

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

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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

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- 1 Enhancing the activity of biocatalysts supported on calcium phosphate by inducing
- 2 mesoporosity with phosphopeptides
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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 N_2 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	

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

43 **1. Introduction**

44 The wet precipitation of calcium and phosphate ions can lead to various calcium phosphate 45 (CaP) polymorphs, including hydroxyapatite (Ca₅(OH)(PO₄)₃H₂O) [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 published [3,4,5]. However, increasing the porosity of an inorganic support by coating it 56 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 molecules. In general, casein adsorption is driven by interactions between the organic 65 phosphate (serine-bound) and the surface of calcium phosphate particles. This adsorption 66

67 leads to a 'brush-like' layer of casein polymers that improves colloidal stability and prevents the aggregation of nanoclusters. Furthermore, it has reported that the crystalline degree of 68 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 mesoporogen by modulating the crystallinity of the inorganic 80 calcium phosphate matrix. The resulting materials were characterized in terms of porous texture, morphology and crystallinity. Additionally, DLS and ³¹P NMR analysis were 81 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 2. Material and Methods 89

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- eta -
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 (CaCl ₂ ·2H ₂ O) and
102	magnesium sulphate heptahydrate (MgSO $_4$ ·7H $_2$ O)) or from Merck for disodium hydrogen
103	phosphate dihydrate (Na ₂ HPO ₄ ·2H ₂ O), sodium dihydrogen phosphate monohydrate
104	(NaH ₂ PO ₄ ·H ₂ O), sodium chloride (NaCl), sodium carbonate (Na ₂ CO ₃) 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 $\lambda_{CuKlpha}$ = 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.

The degree of crystallinity (DOC) of the samples was calculated from the ratio between the total crystalline area divided by the sum of total crystalline area plus total amorphous area extracted from the diffusion background. Then, the amorphous content (%) was determined from 1-DOC (see SI6c). The molar ratio of calcium (Ca) to phosphate (P) was determined by 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 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 127 128 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 Na₂HPO₄. The lactase enzyme stock solution (Enz) was used as received and MQ-water (W) 129 130 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) for CaP-Enz-Mono sample; Enz and 60 g·L⁻¹ of Poly-CGMP (f) for CaP-Enz-Poly sample. Step-133 (ii): 60 mL of CaCl₂ stock solution (b) were added dropwise for 5 min under 1000 rmp to 940 134 135 mL solution obtained in step-(i). Step-(iii): Addition of the resulting mixtures to 1L of respectively 0.3 M Na₂HPO₄ in the case of CaP and CaP-Enz samples, 20 g·L⁻¹ of Mono-CGMP 136 137 stock solution (e) for the CaP-Enz-Mono sample; and 20 g·L⁻¹ of Poly-CGMP stock solution (g)

to prepare CaP-Enz-Poly. In each case the final pH was 7. Those solutions were also added 138 over a period of 5 min under stirring at 1000 rpm. After additional 5 min of stirring, the 139 140 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 were centrifuged at 3000 × g (20 °C, 30 min). The pellets obtained after centrifugation were 143 144 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 (mg·g⁻¹) was calculated from the ratio of the measured amount of protein (mg) to the weight of dry powder (g). The average $mg \cdot g^{-1}$ of 152 153 enzyme loading and standard deviation were obtained from three replicates. Briefly, the powdered catalyst was suspended in 1 mL of PEM buffer (the same as Britton-Robinson 154 155 buffer, see SI3 for details) at 10 g·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 10 minutes at the same temperature of 40 °C. The reaction was then stopped by adding 157 158 Na_2CO_3 (1M), followed by centrifugation at 10,000 × 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 NLU·g⁻¹. The average NLU·g⁻¹

and standard deviation were obtained from three replicates.

163

164 **2.4 Textural characterization by nitrogen sorption**

The BET area (A_{BET}) and the pore size distribution (PSD) were determined using nitrogen adsorption at -196°C in an automatic equipment (ASAP 2020, Micromeritics) [21-24]. All samples were pre-treated before nitrogen adsorption by degassing under secondary 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

The hydrodynamic radius (Rh) of the prepared colloidal CaP suspensions was measured at 171 172 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 distributions based on intensity diffusion were obtained using the general-purpose model in 175 176 the standard Malvern software. To study the colloidal stabilization of CaP particles formed in the presence of Mono-CGMP or Poly-CGMP, two types of experiments were carried out 177 178 without enzyme. In the first series, the concentration of the Poly-CGMP stock solution was fixed at 10 g·L⁻¹ in a 50 mM Na-PB solution and the CaP particles were formed by one-pot 179 precipitation by adding appropriate amounts of CaCl₂ to reach a final concentration of 10, 180 181 50, or 100 mM, respectively. Then, the solution was rapidly transferred into the DLS 182 instrument for analysis. The delay between the addition of CaCl₂ 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

precipitated by adding a fixed amount of 2 μ L 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
- 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-
- dimensional ¹H-³¹P HETCOR correlations, proton to phosphorus-31 polarization transfer was
- achieved by CP with a 2 ms contact time. SPINAL-64 proton decoupling at 60 kHz was
- 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

