



HAL
open science

Enhancing the activity of biocatalysts supported on calcium phosphate by inducing mesoporosity with phosphopeptides

Surender Kumar Dhayal, Martin Lund, J M van den Brink, Ghouti Medjahdi, Alain Celzard, Vanessa Fierro, Carole Gardiennet, Andreea Pasc, Nadia Canilho

► To cite this version:

Surender Kumar Dhayal, Martin Lund, J M van den Brink, Ghouti Medjahdi, Alain Celzard, et al.. Enhancing the activity of biocatalysts supported on calcium phosphate by inducing mesoporosity with phosphopeptides. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2023, 660, pp.130906. 10.1016/j.colsurfa.2022.130906 . hal-03959654

HAL Id: hal-03959654

<https://hal.univ-lorraine.fr/hal-03959654>

Submitted on 1 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

20 **ABSTRACT**

21 Preventing enzyme deactivation while increasing their accessibility by adsorption in
22 mesopores remains a key challenge in the design of biocompatible supported enzymatic
23 catalysts. Herein, it is expected that food-grade monomeric or polymeric glycopeptides
24 containing organic phosphate groups (as casein phosphopeptide derivatives) enhances the
25 entrapment of the enzyme over the one pot method of wet precipitation of calcium
26 phosphate (CaP) leading to a biocompatible hybrid organic/inorganic catalyst.
27 The supported catalyst was prepared by wet precipitation of calcium and phosphorous in
28 the presence of either monomeric glycopeptide or polymeric glycopeptide. These organic
29 glycopeptides had an influence on the electrostatic stabilization during the nucleation of
30 calcium phosphate particles, as tracked by dynamic light scattering and by scanning electron
31 microscopy.
32 The phosphopeptide derivatives, were able to decrease the crystallization degree in calcium
33 phosphate particles, as demonstrated by XRD and solid-state NMR analysis. Furthermore,
34 according to N₂ sorption data, the glycopeptide derivatives induced interparticle mesopores,
35 which might be responsible of the enhanced enzymatic activity of the supported biocatalyst
36 that improved from 52 (+/-19) NLU·g⁻¹ without organic compound to 607 (+/-34) NLU·g⁻¹.

37

38 **KEYWORDS** : biomaterial; biomineralization; hydroxyapatite-like nanoparticles; enzymatic
39 supported catalyst

40

41

42

43 **1. Introduction**

44 The wet precipitation of calcium and phosphate ions can lead to various calcium phosphate
45 (CaP) polymorphs, including hydroxyapatite ($\text{Ca}_5(\text{OH})(\text{PO}_4)_3\text{H}_2\text{O}$) [1]. The precipitation
46 process starts with the nucleation and growth of primary particles, which can then
47 aggregate into larger ones. Depending on the colloidal stability of the particles, inter-grains
48 porosity can be induced, and in general, the higher is the stability, the higher is the porosity.
49 Still, such particles exhibit only a low surface area and low pore volume, especially at the
50 mesoscale, which limits the use of calcium phosphate support as a enzymatic biocatalyst.
51 Strategies to tune the mesoporous texture in these inorganic particle matrices are based on
52 controlling the crystal size and/or reducing the degree of crystallinity, either by using
53 templates (e.g. based on self-assembled mesophases) to guide the crystal growth or by
54 using polymeric coatings [2]. Much work on the immobilization of proteins on biocompatible
55 supports such as porous silica for the development of enzymatic catalysts has also been
56 published [3,4,5]. However, increasing the porosity of an inorganic support by coating it
57 with organic food-grade polymers, for example, remains a straightforward approach
58 compared to post-synthesis adsorption of the enzyme on a porous inorganic material. The
59 coating strategy is much more suitable for bio-applications, especially for the elaboration of
60 supported enzymatic catalysts. Indeed, both trapping and coating can be done *in situ* during
61 the precipitation process of the inorganic matrix. Moreover, it is known that nucleation,
62 growth and crystallinity of inorganic particles can be altered by the presence of a polymer in
63 the synthesis medium [6,7]. The calcium phosphate nanoclusters (CaP) present in casein
64 micelles are one example of particles stabilized by the organic phosphate groups of casein
65 molecules. In general, casein adsorption is driven by interactions between the organic
66 phosphate (serine-bound) and the surface of calcium phosphate particles. This adsorption

67 leads to a 'brush-like' layer of casein polymers that improves colloidal stability and prevents
68 the aggregation of nanoclusters. Furthermore, it has reported that the crystalline degree of
69 calcium phosphate particles can be attenuated by the presence of biomolecule containing
70 organic phosphate during the precipitation reaction [8]. Moreover, when proteins such as
71 enzymes will be involved together with organic phosphate in the precipitation reaction, its
72 will be trapped in the pores of the inorganic matrix [9]. The size of most food-grade enzymes
73 is in the range 2 – 20 nm, so the pore size of the inorganic matrix must be similar or slightly
74 larger. Therefore, for the effective immobilization and retention of active molecules, both
75 surface area and pore size must be controlled. In this study, we report the synthesis of
76 supported biocatalysts by a one-step procedure involving wet precipitation and enzyme
77 entrapment. An oligomeric or polymeric form of a phosphopeptide (*e.g.* casein
78 glycomacropeptide or CGMP), extracted from whey, was added as a source of organic
79 phosphate, to behave as a mesoporegen by modulating the crystallinity of the inorganic
80 calcium phosphate matrix. The resulting materials were characterized in terms of porous
81 texture, morphology and crystallinity. Additionally, DLS and ³¹P NMR analysis were
82 performed to better understand the formation mechanism of the hybrid inorganic-organic
83 calcium phosphate particles. Finally, the catalytic efficiency of the biocatalyst support was
84 determined as a function of the oligomer vs polymer nature of the glycopeptide.

85

86

87

88

89 **2. Material and Methods**

90 **2.1 Materials**

91 The casein glycomacropeptide (CGMP) was purified from a commercial sample (Lacprodan
92 CGMP-10, Arla Food Ingredients, Denmark) [10-13]. After purification, the dry matter
93 contained around 85 % protein of which about 73 % was monomeric CGMP (Mono-CGMP).
94 Polymeric CGMP (Poly-CGMP) was then prepared from this monomer by enzymatic
95 crosslinking with microbial transglutaminase (mTG), following a procedure previously
96 reported (see SI1) [14-18]. The enzyme (Enz), a lactase (Trade name Ha-Lactase, which
97 activity is of 5200 NLU·g⁻¹ of solution, namely 100k NLU·g⁻¹ of protein), was provided by Chr.
98 Hansen A/S, Denmark. The lactase activity was measured by using o-nitrophenyl- β -
99 galactopyranoside (ONPG) as substrate and the catalytic test was done according to the
100 method of Kim *et al.* [19]. The following chemicals were purchased either from Sigma
101 Aldrich for ONPG, dithiothreitol (DTT), calcium chloride dihydrate (CaCl₂·2H₂O) and
102 magnesium sulphate heptahydrate (MgSO₄·7H₂O) or from Merck for disodium hydrogen
103 phosphate dihydrate (Na₂HPO₄·2H₂O), sodium dihydrogen phosphate monohydrate
104 (NaH₂PO₄·H₂O), sodium chloride (NaCl), sodium carbonate (Na₂CO₃) and
105 ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA). All were used as received.
106 The microbial transglutaminase (mTG) used for the enzymatic crosslinking of the monomeric
107 CGMP was from Ajinomoto and had an activity of 1000 U per g of powder, as measured by
108 the hydroxamate colorimetric method proposed by Folk *et al.* [20]. All other chemicals used
109 and mentioned in this work were of analytical grade.

110 The morphology and microstructure of the powdered microparticles were analysed using
111 Scanning Electron Microscopy (SEM, Hitachi microscope, Hitachi S4800). X-ray powder
112 diffraction analysis was performed at 45 kV and 40 mA, with a wavelength of $\lambda_{\text{CuK}\alpha} = 1.5406$
113 Å. The crystalline phases were identified by comparing the X-ray diffraction patterns with
114 the JCPDS powder diffraction files produced by the International Centre for Diffraction Data.

