

1 Article

2 **MARINE-INSPIRED ENZYMATIC MINERALIZATION OF**
3 **DAIRY-DERIVED WHEY PROTEIN ISOLATE (WPI)**
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24 **Abstract:** Whey protein isolate (WPI) is a by-product from the production of cheese and Greek
25 yoghurt comprising β -lactoglobulin (β -lg) (75%). Hydrogels can be produced from WPI solutions
26 through heating; hydrogels can be sterilized by autoclaving. WPI hydrogels have shown
27 cytocompatibility and ability to enhance proliferation and osteogenic differentiation of bone-
28 forming cells. Hence, they have promise in the area of bone tissue regeneration. In contrast to
29 commonly used ceramic minerals for bone regeneration, a major advantage of hydrogels is the ease
30 of their modification by incorporating biologically active substances such as enzymes. Calcium
31 carbonate (CaCO_3) is the main inorganic component of the exoskeletons of marine invertebrates.
32 Two polymorphs of CaCO_3 , calcite and aragonite, have shown the ability to promote bone
33 regeneration. Other authors have reported that the addition of magnesium to inorganic phases has
34 a beneficial effect on bone-forming cell growth. In this study, we employed a biomimetic, marine-
35 inspired approach to mineralize WPI hydrogels with an inorganic phase consisting of CaCO_3
36 (mainly calcite) and CaCO_3 enriched with magnesium using the calcifying enzyme urease. **The**
37 **novelty of this study lies in both the enzymatic mineralization of WPI hydrogels and enrichment of**
38 **the mineral with magnesium.** Calcium was incorporated into the mineral formed to a greater extent
39 than magnesium. Increasing the concentration of magnesium in the mineralization medium led to
40 a reduction in the amount and crystallinity of the mineral formed. Biological studies revealed that
41 mineralized and unmineralized hydrogels were not cytotoxic and promoted cell viability to
42 comparable extents (approximately 74% of standard tissue culture polystyrene). The presence of
43 magnesium in mineral formed had no adverse effect on cell viability. In short, WPI hydrogels, both
44 unmineralized and mineralized with CaCO_3 and magnesium-enriched CaCO_3 , show potential as
45 biomaterials for bone regeneration.

46 **Keywords:** hydrogel; composite; mineralization; enzyme; bioinspired; whey protein isolate.
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49 **1. Introduction**

50 Natural biproducts from industrial processes have tremendous biomimetic properties and are
51 inexpensive as they are infrequently utilized. Whey protein isolate (WPI) is a by-product from the
52 production of cheese and Greek yoghurt comprising β -lactoglobulin (β -lg) (50%) and α -lactalbumin
53 (α -la) (20%) [1]. WPI in solution has been shown to enhance osteogenic differentiation of bone-
54 forming cells and promote cellular proliferation [1]. It was recently discovered that hydrogels can be
55 produced from WPI solutions, whereby gelation is achieved through heating and sterilization is
56 achieved by autoclaving [2-4].

57 With regards to mechanical properties, hydrogels are relatively weak due to the high water
58 content. However, the incorporation of a mineral phase may improve the mechanical properties and
59 therefore promote cellular adhesion, proliferation and osteogenic differentiation [5]. In comparison
60 to ceramic minerals, a major advantage of hydrogels is that they can be modified with ease by
61 incorporating biologically active substances such as enzymes. Similarly, it is possible to modify and
62 enhance properties of hydrogels by enriching the solution with a mineral phase either before or after
63 gelation [6].

64 Marine invertebrates use the mineral calcium carbonate (CaCO_3) in their exoskeletons. CaCO_3
65 occurs naturally as three crystalline polymorphs known as calcite, aragonite and vaterite. Calcite
66 occurs in bivalves and certain sponges, while aragonite occurs in coral and nacre, or „mother of
67 pearl“. All three polymorphs of CaCO_3 may exhibit beneficial biological properties. For example,
68 calcite is a bioactive substance that can form direct connections with bone tissue *in vivo* and could
69 potentially strengthen fractured bone [7]. Aragonite has been employed as a biomaterial for bone
70 regeneration and outperformed calcium phosphates [8], and when added in particle form to WPI
71 hydrogels, has improved mechanical properties and proliferation of osteoblast-like cells [4]. Vaterite
72 coatings have been shown to stimulate the formation of apatite upon incubation in simulated body
73 fluid (SBF) [9]. Hence, one may hypothesize that enzymatic mineralization of WPI hydrogels with
74 polymorphs of CaCO_3 will improve their biological performance *in vitro* and *in vivo*.

75 Several approaches to mineralize hydrogels have been tried (Gkioni et al – reference 5). The most
76 popular approach is the addition of pre-formed ceramic particles (e.g. hydroxyapatite, bioactive
77 glasses) during hydrogel formation. Enzymatic mineralization has certain advantages over addition
78 of pre-formed particles. Firstly, particles are prone to aggregation, resulting in inhomogenous
79 distribution, while enzymatic mineralization can potentially lead to a more homogeneous
80 distribution of mineral and better integration of mineral with the hydrogel network. Secondly, the
81 amount of pre-formed ceramic particles which can be incorporated is limited to approximately 30-
82 50% [5], as the presence of too many particles may impede formation of the hydrogel. Using
83 enzymatic mineralization, larger mineral contents can be achieved.