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

CaP-Enz, CaP-Enz-Mono and CaP-Enz-Poly. Figures 1 and SI5 show the morphologies of the 207 208 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 SI5). 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

3.2 CaP particles formation followed by DLS

To decipher the formation of CaP particles, dynamic light scattering (DLS) analysis was 221 222 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 Na₂HPO₄ as reagent, the precipitation of brushite (CaHPO₄·2H₂O) should be the most favourable solubility product constant (K_{sp}, of 2.6x10⁻⁷ M²). In the conditions chosen for DLS 225 226 experiments, the ion activity product ([Ca²⁺]x[HPO₄-]) 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 same behaviour was observed for the CaP-Enz matrix, indicating that lactase does not act as a stabilizer either. In that case, the electrostatic repulsions from the surface charge are not high enough to prevent particle growth to large dimensions. In the case of Mono-CGMP and Poly-CGMP, different kinetics were observed. Figure 2a depicts the variation of the hydrodynamic radius (R_h) during the formation of CaP particles over time, at three different concentrations of Ca²⁺ ions, i.e., 10, 50 and 100 mM, in the presence of Poly-CGMP at a concentration of 10 g·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 Ca²⁺. Then, at longer 242 time scales (> 10 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 Ca²⁺. 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 than its monomeric form. Moreover, the size distribution representation for the P1 plateau 252 253 overlaps the size range of the P2 plateau distribution. This could be related to ripening,

which is a continuous process along the reaction time. Thus, the particle size distribution
measured at a given time must comprise particles from both the initial stage and the
ripened stage. These results obtained on the CaP and Poly-CGMP system seem to be similar
to those observed in the case of CaP particles stabilized by Fetuin-A. Indeed, Fetuin-A is
known to be an inhibitor of CaP mineralization and deposition in the soft tissues of animals
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 'calciprotein' 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 $g \cdot L^{-1}$, the CaP particle size is stabilized at a hydrodynamic radius

of around 100 nm. This clearly demonstrates that the polymeric CGMP acts as a colloidal stabilizer, and it can be speculated that the mode of action is a combination of electrostatic and steric repulsions originating from a combination of a charged backbone and a higher molar mass of the polyelectrolyte. The threshold of 10 g·L⁻¹ can be designated as the 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 (ABET) 287 and the mesoporous volume (V_{mes}) increased in the case of CaP-Enz-Mono and CaP-Enz-Poly particles compared to CaP and CaP-Enz: 70 and 59 m²·g⁻¹ vs 36 and 31 m²·g⁻¹ for A_{BET}, 288 respectively, and 0.218 vs 0.150 cm³·g⁻¹ in average, for V_{mes}, respectively. This could be due 289 290 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

were clearly identified as monoclinic, prismatic crystals of brushite (CaHPO₄·2H₂O). This result agrees with the existing literature. For instance, under neutral or slightly acidic conditions, wet precipitation of CaP particles leads to the rapid formation of a platelet-like brushite phase (see Fig. 4a) [2]. The intensity and resolution of the crystal peaks corresponding to brushite significantly decreased in the case of CaP-Enz-Poly. The broadened pattern obtained for CaP-Enz-Poly is a typical signature of a more disordered calcium phosphate phase such as reported 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 $(Ca_{10-x}(PO_4)_{6-x}(HPO_4)_x(OH)_{2-x})$ 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