115 The degree of crystallinity (DOC) of the samples was calculated from the ratio between the
116 total crystalline area divided by the sum of total crystalline area plus total amorphous area
117 extracted from the diffusion background. Then, the amorphous content (%) was determined
118 from 1-DOC (see SI6c). The molar ratio of calcium (Ca) to phosphate (P) was determined by
119 ICP-OES elemental analysis.

120 **2.2 Synthetic route: wet precipitation**

121 Four materials have been synthesized by wet precipitation. The sample CaP refers to the pure
122 inorganic matrix of calcium phosphate (CaP) prepared without enzyme (Enz) and CaP-Enz with
123 enzyme. The hybrid particles obtained in the presence of enzyme and mono-CGMP (Mono)
124 or poly-CGMP (Poly) have been abbreviated as CaP-Enz-Mono and CaP-Enz-Poly, respectively.
125 The following stock solutions were used for preparing the different samples mentioned
126 above: (a) 0.1 M sodium phosphate buffer and 0.1 M NaCl at pH 7.0, named Na-PB, (b), 4.5M
127 CaCl₂, (c) 0.3M Na₂HPO₄, (d) 60 g·L⁻¹ of Mono-CGMP in Na-PB, (e) 20 g·L⁻¹ of Mono-CGMP in
128 0.3 M Na₂HPO₄, (f) 60 g·L⁻¹ of Poly-CGMP in Na-PB, and (g) 20 g·L⁻¹ of Poly-CGMP in 0.3 M
129 Na₂HPO₄. The lactase enzyme stock solution (Enz) was used as received and MQ-water (W)
130 was used in all stock solutions. The preparation of the different samples involved three
131 sequential steps. Step-(i): Prepare 940 mL solution by mixing equal volumes of W and Na-PB
132 (a) for CaP sample; Enz and Na-PB (a) for CaP-Enz sample; Enz and 60 g·L⁻¹ of Mono-CGMP (d)
133 for CaP-Enz-Mono sample; Enz and 60 g·L⁻¹ of Poly-CGMP (f) for CaP-Enz-Poly sample. Step-
134 (ii): 60 mL of CaCl₂ stock solution (b) were added dropwise for 5 min under 1000 rpm to 940
135 mL solution obtained in step-(i). Step-(iii): Addition of the resulting mixtures to 1L of
136 respectively 0.3 M Na₂HPO₄ in the case of CaP and CaP-Enz samples, 20 g·L⁻¹ of Mono-CGMP
137 stock solution (e) for the CaP-Enz-Mono sample; and 20 g·L⁻¹ of Poly-CGMP stock solution (g)

138 to prepare CaP-Enz-Poly. In each case the final pH was 7. Those solutions were also added
139 over a period of 5 min under stirring at 1000 rpm. After additional 5 min of stirring, the
140 mixtures were allowed to rest for 30 min and then were stirred at 500 rpm for 2 h at 20 °C.
141 Mixing was stopped to allow particle sedimentation and slow growth and was continued
142 gently after to avoid their agglomeration. At the end of step-(iii), the particle suspensions
143 were centrifuged at $3000 \times g$ (20 °C, 30 min). The pellets obtained after centrifugation were
144 washed 3 times with 2L of MQ-water and dried at 20 °C using laminar airflow. The dried
145 particles were hand grounded using a mortar and pestle and then stored in closed containers
146 at room temperature. At the end of the step-(iii) 25 ± 3 g of dry powder was obtained from 1
147 L of starting suspension.

148 **2.3 Loading and Activity of immobilized lactase**

149 The amount of immobilized lactase in each sample was determined from sodium dodecyl
150 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and bicinchoninic acid (BCA)
151 calibration curves (see SI2). The enzyme loading ($\text{mg}\cdot\text{g}^{-1}$) was calculated from the ratio of the
152 measured amount of protein (mg) to the weight of dry powder (g). The average $\text{mg}\cdot\text{g}^{-1}$ of
153 enzyme loading and standard deviation were obtained from three replicates. Briefly, the
154 powdered catalyst was suspended in 1 mL of PEM buffer (the same as Britton-Robinson
155 buffer, see SI3 for details) at $10 \text{ g}\cdot\text{L}^{-1}$. After separate incubation of catalyst and ONPG
156 substrate solutions at 40 °C, the two preparations were mixed and stirred at 1000 rpm for
157 10 minutes at the same temperature of 40 °C. The reaction was then stopped by adding
158 Na_2CO_3 (1M), followed by centrifugation at $10,000 \times g$ for 5 minutes. The absorbance of the
159 supernatant was measured at 420 nm. A blank measurement was done without any
160 particles in the solution (see SI3). The measured activity was divided by the weight of the

161 dry powder to obtain the specific activity of the material, in $\text{NLU}\cdot\text{g}^{-1}$. The average $\text{NLU}\cdot\text{g}^{-1}$
162 and standard deviation were obtained from three replicates.

163

164 **2.4 Textural characterization by nitrogen sorption**

165 The BET area (A_{BET}) and the pore size distribution (PSD) were determined using nitrogen
166 adsorption at -196°C in an automatic equipment (ASAP 2020, Micromeritics) [21-24]. All
167 samples were pre-treated before nitrogen adsorption by degassing under secondary
168 vacuum at 25°C for at least 48h. The parameters of the analyses are detailed in SI4.

169 **2.5 Dynamic Light Scattering (DLS) on colloidal CaP in the presence of Mono-CGMP or** 170 **Poly-CGMP**

171 The hydrodynamic radius (R_h) of the prepared colloidal CaP suspensions was measured at
172 25°C using a Zetasizer (Nano ZSP, Malvern Instruments, Worcestershire, UK) equipped with
173 a 633 nm laser. The intensity fluctuations in the scattered light were measured at 173° for
174 10 to 30 s. At least ten scans were averaged to obtain the correlation curve. The size
175 distributions based on intensity diffusion were obtained using the general-purpose model in
176 the standard Malvern software. To study the colloidal stabilization of CaP particles formed
177 in the presence of Mono-CGMP or Poly-CGMP, two types of experiments were carried out
178 without enzyme. In the first series, the concentration of the Poly-CGMP stock solution was
179 fixed at $10\text{ g}\cdot\text{L}^{-1}$ in a 50 mM Na-PB solution and the CaP particles were formed by one-pot
180 precipitation by adding appropriate amounts of CaCl_2 to reach a final concentration of 10,
181 50, or 100 mM, respectively. Then, the solution was rapidly transferred into the DLS
182 instrument for analysis. The delay between the addition of CaCl_2 and the start of analysis
183 was one minute. In the second series of experiments, only Poly-CGMP solutions were

184 prepared between 1 and 20 g·L⁻¹ in a 100 mM Na-PB solution. The CaP particles were
185 precipitated by adding a fixed amount of 2 μL CaCl₂ (5M), e.g. 10 mM Ca²⁺ as final
186 concentration, to 1 mL of Poly-CGMP in Na-PB. The solution was kept at rest for 30 minutes
187 at 25 °C, before the DLS measurements. The average and standard deviation were
188 calculated from the data obtained from three replicates.

189 **2.6 ¹H and ³¹P solid-state NMR**

190 The experiments were performed on a Bruker Biospin Avance III spectrometer, at a
191 magnetic field of 7 T corresponding to a ¹H resonance frequency of 300 MHz, using a 4 mm
192 probe. The spinning frequency was set to 12.5 kHz, unless otherwise stated. A 300 s recycle
193 delay was used for ³¹P direct excitation spectra (single-pulse experiments). Proton to
194 phosphorus cross-polarization (CP) 1D spectra were recorded with ¹H and ³¹P resonance
195 frequency fields of 47 and 34 kHz respectively, using a contact time of 1 ms. In two-
196 dimensional ¹H-³¹P HETCOR correlations, proton to phosphorus-31 polarization transfer was
197 achieved by CP with a 2 ms contact time. SPINAL-64 proton decoupling at 60 kHz was
198 applied during 40 ms ³¹P acquisition. ³¹P and ¹H chemical shifts are relative to external 85%
199 H₃PO₄ and TMS standards. Deconvolution and low spinning frequency spectra simulations
200 were performed with dmfit software [25,26].