84 In previous work, hydrogels have been mineralized enzymatically with calcium phosphate
85 (CaP) using alkaline phosphatase (ALP), the enzyme responsible for mineralization of bone tissue.
86 One advantage of using urease instead of ALP is the superior thermal stability of urease at higher
87 temperatures such as 70 °C, at which WPI hydrogels are formed.

88 The reaction steps of mineral precipitation using urease have been described previously [10].
89 Briefly, urea diffuses into the hydrogel, where it is converted under the action of urease into ammonia
90 and bicarbonate ions. After dissociation of bicarbonate ions to hydrogen and carbonate ions, the
91 carbonate ions react with calcium, which has also diffused into the hydrogel, to form CaCO_3 . The
92 hydrogen ions formed by the dissociation of the bicarbonate ions are neutralized by the ammonia,
93 resulting in a sufficiently high pH to allow CaCO_3 precipitation.

94 Another beneficial enriching agent is magnesium, which can be found in the calcareous
95 exoskeletons of certain marine organisms and may provide benefits to mammals [11]. Previous work
96 has shown that the presence of magnesium in CaP has promoted adhesion and proliferation of
97 osteoblastic cell lines [12]. During enzymatic mineralization of hydrogels with CaCO_3 , magnesium
98 ions can be added to the mineralization medium which may lead to an increase in cell number [13,14].

99 In previous work, Gellan Gum (GG) hydrogels have been mineralized with CaCO₃ and/or calcium
100 magnesium carbonate by incorporation of the enzyme urease into the hydrogel network followed by
101 incubation in a solution containing the enzyme substrate, urea, and calcium and magnesium ions
102 [14,15].

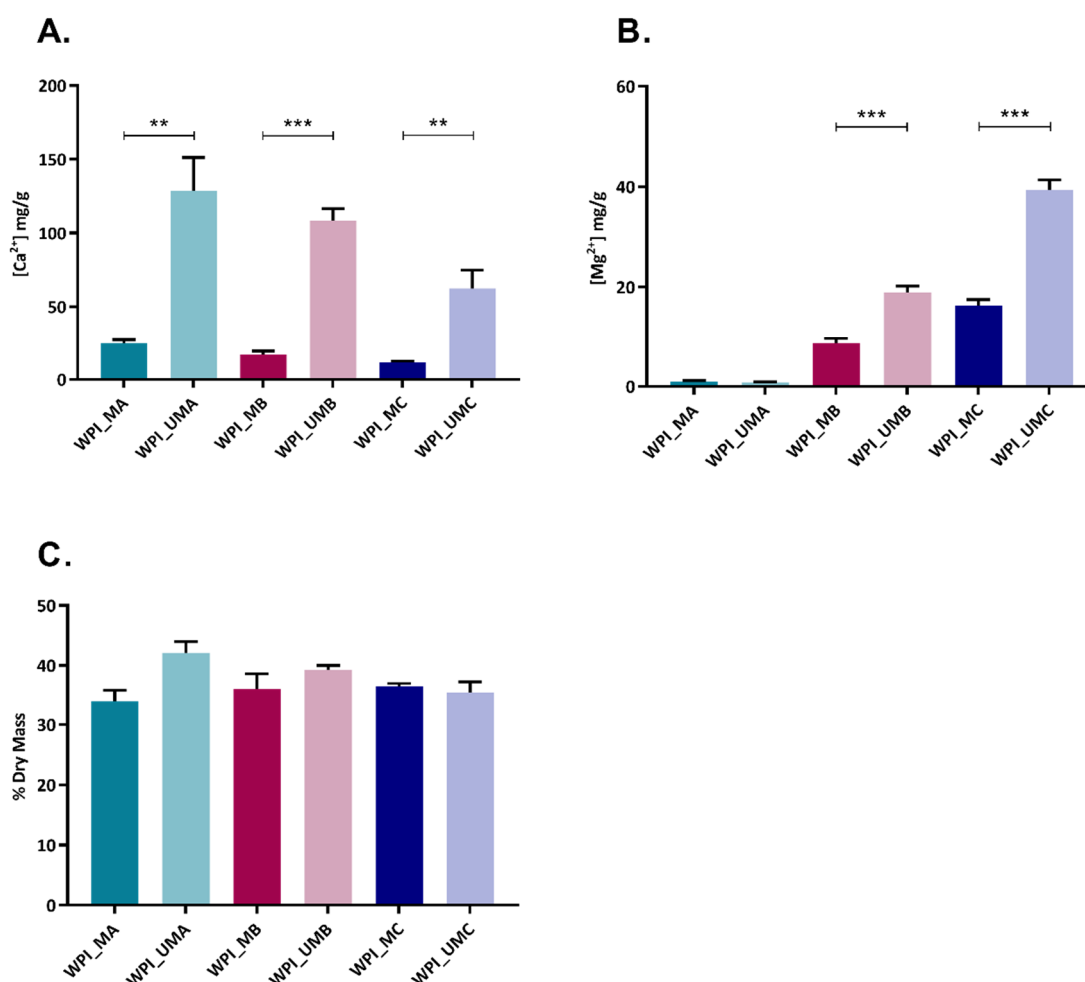
103 In the following study, the enzymatic mineralization approach was extended to WPI hydrogels.
104 WPI hydrogels were mineralized with CaCO₃ or magnesium-enriched CaCO₃ via enzymatic
105 mineralization with urease and incubation in solutions containing calcium and magnesium ions. **The
106 novelty of this study lies in both the enzymatic mineralization of WPI hydrogels and enrichment of
107 the mineral with magnesium. It was thus demonstrated that urease retained activity after
108 incorporation into WPI hydrogels, which involved gelation at 70 °C.** Three different Ca:Mg
109 concentration ratios were compared. The resulting hydrogels were subjected to physicochemical,
110 morphological and biological characterization to examine the influence of Ca:Mg ratio.

