 25%
470 beads. The freeze-drying altered the microstructure of the beads, therefore it took longer for
471 the pepsin to break down the structure.

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473

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520 Figure 1



521

522

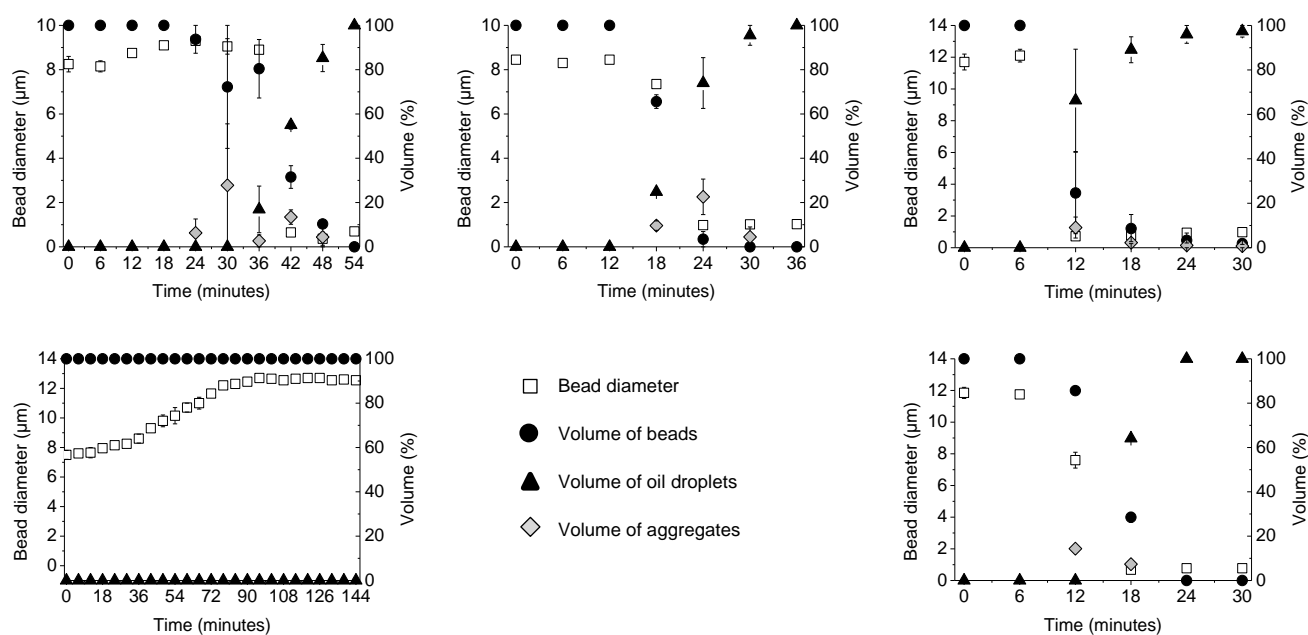
523 Figure 1: SEM images of sections of protein microbeads. Left-hand side, up-down: 25%,

524 20%, 15% heat-set. Right-hand side, up-down: rapidly heated 25% heat-set, freeze-dried 25%

525 heat-set, 10% cold-set.