201

202 **3. Results and discussion**

203 **3.1 CaP-based Biocatalyst Morphology**

204 The main goal of this study was to investigate the formation of calcium phosphate-supported
205 biocatalysts for food applications. For this purpose, organic phosphate sources (CGMP),
206 namely phosphopeptides, were used to produce hybrid materials previously labelled CaP,

207 CaP-Enz, CaP-Enz-Mono and CaP-Enz-Poly. Figures 1 and S15 show the morphologies of the
208 aforementioned systems (CaP, CaP-Enz, CaP-Enz-Mono and CaP-Enz-Poly), as obtained by
209 scanning electron microscopy. Both pure CaP and CaP-Enz particles had platelets of brushite-
210 like shape, which suggests that the enzyme has no influence on the formation of CaP particles
211 (Figure 1a, 1b and Figure S15). On the contrary, the addition of phosphopeptides significantly
212 influenced the morphology of the resulting hybrid particles. Indeed, the platelets size
213 decreased when using monomeric CGMP (Figure 1c) to completely disappear when using
214 polymeric CGMP (Figure 1d). Moreover, aggregated, almost spherical nanoparticles appeared
215 in the samples CaP-Enz-Mono and CaP-Enz-Poly. The sphericity of the particles seems to be
216 better defined in the presence of the polymer compound and the size of the primary particles
217 is around 50 – 100 nm, which is close to the R_h of the pure polyglycopeptide in aqueous
218 solution [27].

219

220 **3.2 CaP particles formation followed by DLS**

221 To decipher the formation of CaP particles, dynamic light scattering (DLS) analysis was
222 performed in the presence of Mono-CGMP and Poly-CGMP to evidence their influence in the
223 colloidal stabilization of CaP particles. As the synthesis is performed at pH 7 in the presence
224 of Na_2HPO_4 as reagent, the precipitation of brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) should be the most
225 favourable solubility product constant (K_{sp} , of $2.6 \times 10^{-7} \text{ M}^2$). In the conditions chosen for DLS
226 experiments, the ion activity product ($[\text{Ca}^{2+}]_x[\text{HPO}_4^-]$) is high and therefore the
227 supersaturation might explain the rapid precipitation of CaP at room temperature (20 °C).
228 According to the DLS data (not shown), the pure CaP particles rapidly grew (within minutes)
229 to a size larger than 1 μm and subsequently settled down under the influence of gravity. The

230 same behaviour was observed for the CaP-Enz matrix, indicating that lactase does not act as
231 a stabilizer either. In that case, the electrostatic repulsions from the surface charge are not
232 high enough to prevent particle growth to large dimensions. In the case of Mono-CGMP and
233 Poly-CGMP, different kinetics were observed. Figure 2a depicts the variation of the
234 hydrodynamic radius (R_h) during the formation of CaP particles over time, at three different
235 concentrations of Ca^{2+} ions, i.e., 10, 50 and 100 mM, in the presence of Poly-CGMP at a
236 concentration of $10\text{ g}\cdot\text{L}^{-1}$.

237 In the presence of Mono-CGMP (data not shown), the formation rate of CaP particles is
238 slightly slowed down compared to pure CaP or CaP-Enz particles, which formed
239 instantaneously. The monomeric CGMP does not seem to be an efficient particle growth
240 inhibitor or colloidal stabilizer because large CaP particles (> 500 and 1000 nm) were formed
241 within around 10 minutes in the presence of 10, 50 and 100 mM of Ca^{2+} . Then, at longer
242 time scales ($> 10\text{ min}$), a settlement phenomenon appeared. However, under the same
243 conditions, CaP particles formation was found to be significantly delayed in the presence of
244 the polyglycopeptide (Figure 2a). In fact, looking at the R_h values extracted from scattering
245 intensity over time, below 10 minutes, the radius of the scattering objects has a size
246 between 60 and 75 nm (corresponding to the P1 plateau), which corresponds to the R_h of
247 the polyglycopeptide [27]. Then, suddenly, a new plateau of size around 90 – 110 nm was
248 reached (referred to as the P2 plateau) for any concentration of Ca^{2+} . According to the
249 representations of the intensity size distribution data (Figure 2b, 2c), the CaP clusters are
250 stabilized by the polymeric CGMP, leading to highly monodisperse nanoparticles. This
251 indicates that the Poly-CGMP provides much greater colloidal stability to the CaP particles
252 than its monomeric form. Moreover, the size distribution representation for the P1 plateau
253 overlaps the size range of the P2 plateau distribution. This could be related to ripening,

254 which is a continuous process along the reaction time. Thus, the particle size distribution
255 measured at a given time must comprise particles from both the initial stage and the
256 ripened stage. These results obtained on the CaP and Poly-CGMP system seem to be similar
257 to those observed in the case of CaP particles stabilized by Fetuin-A. Indeed, Fetuin-A is
258 known to be an inhibitor of CaP mineralization and deposition in the soft tissues of animals
259 and humans [28].

260 Wald *et al.* [28] found that in the presence of Fetuin-A, the CaP/Fetuin-A particles formed
261 had initially a R_h of 40 – 60 nm which ripened to a R_h of around 100 nm after a lag period of
262 about 300 minutes. They also argued that particle size was mainly governed by the
263 concentrations of the mineral ion and Fetuin-A. Increasing the concentration of Fetuin-A led
264 to smaller particles, while increasing the concentration of the mineral ion accelerated the
265 particle ripening process at fixed amount of Fetuin-A. Thus, they proposed that the size and
266 stability of the ‘calcioprotein’ particles could be controlled by a combination of these
267 physicochemical parameters. It should be noted that Fetuin-A has several phosphoserine
268 residues and O- and N-glycosylated chemical groups (see *e.g.* P02765 in www.uniprot.org).

269 In our case, the monomeric CGMP prepared from κ -casein phosphorylated serine residues
270 and the polyglycopeptide synthesized from the monomeric form is a polyelectrolyte.
271 Therefore, it has a higher density of phosphoserines per molecule, which allows binding to
272 the surface of inorganic CaP in a way analogous to caseins micelles for CaP nanoclusters
273 [29]. Moreover, by analogy with Fetuin-A, the polymeric CGMP also has O-glycosylated
274 residues, which are known to control the size and stability of CaP particles by varying the
275 Poly-CGMP concentration, as presented in Figure 3. Thus, according to Figure 3, the R_H
276 values (measured after 10 min of reaction) decrease with increasing Poly-CGMP amount in
277 the solution. Then, above $10 \text{ g}\cdot\text{L}^{-1}$, the CaP particle size is stabilized at a hydrodynamic radius

278 of around 100 nm. This clearly demonstrates that the polymeric CGMP acts as a colloidal
279 stabilizer, and it can be speculated that the mode of action is a combination of electrostatic
280 and steric repulsions originating from a combination of a charged backbone and a higher
281 molar mass of the polyelectrolyte. The threshold of $10 \text{ g}\cdot\text{L}^{-1}$ can be designated as the
282 maximum surface concentration of the polymer adsorbed on the CaP particles formed.

283

284 **3.3 Textural and structural properties**

285 Nitrogen adsorption isotherms have led to a better understanding of the textural properties
286 of the dried materials (see SI4 Table and Figures). The results show that the specific area (A_{BET})
287 and the mesoporous volume (V_{mes}) increased in the case of CaP-Enz-Mono and CaP-Enz-Poly
288 particles compared to CaP and CaP-Enz: 70 and 59 $\text{m}^2\cdot\text{g}^{-1}$ vs 36 and 31 $\text{m}^2\cdot\text{g}^{-1}$ for A_{BET} ,
289 respectively, and 0.218 vs 0.150 $\text{cm}^3\cdot\text{g}^{-1}$ in average, for V_{mes} , respectively. This could be due
290 to voids formed between aggregated nanoparticles or, in other words, due to interparticle
291 porosity. In the case of platelet-shaped particles, these voids are almost negligible at the
292 mesoscale. On the contrary, less ordered aggregated nanoparticles, trapped in an organic
293 network, might induce the observed mesoporosity (Table SI4). Furthermore, the pore size
294 distribution (PSD) became much more monodisperse in the presence of Mono-CGMP or Poly-
295 CGMP, and it was centred on 15 nm (Figure SI4 a, b). These results highlight the fact that the
296 porous texture in the mesopore range (2-50 nm) of the inorganic carrier matrix can be tailored
297 by the monomer or the polymer to obtain pore sizes suitable for enzyme entrapment (herein
298 Ha-Lactase, 14 nm in size). In order to understand whether the CGMP (monomer or polymer)
299 interferes with the inorganic particles in the crystallisation process, the variation of
300 crystallinity in the CaP, CaP-Enz, CaP-Enz-Mono and CaP-Enz-Poly samples was measured by
301 XRD (See 4a,b). Thus, the platelet-shaped particles observed for the CaP or CaP-Enz systems

302 were clearly identified as monoclinic, prismatic crystals of brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$). This result
303 agrees with the existing literature. For instance, under neutral or slightly acidic conditions,
304 wet precipitation of CaP particles leads to the rapid formation of a platelet-like brushite phase
305 (see Fig. 4a) [2]. The intensity and resolution of the crystal peaks corresponding to brushite
306 significantly decreased in the case of CaP-Enz-Poly. The broadened pattern obtained for CaP-
307 Enz-Poly is a typical signature of a more disordered calcium phosphate phase such as reported
308 by Sun et al. [30].