111 2. Results

112 *Influence of mineralization medium on extent and elemental composition of mineral formed*

113 We aimed to enhance the properties of WPI hydrogels by mineralization with CaCO₃ or
114 magnesium-enriched CaCO₃. The effects of Ca²⁺ and Mg²⁺ content of the mineralization media on
115 extent of mineral formed were investigated. As expected, a dose dependent effect was observed
116 depending in which medium the urease WPI hydrogel was incubated (Table 1, Fig. 1A and B).
117 Notably, WPI hydrogels containing urease were able to retain higher amounts of Ca²⁺ and Mg²⁺ than
118 control hydrogels, suggesting a greater extent of mineralization. When WPI hydrogels containing
119 urease were incubated with calcium and magnesium in equimolar concentrations (WPI_U_MC),
120 calcium content was five-fold that of magnesium, indicating that calcium was preferentially
121 incorporated into the hydrated polymer. The increase in dry mass as a result of enzymatic
122 mineralization was highest for hydrogels incubated in calcium only (WPI_U_MA) (Fig. 1C).

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Figure 1 Mass of elemental calcium (A) and magnesium (B) per unit mass of hydrogel and dry mass percentage (C) of WPI hydrogels without urease (WPI) and with urease (WPI_U) incubated in different media: MA, MB, MC, n = 3, * p<0.05, ** p<0.01, *** p<0.001.

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Physicochemical characterization of mineral formed by FTIR, Raman, XRD and SEM

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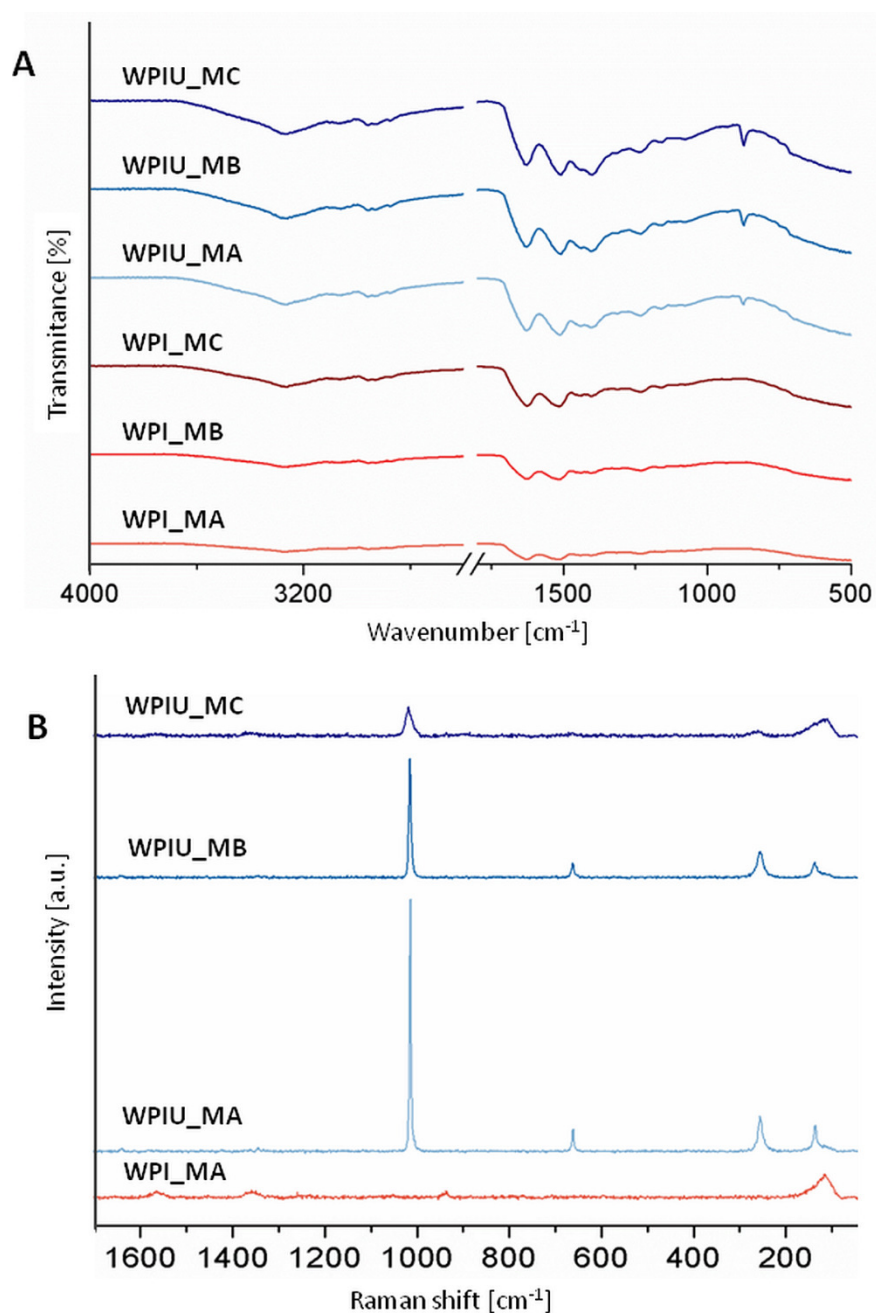
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FTIR spectra (Fig. 3A) of urease-free hydrogels indicated characteristic bands for WPI at 1650, 1570 and 1350-1200 cm⁻¹ [16]. In contrast, hydrogels containing urease showed bands characteristic of calcium carbonate. There was a broad band at 1400 cm⁻¹, which is indicative of calcite and corresponds to ν_3 antisymmetric stretching of carbonate group [17]. Moreover, a band at approximately 1500 cm⁻¹ was detected, which might indicate the presence of vaterite. This band was more intense in WPI_U_MA than in samples incubated in MB and MC. A band at approximately 1080 cm⁻¹ was also observed in all hydrogels with urease and might indicate that vaterite was formed within these hydrogels [18]. In all hydrogels with urease, bands at 870 and 715 cm⁻¹ correspond to ν_2 out-of-plane bending and ν_4 in-plane bending, respectively. These bands are characteristic for calcite [17]. These results show strong evidence that, in WPI hydrogels containing urease, the minerals calcite and vaterite were formed.