526

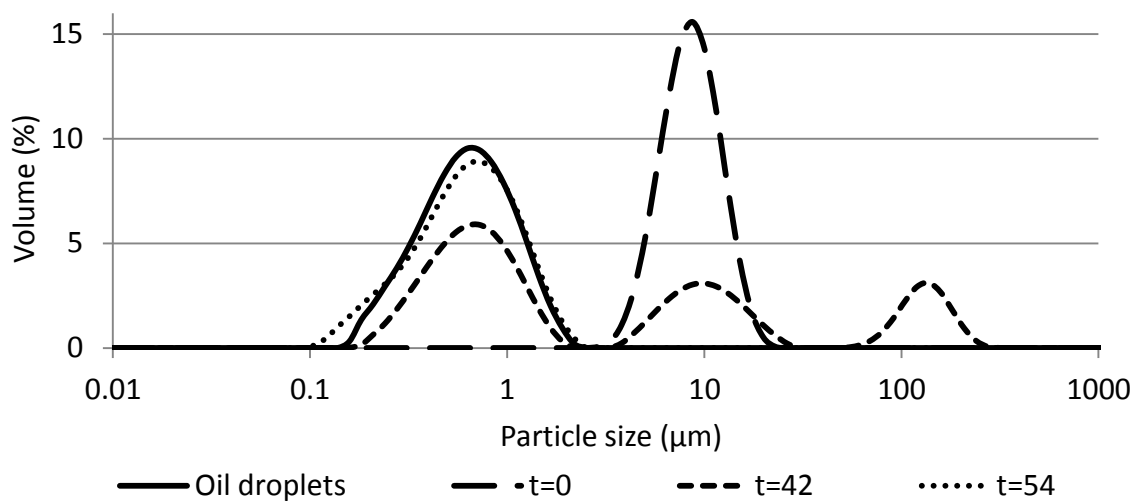
527 Figure 2



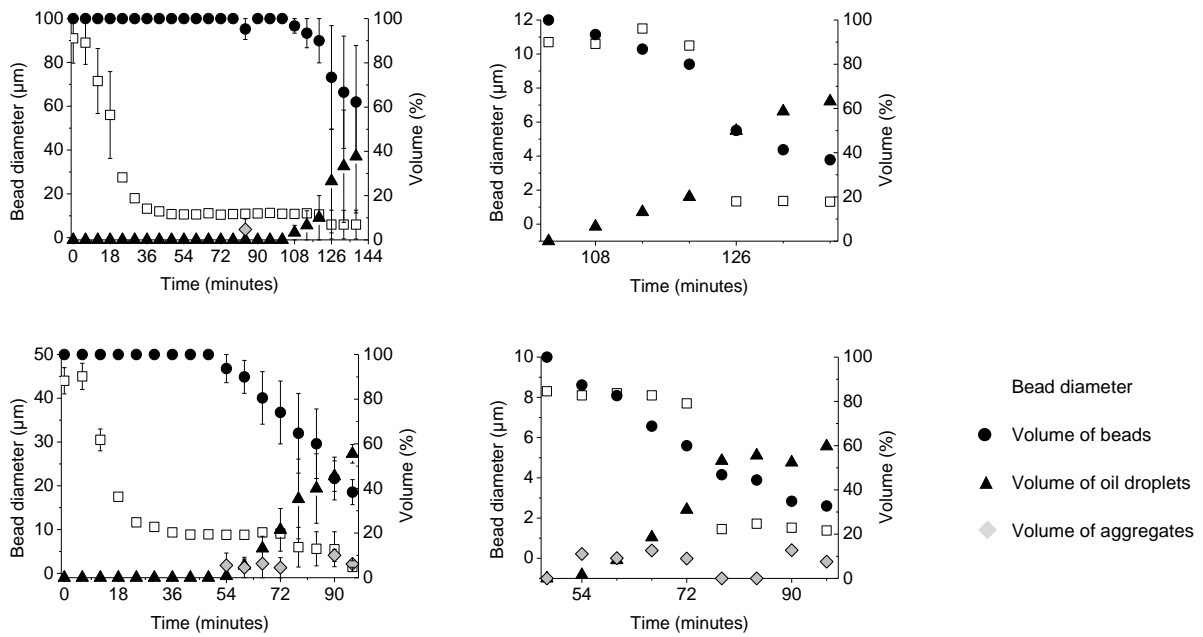
528

529 Figure 2: Breakdown of WPI beads made with different WPI concentrations and process
 530 conditions. Top left to right: 25%, 20%, 15% heat-set. Bottom left, right: rapidly heated 25%
 531 heat-set, 10% cold-set.

532 Figure 3



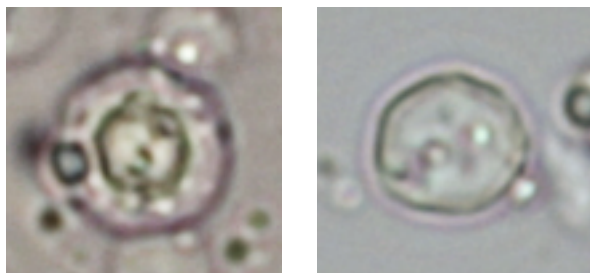
535 Figure 4



536 Figure 4: Breakdown of WPI beads. Top: left: freeze-dried beads, right: detail of a single
 537 measurement. Bottom: left: hydrated freeze-dried beads, right: detail of a single measurement.