309 However, the most pronounced crystal peaks still present in the signal of the CaP-Enz-Poly
310 sample mainly fitted the hydroxyapatite (Hap) crystalline form ($\text{Ca}_{10-x}(\text{PO}_4)_{6-x}(\text{HPO}_4)_x(\text{OH})_{2-x}$)
311 (see Fig4b). Finally, the ratio between the areas under the broadened bands and the sharp
312 peaks gave an estimate of the degree of disorder in each sample prepared. Thus, it appears
313 that the proportion of disordered phase in the CaP particles increased from 15% without
314 CGMP to around 45 % in the presence of the phosphopeptide [7]. This clearly indicates that
315 polymeric CGMP disturbed the crystallization process of CaP nanoparticles. Indications on the
316 nature of phases can also be obtained from the Ca/P molar ratio determined by elemental
317 analysis (see Table SI7). Indeed, it is known from the literature that the Ca/P molar ratio can
318 vary between 1.5 and 1.67 for Hap and calcium-depleted Hap, while it is 1 for brushite and
319 varies from 1.2 to 2.2 for amorphous calcium phosphate, respectively [1,2]. A Ca/P molar ratio
320 of 1.08 was determined in the case of CaP particles, thus confirming the presence of brushite,
321 and of 1.27 in the case of CaP-Enz-Poly particles (1.25 for CaP-Mono), which could correspond
322 to amorphous Hap (see SI7).

323 **3.4 Solid-state NMR**

324 Solid-state NMR (ssNMR) was used to further characterize the different types of phosphates
325 and phosphorus environments in CaP, CaP-Enz, CaP-Enz-Mono and CaP-Enz-Poly. Direct ^{31}P
326 excitation with a long recycle delay allows quantitative analysis of different phosphorus-
327 containing species, whereas cross-polarization (CP) experiments enhance the ^{31}P signals of
328 near-proton and immobilized phosphorus atoms, as shown in Figure 5. ^{31}P chemical shifts as
329 well as the relative signal intensity in the two types of 1D spectra enabled a first
330 identification of different phosphorus species.

331 Additional information was obtained by recording the ^{31}P low spinning frequency spectra
332 and simulating these spectra with different anisotropy parameters. The experimental details
333 and simulations are given in SI8. The 1D ^{31}P spectra of CaP, CaP-Enz, CaP-Enz-Mono and CaP-
334 Enz-Poly samples (Figure 5) exhibit two major peaks at 3-3.3 ppm and 1.5 ppm. An
335 additional signal around -0.2 ppm is also present, excepted for CaP.

336 The component at 1.5 ppm corresponds to protonated phosphates according to chemical
337 shifts reported in the literature. Its protonation state is confirmed by the high relative
338 intensity in the CP spectra. Its contribution is similar in CaP and CaP-Enz, 56% and 51%,
339 respectively, but largely decreases in the presence of mono- or poly-CGMP (see Tables SI8-1,
340 SI8-2, Fig. SI8-1 and Fig.SI8-2). CaP, CaP-Enz, CaP-Mono spinning sidebands manifolds in 1D
341 ^{31}P low spinning frequency CP spectra, can be simulated using δ_{aniso} and η values of 62 ppm
342 and 0.8, respectively (see Fig. SI8-3 and Tab. SI8-3). These parameters are typical for
343 brushite [31-33]. The broader signal of CaP-Enz-Poly is more difficult to deconvolute but
344 gives an δ_{aniso} value around 45 ppm and is therefore probably not related to brushite (SI8-4).

345 These observations are consistent with the XRD data. Confirmation of brushite phase comes
346 from 2D ^1H - ^{31}P correlations: strong cross-signals between ^{31}P at 1.5 ppm and ^1H at 10.7 ppm
347 in CaP-Enz are indeed typical for brushite (see SI8.5) [34,35]. The ^{31}P signal at 3-3.3 ppm is

348 broad (2 to 2.5 ppm FWHM) and its relative intensity is lower in CP spectra. The contribution
349 of this peak to the total ^{31}P signal is 44% in CaP and CaP-Enz samples, and
350 65% in CaP-Enz-Mono and 67% in CaP-Enz-Poly samples (see Table SI8.1). Its anisotropy
351 contribution is not visible in the low spinning frequency experiments of CaP and CaP-Enz,
352 whereas in CaP-Mono and CaP-Enz-Mono it shows an anisotropy parameter around -20 or -
353 30 ppm (see Figure SI8.4), consistent with inorganic colloidal calcium phosphate in a
354 hydroxyapatite-like environment. In the 2D ^1H - ^{31}P spectra of CaP-Enz-Poly, a correlation
355 between the ^{31}P signal at 3-3.3 ppm and a ^1H hydroxyl group resonance at -0.2 ppm is again
356 a hallmark of hydroxyapatite [26,31,36]. This correlation is more intense in CaP-Enz-Poly, in
357 agreement with XRD data indicating a hydroxyapatite phase representing 96% of the
358 crystalline phase (data from area integration of crystal peaks, not shown). The C-terminal
359 peptide of κ -casein is flexible and is known to remain a flexible tail in casein micelles, in
360 contrast to α - and β -caseins, which participate in micelle formation and show an
361 immobilized phosphoserine (SerP) ^{31}P signal [35]. Therefore, monomeric CGMP should not
362 interact extensively with calcium phosphate particles. The ^{31}P data of CaP-Mono and CaP-
363 Enz-Mono (brushite signal, only a small amount of Hap-like particles) are closer to CaP and
364 CaP-Enz than to CaP-Enz-Poly. On the contrary, the CaP-Enz-Poly sample exhibits a higher
365 proportion of Hap-like phosphate. The broad ^{31}P signal of CaP-Enz-Poly around 1.8-2 ppm
366 present in 1D CP spectra (left peak, Figure 5) and in 2D HETCOR (SI8-5) correlation can be
367 tentatively assigned to immobilized phosphoserine from poly-CGMP [35]. The
368 immobilization of these SerP groups could be ascribed to their participation in the
369 interaction with calcium phosphate particles [35]. Peptide immobilization, and thus SerP
370 participation in colloid formation, seems only observable in the presence of polymeric
371 CGMP.

372 The detailed characterization of the inorganic particle seems to indicate that the presence
373 of peptide leads to less crystalline particles (XRD) and to the appearance of hydroxyapatite-
374 like calcium phosphates (XRD and NMR). The immobilization of the peptide phosphoserine
375 groups by participation in the interaction with the calcium phosphate particles has been
376 tentatively evidenced by NMR only for CaP-Enz-Poly. The phosphoglycopeptide polymer
377 appears to be necessary to allow enzyme retention with good activity, to increase the
378 proportion of hydroxyapatite-like particle and to prevent the formation of the brushite
379 phase.

380 **3.5 Enzyme activity of the Hap-like catalytic supports**

381 The Figure 6 compares for the different samples: i- the amount of lactase immobilized in the
382 different samples prepared by in situ wet precipitation as described above, and ii- the
383 respective catalytic activities. Thus, the CaP-Enz and CaP-Enz-Mono particles contained
384 respectively 52 ± 19 and 34 ± 21 mg of immobilized lactase per g of dry powder, while the
385 CaP-Enz-Poly particles had an amount of encapsulated lactase of $125 \pm 4 \text{ mg}\cdot\text{g}^{-1}$, which is
386 more than twice the amount immobilized in the CaP-Enz and CaP-Enz-Mono matrices. The
387 amount of enzyme immobilized in CaP-Enz and CaP-Enz-Mono particles is of the same order
388 of magnitude as that typically observed for silica matrix ($10 - 70 \text{ mg}\cdot\text{g}^{-1}$) [9]. In addition,
389 according to the standard deviation values, the lactase encapsulation process seems to be
390 highly reproducible in the case of CaP-Enz-Poly particles compared to the other two
391 samples.