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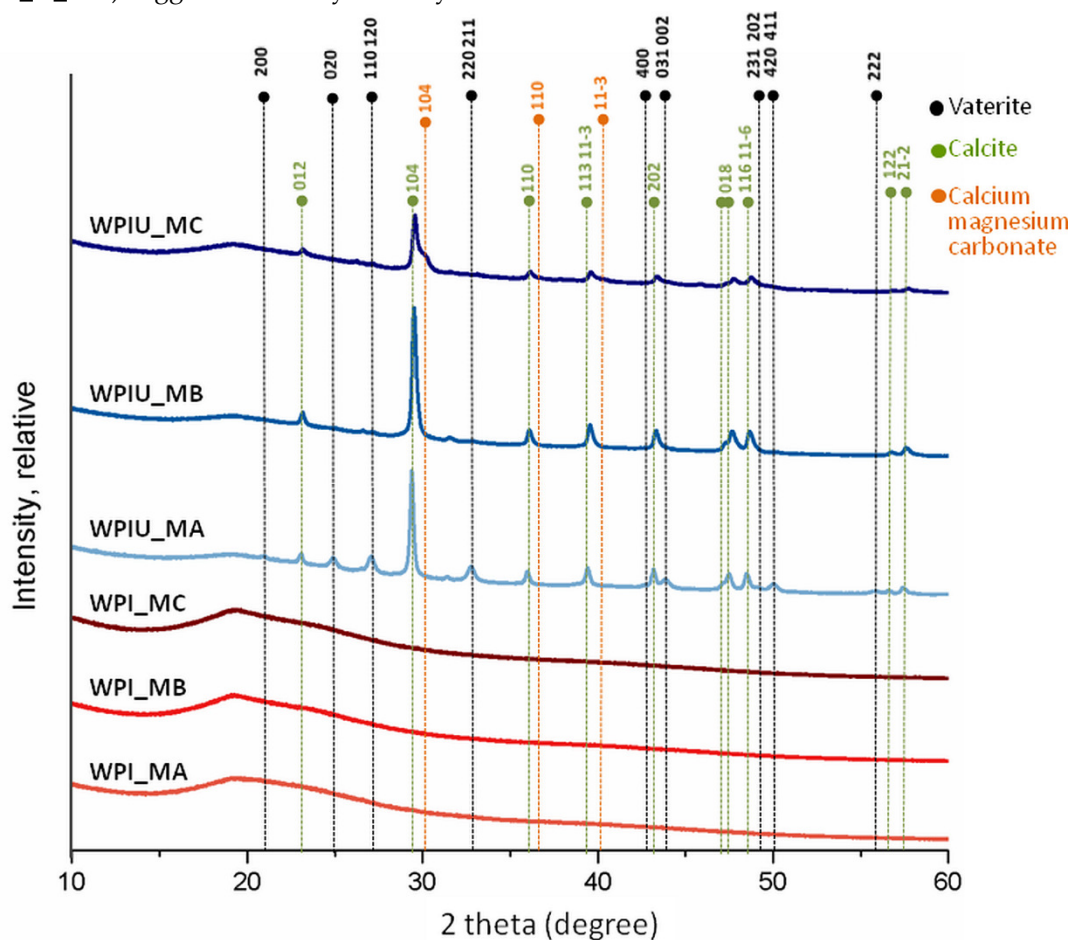
143 Figure 2 FTIR (A) and Raman spectra (B) of WPI hydrogels without and with urease WPI_U incubated
 144 in different media: MA, MB, MC.