538

539 Figure 5

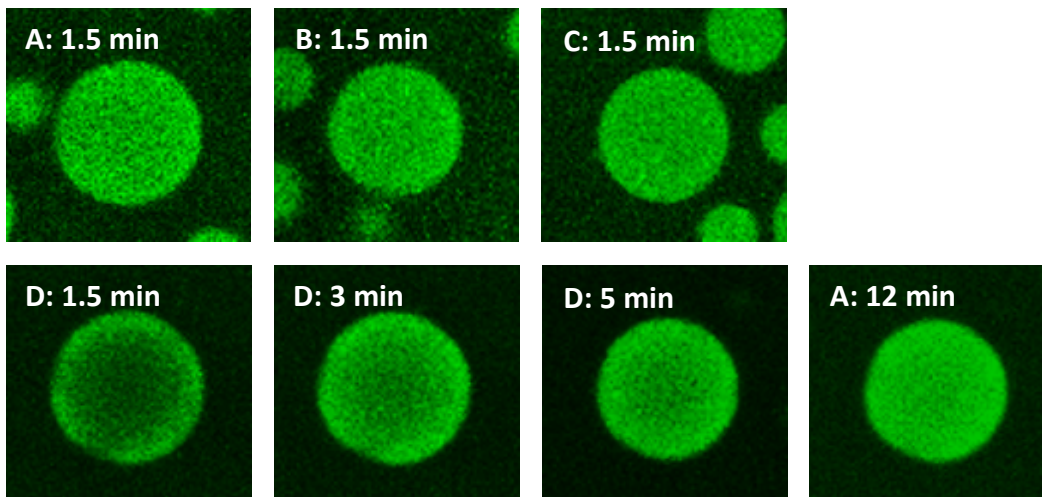


540

541 Figure 5: Microscopic observations of freeze-dried WPI beads during gastric treatment. Left:

542 after 60 minutes in SGF, Right: after overnight stirring in water.

543 Figure 6



544

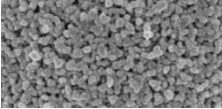
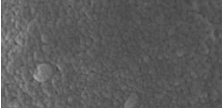
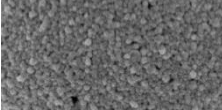
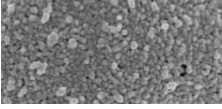
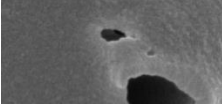
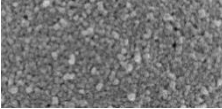
545 Figure 6: Diffusion of Dextran-FITC through 25% heat-set (A), 25% rapidly heated (B) 25%
546 freeze-dried, hydrated (C) and 10% cold-set (D) beads over time in minutes.

547 Table 1: The expected amount and the measured amount of protein in the beads are given.

	Expected protein content %(w/w)	Measured protein content %(w/w)
25%, heat-set	82.46	81.02 (± 0.36)
20%, heat-set	79.00	77.45 (± 0.36)
15%, heat-set	73.83	70.70 (± 0.08)
10%, cold-set	65.29	55.95 (± 0.10)
25%, quick heat-set	82.46	79.98 (± 0.19)

548

549 Table 2: Summary of rate and way of breakdown of WPI beads with different protein
 550 concentrations, gelation methods and further processing, in order of breakdown rate.

Bead type (in order of breakdown rate)	Microstructure	Type of breakdown	Start release (minutes)	Complete breakdown (minutes)
15% protein Heat-set		Reaction limited	6	18
10% protein External – Cold-set		Diffusion limited	6	18
20% protein Heat-set		Reaction limited	12	24
25% protein Heat-set		Reaction limited	18	48
25% protein Freeze-dried Hydrated		Reaction limited	54	> 90 ^a
2 hours: average residence time of food in the stomach				
25% protein Freeze-dried		Reaction limited	120	>132 ^b
25% protein Heat-set - Fast heat increase		No breakdown, some swelling	>148	>148 ^c

a: about 60% of the volume at the time mentioned is released oil droplets

b: about 60% of the volume at the time mentioned is released oil droplets

c: about 0% of the volume at the time mentioned is released oil droplets