392 Consequently, the supported enzyme presented variable catalytic performance, as also
393 shown in Figure 6. It can be seen that the activity of the CaP-Enz-Mono sample is about
394 three times higher than that of the CaP-Enz sample, despite the similar amounts of

395 immobilized protein. However, the activity of the CaP-Enz-Poly sample was found to be
396 almost 10 times higher than that of the CaP-Enz material. The average specific activity was
397 found to be 1060, 4811 and 4843 NLU·g⁻¹ of protein for CaP-Enz, CaP-Enz-Mono and CaP-
398 Enz-Poly samples, respectively. The specific activity of the immobilized enzyme in the CaP-
399 Enz sample was found to be around 100 times lower than that of the free enzyme (100
400 kNLU·g⁻¹ of protein), while it was only 20 times lower in the case of CaP-Enz- Mono and CaP-
401 Enz-Poly samples. A comparison of the specific activity of immobilized and free enzyme
402 phenomenologically indicates the hindrance to activity due to slower mass transport of the
403 substrate and product through the inorganic matrix. This suggests significant resistance to
404 mass transfer (diffusion) through the particles. The narrowest PSD found for the CaP-Enz-
405 Poly particles could explain the better retention and the higher activity of enzyme in CaP-
406 Enz-Poly particles compared to the other two types of particles. The differences in
407 enzymatic activity between the materials CaP-Enz, CaP-Enz-Mono and CaP-Enz-Poly might
408 be due to some differences of particles mesoporosity as well as to enzyme denaturation
409 caused by adsorption on the inorganic matrix surface. Indeed, a direct interaction of the
410 enzyme with the surface of pure CaP might lead to a partial denaturation of the enzyme,
411 e.g. due to some changes in the quaternary/tertiary structure. A specific activity about five
412 times higher for CaP-Enz-Mono and CaP-Enz-Poly than for CaP-Enz particles indicates some
413 protective effect in the case of monomeric and polymeric CGMP. However, CaP-Enz-Poly
414 had a much higher total activity (607 ± 34 NLU·g⁻¹ of dry powder) than monomeric CGMP-
415 coated particles (161 ± 70 NLU·g⁻¹ of dry powder), which indicates that the difference
416 between the activities of these two types of particles is mainly caused by the different
417 amounts of encapsulated enzyme (Figure 6). This suggests that the monomer or polymer
418 preferentially adsorbs on the CaP surface and that the enzyme is therefore mainly trapped

419 in the organic network where the quaternary/tertiary structure of the lactase might be
420 better preserved. Similar observations were made on lactase immobilized in silica matrix,
421 where the enzyme encapsulated in macropores was found to be more active than the
422 enzyme adsorbed in mesopores [4]. The authors proposed that the difference in activity was
423 related to the quaternary structure of lactase (monomers vs dimers or tetramers).

424 **4. Conclusion**

425 In conclusion, through the careful selection of precipitation conditions, phosphopolyptide
426 polymers have been found to be efficient controllers of crystal growth, crystallinity and
427 colloidal stability of calcium phosphate particles (CaP) when produced by a wet precipitation
428 method. Changes in the crystalline phase, from brushite to hydroxyapatite, crystal size and
429 disorder led to changes in the mesoporous texture of these particles and made them
430 suitable for trapping small bioactive macromolecules such as enzymes while preventing
431 significant loss of catalytic activity. The polymeric form was found to be more suitable for
432 encapsulation since it produces a good interaction with the CaP surface and imparts
433 excellent colloidal stability to the particles. Based on this mechanism, it can be concluded
434 that any bio-macromolecule with organic phosphates and flexible structure can be used to
435 modulate the mesoporosity of calcium-based inorganic particles. The underlying
436 fundamental mechanism seems to be the ionic interactions between the charged
437 macromolecule and the charged inorganic surface. Thus, extending the proposal further,
438 any (bio)macromolecule with strong ionic groups present in its chain that can ionically
439 interact with an inorganic surface should modify its mesoporous texture. Of course, there
440 could be variations originating from other parameters such as the flexibility of the chain, the
441 distribution of charged groups and the presence of other groups that interact with the

442 surface via other types of weak interactions. Using biopolymers for controlling/modulating
443 the mesoporous texture of inorganic matrices seems to be a viable option for practical
444 applications. Modified mesoporosity with a suitable pore size can be also used for the
445 immobilization of various bioactive molecules, macromolecules or cells targeting
446 straightforward applications for food packaging, (ref
447 <https://doi.org/10.3390/polym13234198>) pharmaceuticals (ref notre Adv Colloids and
448 Interface science) or functional food (ref <https://doi.org/10.1111/1541-4337.13049>).

449 **Conflicts of interest**

450 There are no conflicts to declare.

451 **Funding**

452 This work was supported by the People Programme (Marie Curie Actions) of the European
453 Union's Seventh Framework Programme FP7/2007-2013/ under REA (grant agreement n°
454 606713). A.C and A.P. acknowledge financial support from the "Impact Biomolecules"
455 project of the "Lorraine Université d'Excellence" (Investissements d'avenir – ANR 15-004).

456 **Acknowledgements**

457 SKD *et al* would like to thank Mr. Jean Cauzid for SEM analysis and Mr. Jérôme Marin for
458 elemental analysis measurements.

459 **References**

460 [1] S. Schweizer, A. Taubert, Macromol. Biosci., 2007, 7, 1085.
461 <https://doi.org/10.1002/mabi.200600283>

- 462 [2] K. Lin, C. Wu, J. Chang, *Acta Biomaterialia*, 2014, 10, 4071.
463 <https://doi.org/10.1016/j.actbio.2014.06.017>
- 464 [3] I.-A. Pavel, S.F. Prazeres, G. Montalvo, C. Garcia Ruiz, V. Nicolas, A. Celzard, F. Dehez, L.
465 Canabady-Rochelle, N. Canilho, A. Pasc, *Langmuir*, 2017, 33, 3333.
466 <https://doi.org/10.1021/acs.langmuir.7b00134>
- 467 [4] I.-A. Pavel, M. Girardon, S. El Hajj, S. Parant, F. Amadei, S. Kaufmann, M. Tanaka, V. Fierro,
468 A. Celzard, N. Canilho, A. Pasc, *J Mater Chem B*, 2018, 6, 5633.
469 <https://doi.org/10.1039/C8TB01114A>
- 470 [5] Hou, N. Ghéczy, D. Messmer, K. Szymańska, J. Adamcik, R. Mezzenga, A.B. Jarzębski, P.
471 Walde, *ACS Omega*, 2019, 4, 7795. <https://doi.org/10.1021/acsomega.9b00286>
- 472 [6] K.J. Cross, N.L. Huq, J.E. Palamara, J.W. Perich, E.C. Reynolds, *J. Biol. Chem.*, 2005, 280,
473 15362. <https://doi.org/10.1074/jbc.M413504200>
- 474 [7] C. Combes, C. Rey, *Acta Biomaterialia*, 2010, 6, 3362.
475 <https://doi.org/10.1016/j.actbio.2010.02.017>
- 476 [8] C. Holt, J.A. Carver, H. Ecroyd, D.C. Thorn, *J Dairy Sci.*, 2013, 96, 1.
477 <https://doi.org/10.3168/jds.2013-6831>
- 478 [9] Pierre, A. C. *Biocatal. Biotransformation*, 2004, 22, 145.
479 <https://doi.org/10.1080/10242420412331283314>
- 480 [10] B. Dziuba, M. Dziuba, *Acta Scientiarum Polonorum, Technologia Alimentaria*, 2014, 13, 5.