145

146 Raman spectra of control WPI_MA hydrogels (Fig. 2 B) showed bands at 1658 cm⁻¹ and 1453 cm⁻¹,
 147 which are associated with amide I and CH₂ bending, respectively [19]. There were also bands at
 148 approximately 1000 cm⁻¹ relating to phenylalanine [20,21]. Bands typical for CaCO₃ were not
 149 observed. In contrast, WPI_U_MA and WPI_U_MB showed intense bands at 1085 cm⁻¹, which are
 150 typical for CaCO₃ and correspond to ν_1 symmetric stretching [22]. Raman bands were also observed
 151 at 711 cm⁻¹ relating to ν_4 in-plane stretching and both hydrogels showed bands at 281 cm⁻¹ and 154
 152 cm⁻¹ consistent with lattice vibration modes [22]. Sharp peaks in WPI_U_MA and WPI_U_MB were
 153 indicative of crystalline structure. In WPI_U_MC Raman bands at 1085 cm⁻¹ were broader and less
 154 intense, suggesting increased amorphicity and thus incorporation of magnesium in the calcite lattice.
 155 It was found that increased magnesium concentration reduced the crystallinity of CaCO₃. This

156 observation was confirmed by two other bands typical for calcite (at 281 cm^{-1} and 154 cm^{-1}), which
 157 were also broader in WPI_U_MC than in WPI_U_MA and WPI_U_MB [17,23,24]. The decrease in
 158 intensity and band sharpness in the order WPIU_MA > WPIU_MB > WPIU_MC suggests a decrease
 159 in crystallinity in the same order.

160
 161 XRD diffractograms of control samples (urease-free hydrogels) displayed a peak at a 2 theta
 162 value of approximately 19.5, which is a characteristic peak for WPI (Fig. 3) [20]. However, in WPI
 163 hydrogels with urease (WPI_U) this peak was less intense, which might suggest that the proportion
 164 of mineral present in this sample was higher than the proportion of WPI. Minerals formed in all WPI
 165 hydrogels containing urease, displaying peaks at 23.2, 29.5, 36.1, 39.5, 43.3, 47.7, 48.6 and 57.5, which
 166 are characteristic for calcite [13,25]. In addition, WPI_U_MA hydrogels displayed peaks characteristic
 167 for vaterite at 24.9, 27.0 and 32.8 [26]. A peak near 30 degrees in WPI_U_MC hydrogels may indicate
 168 the presence of calcium magnesium carbonate. XRD may be also used to identify the degree of
 169 crystallinity of material. Crystalline materials show sharp peaks whereas amorphous produce a
 170 broad background pattern. Therefore, it could be concluded that WPI hydrogels with urease
 171 incubated in media MA and MB (WPIU_MA and WPIU_MB) are crystalline, as indicated by sharp
 172 peaks [13,25]. The lower intensity and sharpness of peaks of hydrogels incubated in medium MC
 173 (WPI_U_MC) suggests lower crystallinity.



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175 Figure 3 XRD diffractograms of WPI hydrogels incubated in different media MA, MB and MC

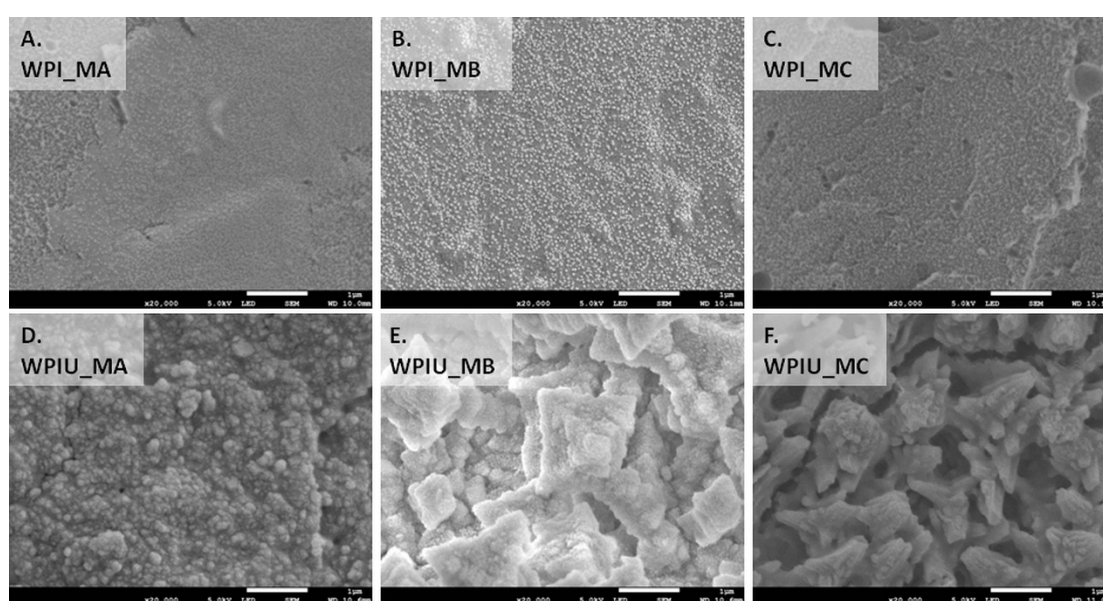
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177 The morphology of the different WPI hydrogels was studied by SEM (Figure 4). As expected,
 178 WPI hydrogels without urease displayed surfaces devoid of mineral deposits (Figure 4A-C). The
 179 surface of hydrogels incubated in medium MA was smooth, whereas hydrogels incubated in medium
 180 MB and MC had some deposits on the surface, which might have been polymer residues. In contrast,