Solid-state NMR (ssNMR) was used to further characterize the different types of phosphates 324 and phosphorus environments in CaP, Cap-Enz, CaP-Enz-Mono and CaP-Enz-Poly. Direct ³¹P 325 326 excitation with a long recycle delay allows quantitative analysis of different phosphoruscontaining species, whereas cross-polarization (CP) experiments enhance the ³¹P signals of 327 near-proton and immobilized phosphorus atoms, as shown in Figure 5. ³¹P chemical shifts as 328 329 well as the relative signal intensity in the two types of 1D spectra enabled a first 330 identification of different phosphorus species. Additional information was obtained by recording the ³¹P low spinning frequency spectra 331 332 and simulating these spectra with different anisotropy parameters. The experimental details 333 and simulations are given in SI8. The 1D ³¹P 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 ³¹P 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). These observations are consistent with the XRD data. Confirmation of brushite phase comes 345 from 2D ¹H-³¹P correlations: strong cross-signals between ³¹P at 1.5 ppm and ¹H at 10.7 ppm 346 347 in CaP-Enz are indeed typical for brushite (see SI8.5) [34,35]. The ³¹P 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 of this peak to the total ³¹P signal is 44% in CaP and CaP-Enz samples, and 349 350 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, whereas in CaP-Mono and CaP-Enz-Mono it shows an anisotropy parameter around -20 or -352 353 30 ppm (see Figure SI8.4), consistent with inorganic colloidal calcium phosphate in a hydroxyapatite-like environment. In the 2D ¹H-³¹P spectra of CaP-Enz-Poly, a correlation 354 between the ³¹P signal at 3-3.3 ppm and a ¹H hydroxyl group resonance at -0.2 ppm is again 355 356 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 -case in is flexible and is known to remain a flexible tail in case in micelles, in contrast to α - and β -caseins, which participate in micelle formation and show an 360 immobilized phosphoserine (SerP) ³¹P signal [35]. Therefore, monomeric CGMP should not 361 362 interact extensively with calcium phosphate particles. The ³¹P 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 proportion of Hap-like phosphate. The broad ³¹P signal of CaP-Enz-Poly around 1.8-2 ppm 365 366 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 interaction with calcium phosphate particles [35]. Peptide immobilization, and thus SerP 369 370 participation in colloid formation, seems only observable in the presence of polymeric 371 CGMP.

The detailed characterization of the inorganic particle seems to indicate that the presence 372 373 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 respective catalytic activities. Thus, the CaP-Enz and CaP-Enz-Mono particles contained 383 384 respectively 52 ± 19 and 34 ± 21 mg of immobilized lactase per g of dry powder, while the CaP-Enz-Poly particles had an amount of encapsulated lactase of $125 \pm 4 \text{ mg} \cdot \text{g}^{-1}$, which is 385 386 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 of magnitude as that typically observed for silica matrix $(10 - 70 \text{ mg} \cdot \text{g}^{-1})$ [9]. In addition, 388 389 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.

Consequently, the supported enzyme presented variable catalytic performance, as also
shown in Figure 6. It can be seen that the activity of the CaP-Enz-Mono sample is about
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 almost 10 times higher than that of the CaP-Enz material. The average specific activity was 396 found to be 1060, 4811 and 4843 NLU·g⁻¹ of protein for CaP-Enz, CaP-Enz-Mono and CaP-397 398 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 kNLU·g⁻¹ of protein), while it was only 20 times lower in the case of CaP-Enz- Mono and CaP-400 401 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-Poly particles could explain the better retention and the higher activity of enzyme in CaP-405 406 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 had a much higher total activity (607 ± 34 NLU.g⁻¹ of dry powder) than monomeric CGMP-414 coated particles (161 ± 70 NLU.g⁻¹ of dry powder), which indicates that the difference 415 416 between the activities of these two types of particles is mainly caused by the different amounts of encapsulated enzyme (Figure 6). This suggests that the monomer or polymer 417 preferentially adsorbs on the CaP surface and that the enzyme is therefore mainly trapped 418

in the organic network where the quaternary/tertiary structure of the lactase might be
better preserved. Similar observations were made on lactase immobilized in silica matrix,
where the enzyme encapsulated in macropores was found to be more active than the
enzyme adsorbed in mesopores [4]. The authors proposed that the difference in activity was
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, phosphopolypeptide polymers have been found to be efficient controllers of crystal growth, crystallinity and 426 427 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 interact with an inorganic surface should modify its mesoporous texture. Of course, there 439 440 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) pharmaceutics (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 <u>https://doi.org/10.1002/mabi.200600283</u>

- 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 Α. Celzard, Ν. Canilho, Α. Pasc, J Mater Chem Β, 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. <u>https://doi.org/10.1021/acsomega.9b00286</u>
- 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 <u>https://doi.org/10.1016/j.actbio.2010.02.017</u>
- 476 [8] C. Holt, J.A. Carver, H. Ecroyd, D.C. Thorn, J Dairy Sci., 2013, 96, 1.
 477 <u>https://doi.org/10.3168/jds.2013-6831</u>
- 478 [9] Pierre, A. C. Biocatal. Biotransformation, 2004, 22, 145.
 479 <u>https://doi.org/10.1080/10242420412331283314</u>
- 480 [10] B. Dziuba, M. Dziuba, Acta Scientiarum Polonorum, Technologia Alimentaria, 2014, 13, 5.