- 481 [11] M.J. Martinez, V.M. Pizones Ruiz-Henestrosa, C. Carrera Sánchez, J.M. Rodríguez Patino,
482 A.M.R. Pilosof, Colloids Surf. B, 2012, 95, 214.
483 http://hdl.handle.net/20.500.12110/paper_09277765_v95_n_p214_Martinez
- 484 [12] R. Morales, M.J. Martinez, A.M.R. Pilosof, International Dairy Journal, 2015, 49, 30.
485 <https://doi.org/10.1016/j.idairyj.2015.04.006>
- 486 [13] R. Silva Diniz, J.S. dos Reis Coimbra, A.V.N. de Carvalho Teixeira, A. Ribeiro da Costa, I.J.
487 Boggione Santos, G. Costa Bressan, A.M. da Cruz Rodrigues, L.H. Meller da Silva, Food
488 Research International, 2014, 64, 157. <https://doi.org/10.1016/j.foodres.2014.05.079>
- 489 [14] A. Tolkach, U. Kulozik, J. Food Eng., 2005, 67, 13.
490 <https://doi.org/10.1016/j.jfoodeng.2004.05.058>
- 491 [15] C. Thomä-Worringer, J. Sørensen, R. López-Fandiño, International Dairy Journal, 2006,
492 16, 1324. <https://doi.org/10.1016/j.idairyj.2006.06.012>
- 493 [16] M.E. Farías, M.J. Martinez, A.M.R. Pilosof, International Dairy Journal, 2010, 20, 79.
494 <https://doi.org/10.1016/j.idairyj.2009.09.002>
- 495 [17] E. Rojas, G. Torres, Food Science and Technology, 2013, 33, 14.
496 <https://doi.org/10.1590/S0101-20612013005000027>
- 497 [18] V. Narwal, N. Sharma, R. Sharma, Y.S. Rajput, B. Mann, International Journal of Dairy
498 Technology, 2017, 70, 1. <https://doi.org/10.1111/1471-0307.12452>
- 499 [19] S.H. Kim, K.P. Lim, H.S. Kim, J. Dairy Sci., 1997, 80, 2264.
500 [https://doi.org/10.3168/jds.S0022-0302\(97\)76175-7](https://doi.org/10.3168/jds.S0022-0302(97)76175-7)

501 [20] J.E. Folk, P.W. Cole, *Biochim. Biophys. Acta*, 1966, 122, 244.
502 [https://doi.org/10.1016/0926-6593\(66\)90066-X](https://doi.org/10.1016/0926-6593(66)90066-X)

503 [21] S. Brunauer, P.H. Emmet, E. Teller, *J. Am. Chem. Soc.*, 1938, 309.
504 <https://doi.org/10.1021/ja01269a023>

505 [22] M.M. Dubinin, 1989, *Carbon* 27, 457. [https://doi.org/10.1016/0008-6223\(89\)90078-X](https://doi.org/10.1016/0008-6223(89)90078-X)

506 [23] M. Kruk, M. Jaroniec and M. Sayari, *Langmuir*, 1997, 13, 6267.
507 <https://doi.org/10.1021/la970776m>

508 [24] P. Barret, L. G. Joyner and P. P. Halenda, *J. Am. Chem. Soc.*, 1951, 73, 373.
509 <https://doi.org/10.1021/ja01145a126>

510 [25] D. Massiot, F. Fayon, M. Capron, I. King, S. Le Calvé, B. Alonso, J. O. Durand, B. Bujoli, Z.
511 H. Gan and G. Hoatson, *Magn. Reson. Chem.*, 2002, 40, 70. <https://doi.org/10.1002/mrc.984>

512 [26] W.P. Rothwell, J.S. Waugh, J.P. Yesinowski, *J. Am. Chem. Soc.*, 1980, 102, 2637.
513 <https://doi.org/10.1021/ja00528a020>

514 [27] International Patent Number W02018/134066 A1.

515 [28] J. Wald, S. Wiese, T. Eckert, W. Jahnen-Dechent, W. Richtering, A. Heiss, *Soft Matter*,
516 2011, 7, 2869. <https://doi.org/10.1039/C0SM01191F>

517 [29] C.G. de Kruif, T. Huppertz, V.S. Urban, A.V. Petukhov, *Adv. Colloid Interface Sci.*, 2012, 36,
518 171. <https://doi.org/10.1016/j.cis.2012.01.002>

519 [30] L. Sun, L.C. Chow, S.A. Frukhtbeyn, J.E. Bonevich, *J Res Natl Inst Stand Technol.* 2010, 115
520 (4), 243. <https://doi.org/10.6028/jres.115.018>

- 521 [31] M. Bak, J.K. Thomsen, H.J. Jakobsen, S.E. Petersen, T.E. Petersen, N. C. Nielsen, J Urol.,
522 2000, 164, 856. <https://doi.org/10.1097/00005392-200009010-00062>
- 523 [32] Z. Hinedi, S. Goldberg, A. Chang, J. Yesinowski, J. Colloid Interface Sci., 1992, 152, 141.
524 [https://doi.org/10.1016/0021-9797\(92\)90015-E](https://doi.org/10.1016/0021-9797(92)90015-E)
- 525 [33] J.P. Yesinowski, H. Eckert, J. Am. Chem. Soc. 1987, 109, 6274.
526 <https://doi.org/10.1021/ja00255a009>
- 527 [34] R. Mathew, P.N. Gunawidjaja, I. Izquierdo-Barba, K. Jansson, A. García, D. Arcos, M.
528 Vallet-Regí, M. Edén, J. Phys. Chem. C, 2011, 115, 20572. <https://doi.org/10.1021/jp206237n>
- 529 [35] J.K. Thomsen, H.J. Jakobsen, N.C. Nielsen, T.E. Petersen, L.K. Rasmussen, Eur. J.
530 Biochem., 1995, 230, 454. <https://doi.org/10.1111/j.1432-1033.1995.0454h.x>
- 531 [36] M. Bak, L.K. Rasmussen, T.E. Petersen, N.C. Nielsen, J. Dairy Sci., 2001, 84, 1310.
532 [https://doi.org/10.3168/jds.S0022-0302\(01\)70160-9](https://doi.org/10.3168/jds.S0022-0302(01)70160-9)
- 533 [37] B. M. Fung, A. K. Khitrin and K. Ermolaev, J Magn Reson., 2000, 142, 97.
534 <https://doi.org/10.1006/jmre.1999.1896>
- 535

536 **Figure Captions**

537 **Figure 1.** SEM images of dry particles showing platelets (a) CaP, (b) CaP-Enz, or nanoparticles
538 (c) CaP-Enz-Mono and (d) CaP-Enz-Poly.

539 **Figure 2.** Mean hydrodynamic radius (R_H) of the CaP particles formed in the presence of Poly-
540 CGMP: (a) R_H recorded over time; (b) size distribution recorded for P1 plateau; (c) size
541 distribution recorded for P2 plateau. The Ca^{2+} concentrations in mM used for making the
542 particles are shown in the legend.

543 **Figure 3.** Mean hydrodynamic radius (R_H) of the CaP particles formed in the presence of
544 different bulk concentrations of Poly-CGMP, in 10 mM of Ca^{2+} .

545 **Figure 4:** XRD showing the presence of brushite in the CaP sample and the formation of highly
546 amorphous structures in other samples, with predominance of hydroxyapatite.