181 WPI hydrogels containing urease displayed microstructures rich in mineral deposits (Figure 4D-L).
182 In mineralized hydrogels incubated in medium MA (WPI_U_MA), some porous cube-like deposits
183 typical for calcite and spherical deposits typical for vaterite were observed [27,28]. Moreover, some
184 undefined elongated, rod-like deposits were also detected on the surface of hydrogels incubated in
185 medium MA. Cuboid deposits typical for calcite were also observed in WPI hydrogels incubated in
186 media containing magnesium (MB and MC). However, there were also some undefined deposits in
187 samples incubated in medium with the highest concentration of magnesium (MC), which were not
188 described in previous studies [14,27]. This finding suggests a lower degree of crystallinity of the
189 mineral formed in such samples (WPI_U_MC). In mineralized hydrogels incubated in medium MB
190 (WPI_U_MB), besides rhombohedral crystals of calcite, there were also rod-like crystals covered with
191 spherical deposits. Plate-like deposits, which were reported in previous work on GG hydrogels and
192 are characteristic of hydromagnesite, were not observed in WPI hydrogels [14].

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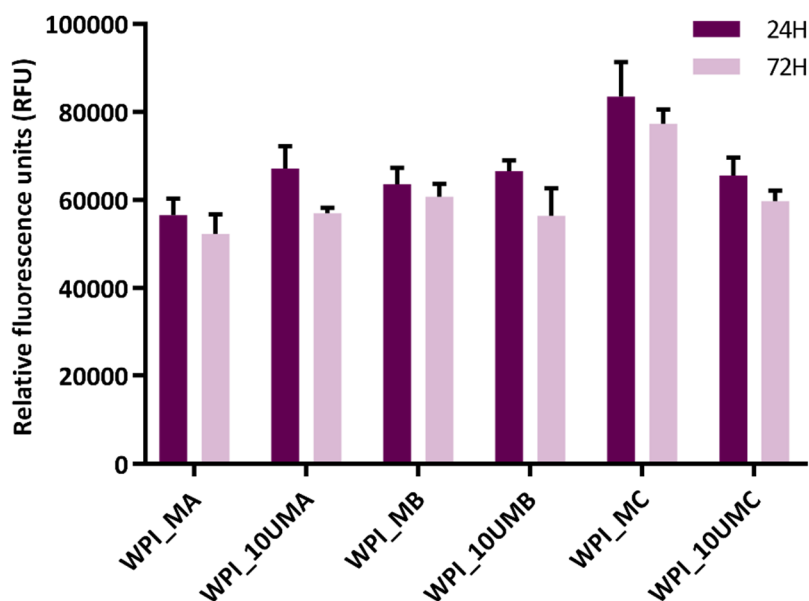
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196 Figure 4 SEM images of WPI and WPI_U hydrogels incubated in medium MA (A, D), MB (B, E) and
197 MC (C, F). Scale bar is representative of 1 μm (A, B, C) and 10 μm (D, E, F)

198 *Cytotoxicity studies and release of Ca and Mg into cell culture medium*

199 Cell viability experiments utilizing the AlamarBlue assay (Fig. 5) indicated that none of the
200 hydrogels were immediately cytotoxic. Cells were viable following 24 hours incubation on the WPI
201 hydrogels. Interestingly, cell viability increased on WPI hydrogels which lacked urease where the
202 calcium and magnesium concentrations decreased and increased, respectively (WPI_MA vs WPI_MB
203 vs WPI_MC). In contrast, viability remained similar for all WPI hydrogels mineralized with urease,
204 independently of the mineralization medium used. For the most part, cell viability remained
205 comparable after 72 hours, indicating that the cells were able to adhere and remain viable.

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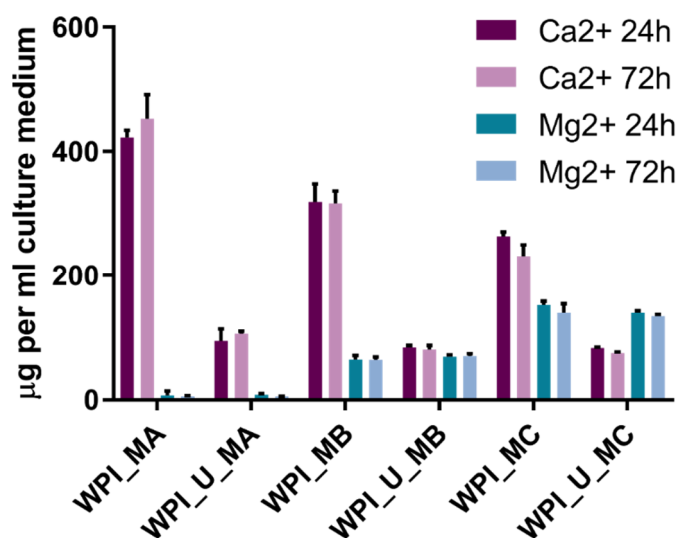
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Figure 5. Cell viability of mouse osteoblast (MC3T3-E1 cells) seeded onto hydrogels over 24 and 72 hours. Background fluorescence of AlamarBlue and cell culture media was subtracted from each experimental hydrogel. $n = 3$, error bars are representative of SD.