[11] M.J. Martinez, V.M. Pizones Ruiz-Henestrosa, C. Carrera Sánchez, J.M. Rodríguez Patino,
A.M.R. Pilosof, Colloids Surf. B, 2012, 95, 214.
<u>http://hdl.handle.net/20.500.12110/paper_09277765_v95_n_p214_Martinez</u>

- 484 [12] R. Morales, M.J. Martinez, A.M.R. Pilosof, International Dairy Journal, 2015, 49, 30.
- 485 <u>https://doi.org/10.1016/j.idairyj.2015.04.006</u>
- 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. <u>https://doi.org/10.1016/j.foodres.2014.05.079</u>
- 489 [14] A. Tolkach, U. Kulozik, J. Food Eng., 2005, 67, 13.
 490 <u>https://doi.org/10.1016/j.jfoodeng.2004.05.058</u>
- 491 [15] C. Thomä-Worringer, J. Sørensen, R. López-Fandiño, International Dairy Journal, 2006,
- 492 16, 1324. <u>https://doi.org/10.1016/j.idairyj.2006.06.012</u>
- 493 [16] M.E. Farías, M.J. Martinez, A.M.R. Pilosof, International Dairy Journal, 2010, 20, 79.
- 494 <u>https://doi.org/10.1016/j.idairyj.2009.09.002</u>
- 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. <u>https://doi.org/10.1111/1471-0307.12452</u>
- 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

- 501 [20] J.E. Folk, P.W. Cole, Biochim. Biophys. Acta, 1966, 122, 244.
 502 <u>https://doi.org/10.1016/0926-6593(66)90066-X</u>
- 503 [21] S. Brunauer, P.H. Emmet, E. Teller, J. Am. Chem. Soc., 1938, 309. 504 <u>https://doi.org/10.1021/ja01269a023</u>
- 505 [22] M.M. Dubinin, 1989, Carbon 27, 457. <u>https://doi.org/10.1016/0008-6223(89)90078-X</u>
- 506 [23] M. Kruk, M. Jaroniec and M. Sayari, Langmuir, 1997, 13, 6267. 507 <u>https://doi.org/10.1021/la970776m</u>
- 508 [24] P. Barret, L. G. Joyner and P. P. Halenda, J. Am. Chem. Soc., 1951, 73, 373.
- 509 <u>https://doi.org/10.1021/ja01145a126</u>
- 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. <u>https://doi.org/10.1002/mrc.984</u>
- 512 [26] W.P. Rothwell, J.S. Waugh, J.P. Yesinowski, J. Am. Chem. Soc., 1980, 102, 2637.
- 513 <u>https://doi.org/10.1021/ja00528a020</u>
- 514 [27] International Patent Number W02018/134066 Al.
- 515 [28] J. Wald, S. Wiese, T. Eckert, W. Jahnen-Dechent, W. Richtering, A. Heiss, Soft Matter,
- 516 2011, 7, 2869. <u>https://doi.org/10.1039/C0SM01191F</u>
- 517 [29] C.G. de Kruif, T. Huppertz, V.S. Urban, A.V. Petukhov, Adv. Colloid Interface Sci., 2012, 36,
- 518 171. <u>https://doi.org/10.1016/j.cis.2012.01.002</u>
- [30] L. Sun, L.C. Chow, S.A. Frukhtbeyn, J.E. Bonevich, J Res Natl Inst Stand Technol. 2010, 115
- 520 (4), 243. <u>https://doi.org/10.6028/jres.115.018</u>

- 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 <u>https://doi.org/10.1016/0021-9797(92)90015-E</u>
- 525 [33] J.P. Yesinowski, H. Eckert, J. Am. Chem. Soc. 1987, 109, 6274.
 526 <u>https://doi.org/10.1021/ja00255a009</u>
- 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. <u>https://doi.org/10.1021/jp206237n</u>
- 529 [35] J.K. Thomsen, H.J. Jakobsen, N.C. Nielsen, T.E. Petersen, L.K. Rasmussen, Eur. J.
- 530 Biochem., 1995, 230, 454. <u>https://doi.org/10.1111/j.1432-1033.1995.0454h.x</u>
- 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
- 533 [37] B. M. Fung, A. K. Khitrin and K. Ermolaev, J Magn Reson., 2000, 142, 97.
 534 <u>https://doi.org/10.1006/jmre.1999.1896</u>
- 535

536 Figure Captions

Figure 1. SEM images of dry particles showing platelets (a) CaP, (b) CaP-Enz, or nanoparticles
(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 Ca²⁺ concentrations in mM used for making the 542 particles are shown in the legend.

Figure 3. Mean hydrodynamic radius (R_H) of the CaP particles formed in the presence of
 different bulk concentrations of Poly-CGMP, in 10 mM of Ca²⁺.

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

547 Figure 5. 1D ³¹P 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 ³¹P spectra; Bottom signals: direct ³¹P excitation. The vertical dashed lines,

550 corresponding to different ³¹P contributions as deconvoluted in Table SI8.1, are drawn to

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.

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







560 Figure 2





Figure 3.



576 Figure 4.