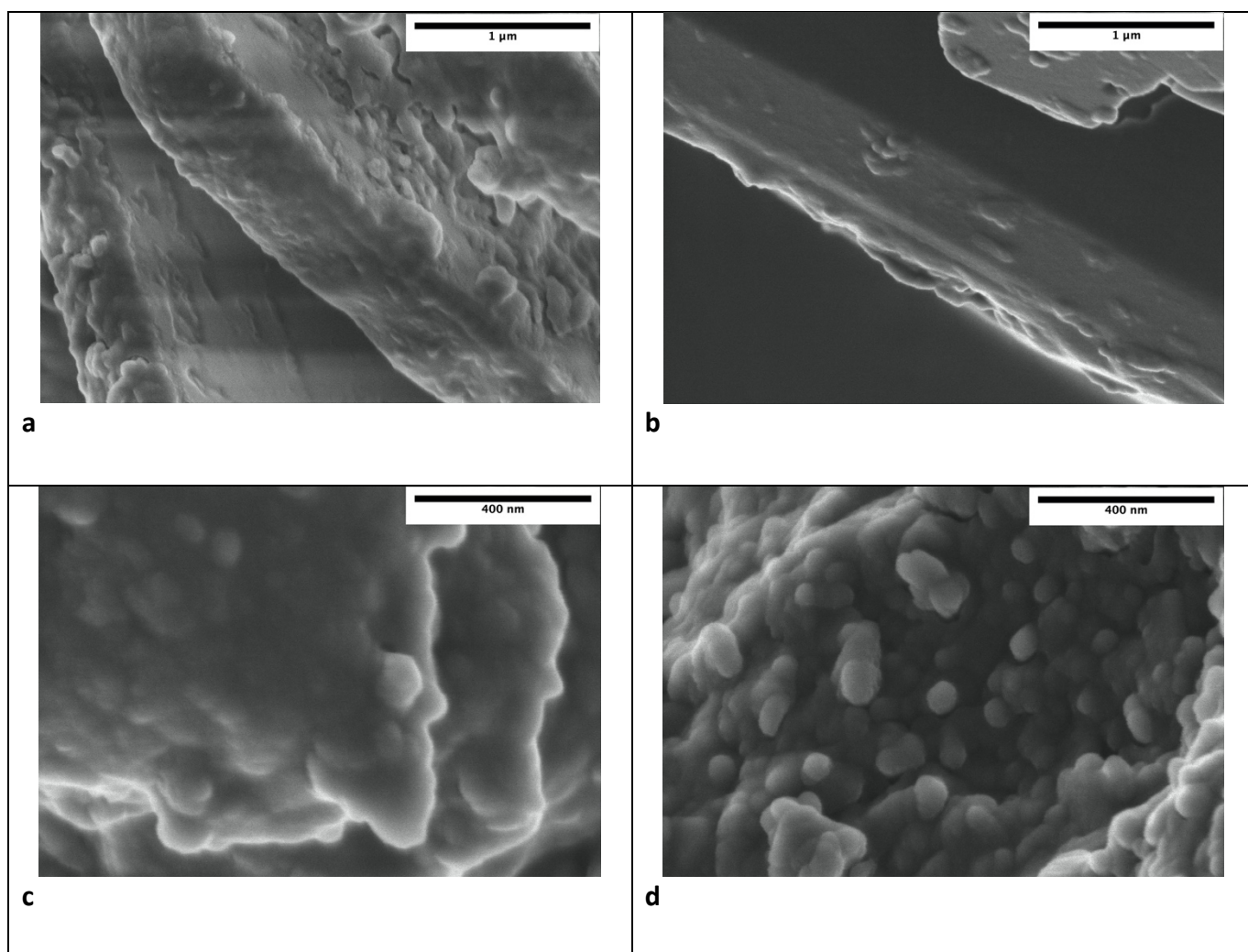
547 **Figure 5.** 1D ^{31}P NMR spectra of CaP (black), CaP-Enz (blue), CaP-Enz-Mono (green) and CaP-
548 Enz-Poly (red) (from bottom to top, respectively) at 12.5 kHz spinning frequency. Top signals:
549 1 ms CP ^{31}P spectra; Bottom signals: direct ^{31}P excitation. The vertical dashed lines,
550 corresponding to different ^{31}P contributions as deconvoluted in Table SI8.1, are drawn to
551 guide the eye. The dotted line corresponds to a putative fourth contribution observed only in
552 CaP-Enz-Poly spectra, as shown in SI8.2.

553 **Figure 6.** Amount of immobilized lactase ($mg \cdot g^{-1}$ of material) and enzyme activity ($NLU \cdot g^{-1}$ of
554 material) in the different types of matrix.