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Figure 6. Concentrations of elemental Ca and Mg in hydrogel extracts after 24 h and 72 h. $n = 3$, error bars show standard deviation.

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The results of measurements of elemental Ca and Mg in extraction medium (Fig. 6) revealed that considerably more Ca and Mg were released from hydrogels which did not contain urease, i.e. which were unmineralized. One explanation may be the presence of residual mineralization medium containing Ca and Mg in the hydrogels, allowing these ions to diffuse out more easily from unmineralized samples. In mineralized samples, taking into account the lower amount of Mg present

220 compared to Ca (Table 2), Mg was preferentially released. It was noticeable that the concentrations
221 present in the extraction medium were similar after 24 h and 72 h. This suggests that practically all
222 Ca and Mg release takes place within the first 24 h.
223

224 3. Discussion

225 Enzymatic mineralization of WPI hydrogels was demonstrated directly by FTIR and Raman
226 spectra (Fig. 2), XRD diffractograms (Fig. 3), SEM images (Fig. 4), and indirectly by ICP-OES
227 quantification of elemental magnesium and calcium and measurement of dry mass percentage (Fig.
228 1).

229 The amount of mineral formed was highest when the mineralization medium contained only
230 calcium, i.e. in sample group WPI_U_MA. Furthermore, when both calcium and magnesium were
231 present in the mineralization medium, calcium was preferentially incorporated into mineral formed.
232 These results were consistent with previous studies on urease-mediated mineralization of GG
233 hydrogels [14]. This effect was also observed for CaP formation and can be explained by the
234 mechanism proposed by Martin and Brown [29]. Accordingly, magnesium ions in solution are more
235 hydrated than calcium ions, thus undergo dehydration more slowly, which results in their adsorption
236 on CaCO₃. Subsequently, they are not included in the carbonate mineral and remain on the surface.
237 As a result, the total amount of magnesium the mineral formed is lower.

238 Marine research on exoskeletons of marine invertebrates mineralized with CaCO₃ has revealed
239 similar effects. In studies in which the effect of the Mg:Ca elemental ratio in seawater on the
240 incorporation of magnesium into marine invertebrate exoskeletons and non-skeletal precipitation
241 was investigated [30,31], preferential incorporation of calcium was reported.

242 A decrease of crystallinity in the order WPIU_MA > WPIU_MB > WPIU_MC was observed using
243 Raman spectroscopy (Fig. 2B), XRD (Fig. 3) and SEM (Fig. 4). This can also be explained by the
244 mechanism proposed by Martin and Brown (see above), whereby magnesium ions dehydrate more
245 slowly and hence are not included in the carbonate mineral and remain on the surface [29]. XRD
246 results suggested that small amounts of calcium magnesium carbonate may have formed in
247 WPIU_MC samples (Figure 3). Raz et al. (2000) proposed that calcium magnesium carbonate forms
248 via an amorphous precursor phase, into which hydrated magnesium ions can be more easily
249 incorporated in the amorphous precursor phase and hence are more likely to be incorporation into
250 the final, more crystalline calcium magnesium carbonate phase [32]. Furthermore, magnesium may
251 stabilize the amorphous precursor phase; magnesium has been reported to 'poison' calcite formation
252 by binding to the surface of calcite nuclei and inhibiting further crystal growth as a result of its
253 hydration [33].

254 SEM images (Fig. 4) revealed differences in the morphologies of mineral deposits formed in WPI
255 hydrogels in this study and those formed in GG hydrogels in previous work [14]. **Hydrogels are 3D**
256 **cross-linked polymer networks with entrapped water. The mechanism of WPI hydrogel formation at**
257 **elevated temperature and pressure involves denaturation of its main component β -lg, leading to**
258 **unfolding of the molecule and the formation of disulphide bonds between β -lg molecules and the**
259 **formation of a 3D cross-linked network [34].**

260 **Differences between the structures of WPI hydrogels and GG hydrogels may influences the type**
261 **of mineral formed. The concentration of polymer in WPI hydrogels in this study (50% (w/v)) is much**
262 **higher than in the GG hydrogels used in previous work (0.7% (w/v)) [10,14,15]. A higher**
263 **concentration of polymer would lead to smaller pores in the polymer network. In turn, this would**
264 **hinder diffusion of mineralization medium through the hydrogel which would lower the amount of**
265 **mineral formed. Another consequence of small pores is hindrance of the formation of large crystals.**
266 **Different degrees of compactness of the mineralized hydrogels were observed (Figure 4D, E, F),**
267 **which may be due to the differences in the sizes of the mineral deposits. A higher concentration of**
268 **polymer might however promote mineral formation; diffusion of enzyme out of the hydrogel would**
269 **be impeded, and also more polymer chains mean more potential binding sites for calcium or**
270 **carbonate ions which may serve as nucleation sites for crystal growth.**

271 There is no obvious correlation between amount of Ca and Mg released (Fig. 6) and cell
 272 proliferation (Fig. 5). This demonstrates that the amounts of Ca and Mg released are non-cytotoxic
 273 and may be considered harmless. Ca concentrations of 10 mM and above have been reported to be
 274 toxic for osteoblasts, while concentrations in the range 2-4 mM have been reported to be beneficial
 275 for proliferation [35]. The Ca (and Mg) concentrations in cell culture media are in the range 0-10 mM
 276 (Fig. 6), however no obvious positive effect was observed. It is to be expected that protein is released
 277 from WPI hydrogels; one may speculate that the released protein may be binding Ca and Mg and
 278 hindering any stimulatory effect of these ions.