555

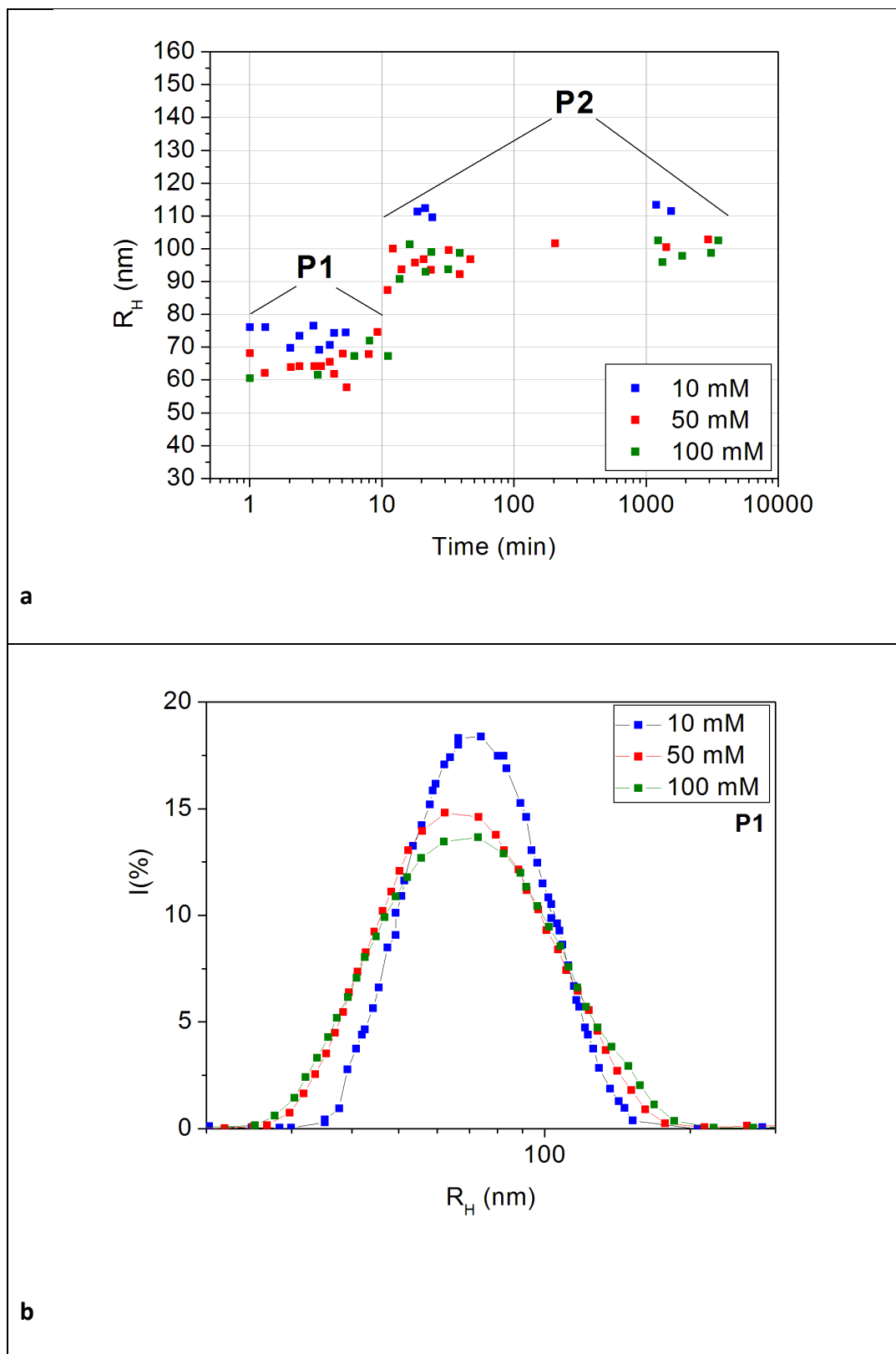
556 **Figure 1**

557



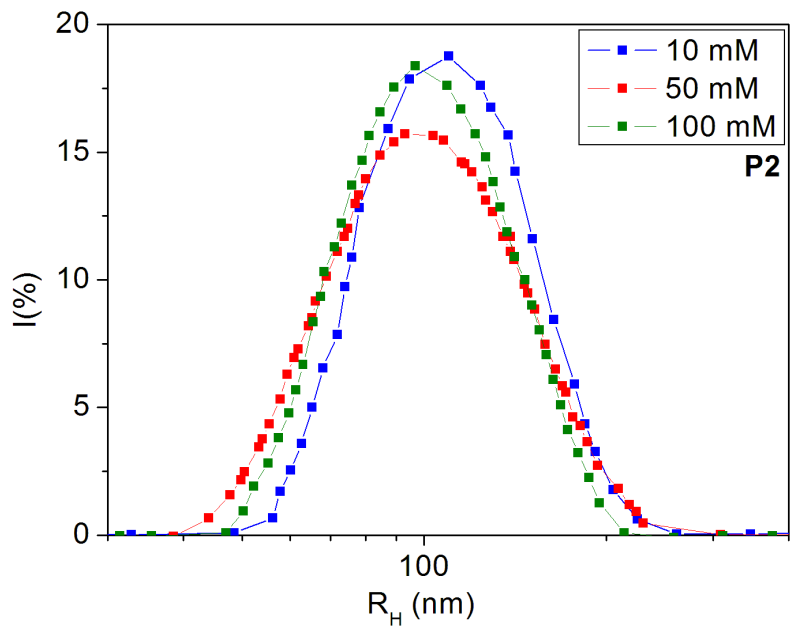
558

559



c

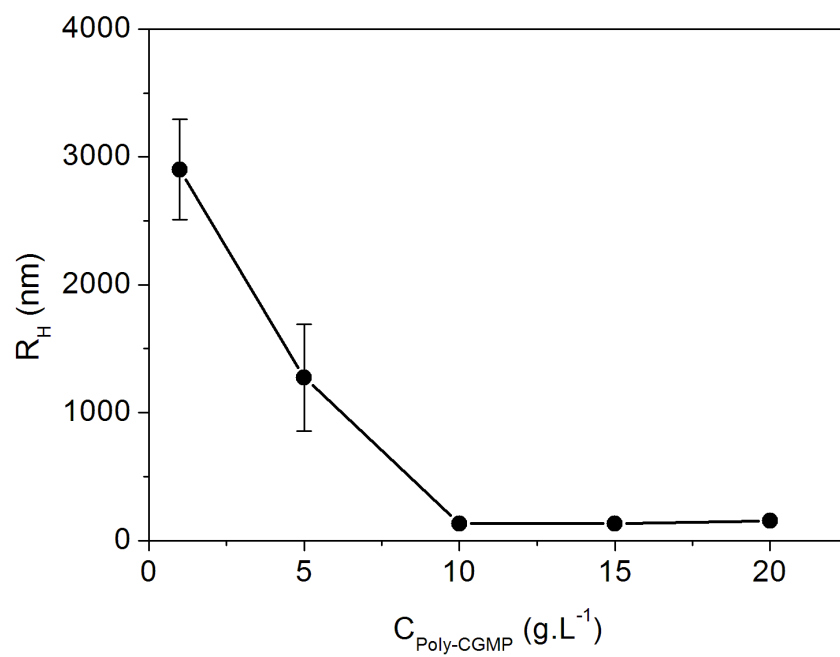
561



571

572

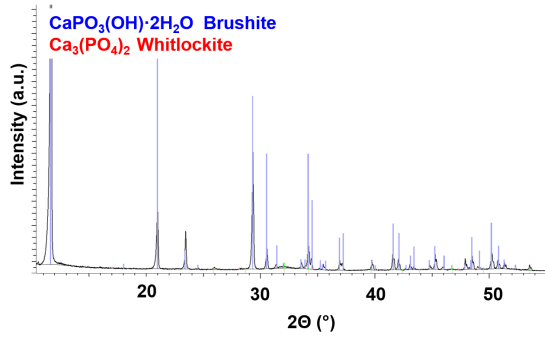
573 **Figure 3.**



574

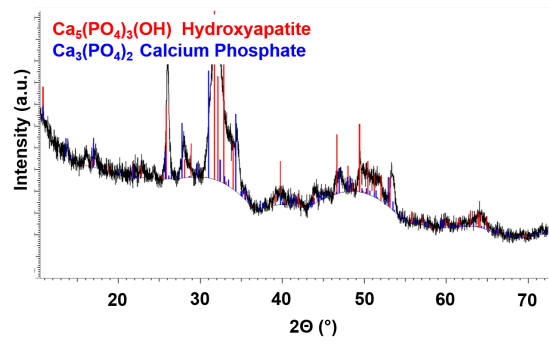
575

576 **Figure 4.**



577

(a)

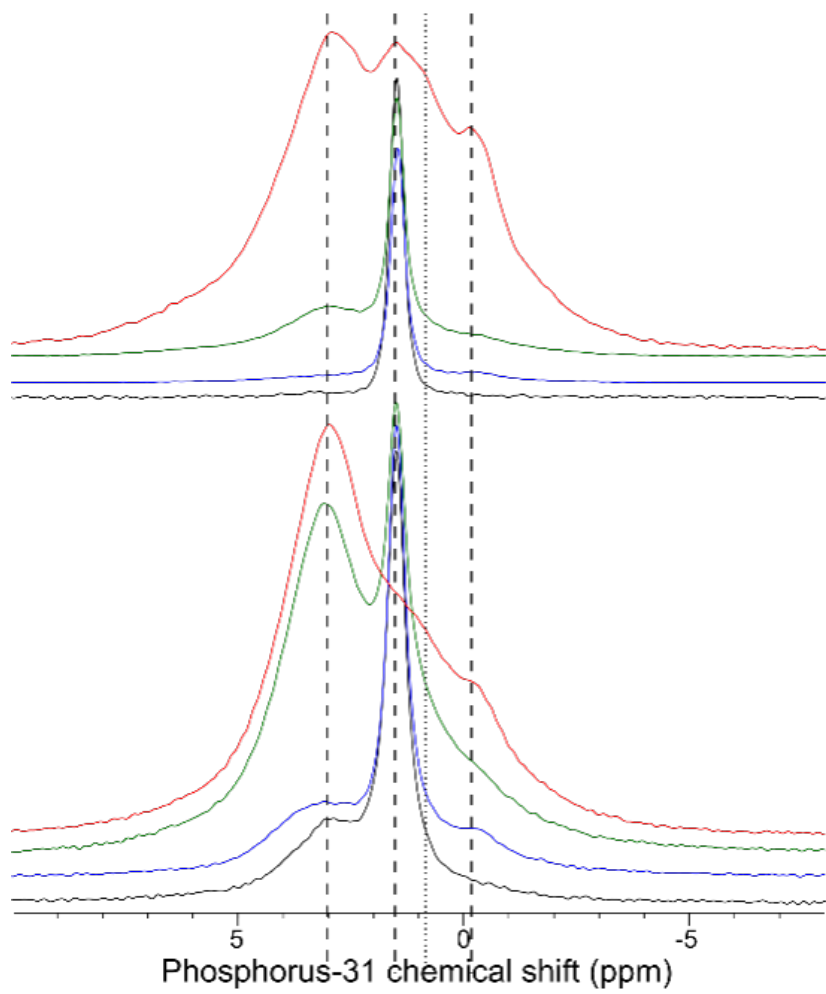


578

(b)

579

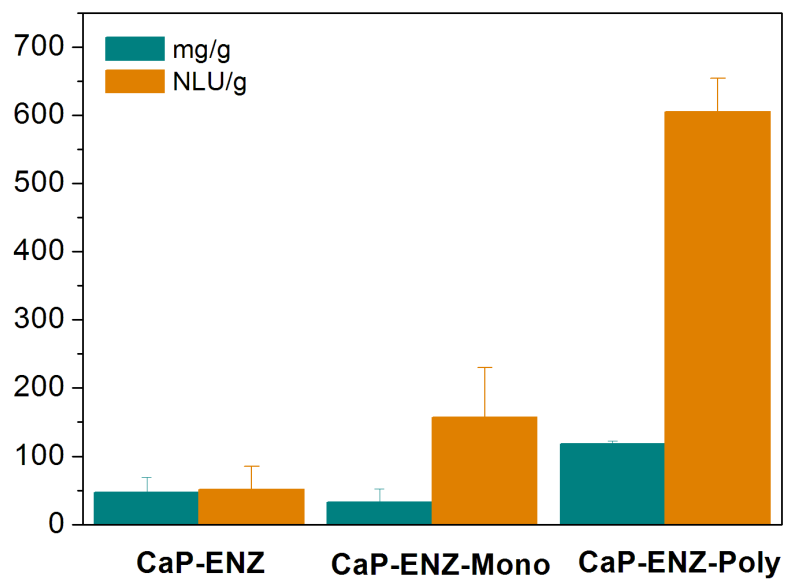
580 **Figure 5.**



581

582

583 **Figure 6.**



584

585