279 Previous work on GG hydrogels mineralized with calcium and magnesium carbonates by
 280 enzymatic mineralization demonstrated that hydrogels mineralized with calcite improved the
 281 adhesion and proliferation of osteoblast-like MC3T3-E1 cells, and that hydrogels mineralized with
 282 calcite containing small amounts of magnesium had no appreciable negative effect [14]. Similarly, a
 283 previous study on GG hydrogels mineralized with calcium and magnesium carbonates by alternate
 284 soaking in solutions of calcium/magnesium and carbonate ions also demonstrated comparable
 285 proliferation and differentiation of osteoblast-like MC3T3-E1 cells on hydrogels mineralized with
 286 predominantly calcite and vaterite and those mineralized with predominantly calcite and vaterite
 287 containing small amounts of magnesium [15].

288 The results of this study suggest that magnesium as a dopant of CaCO₃ does not provide an
 289 appreciable positive biological effect on cell viability, in contrast to reports of a positive effect of
 290 magnesium as a dopant of CaP. The reasons for this remain unclear and are outside the scope of this
 291 work. **In this study, no appreciable effect of mineralization on swelling was observed. However, such**
 292 **effects are worthy of investigation in future work.**
 293

294 4. Materials and Methods

295 *Production of urease WPI hydrogels containing urease*

296 WPI hydrogels were produced as previously described [2-4]. Briefly, WPI powder (Daisco,
 297 USA) was added to ddH₂O for a final concentration of 50% (w/v). The solution was incubated in an
 298 ultrasonic bath for 30 minutes to ensure the powder had fully dissolved. Urease extracted from
 299 *Canavalia ensiformis* (Sigma Aldrich, U1500) was added to WPI solution at a concentration of 10 mg
 300 urease/ml solution prior to the solution being heated to 70°C for 8 min for gelation.
 301

302 *Mineralization of urease WPI hydrogels*

303 The urease WPI hydrogels were mineralized using three different media at room temperature
 304 over 7 days. In these media, the concentration of urea (0.17M) was kept constant whilst the ratio of
 305 CaCl₂:MgCl₂ was varied (Table 1). Unmineralized control samples were prepared in a similar fashion
 306 by incubating urease-free WPI hydrogels in the mineralization media.

307 Table 1 Mineralization media composition

Medium	Concentration (mol/dm ³)			Ratio
	CaCl ₂	MgCl ₂	Urea	
MA	0.2700	0	0.1700	1:0
MB	0.2025	0.0675	0.1700	0.75:0.25
MC	0.1350	0.1350	0.1700	0.5:0.5

308

309 *Determination of mineral formation and elemental composition*

310 To assess the extent of mineral formation, the samples were dried at 60 °C for 48 hours. Samples
 311 were weighed before and after the drying process to calculate the dry mass percentage, or (weight

312 after drying/weight before drying) $\times 100\%$, which the mass percentage attributable to polymer and
313 mineral and not water and serves as a measure of extent of mineralization.

314 Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to determine the
315 calcium and magnesium content of samples using a 5100 Synchronous Vertical Dual View
316 spectrometer (Agilent, **Cheadle, UK**). Prior to analysis, solid samples were dried and ground before
317 being diluted in 1 % nitric acid and diluted 10-fold. No additional steps were necessary for liquid
318 samples (cell culture medium), which were diluted in 1 % nitric acid immediately.
319

320 *Physiochemical and morphological characterisation: Raman, XRD, SEM, FTIR*

321 Samples were dried at 60 °C for 48 hours prior to analysis. Raman spectra were collected using
322 a confocal Raman system (InVia, Renishaw plc, Wotton-Under edge, UK) equipped with a near
323 infrared laser. A laser power of ~15 mW was used to prevent any damage to the samples. Spectral
324 collection exposure time was set to 1 second with one acquisition. Spectra were collected from each
325 sample over the spectral range of 60-1800 cm^{-1} . Pre-processing such as baseline correction was carried
326 out using Wire 4.0 software (Renishaw plc, Wotton-Under edge, UK).

327 X-ray diffraction (XRD) measurements were performed with Rigaku, SmartLab 9kW with DteX-
328 250 detector and Cu rotating anode source (**Rigaku, Tokyo, Japan**). Diffractometer was set to 45 kV
329 and 200 mA. 5 degree soller slits and 2 theta scans in Bragg Brentano configuration were used.

330 Morphological characterization of hydrogels was performed using scanning electron
331 microscopy (SEM). Firstly, samples were coated with a 9 nm layer of gold for 3 min at 20 mA and
332 1×10^{-1} mBar using a Quorum Q150RES sputter coater (Quorum Technologies Ltd, **Lewes, UK**). SEM
333 analysis was performed using secondary electron detector JSM-7800F (Jeol UK Ltd., Welwyn Garden
334 City). Images were acquired with an accelerating voltage of 5 kV at a working distance of about 10
335 mm.

336 The chemical structure of samples was examined using Fourier transform infrared spectroscopy
337 (FTIR) (Agilent Technology, **Cheadle, UK**) in Attenuated Total Reflectance (ATR) mode. Spectra were
338 collected in the 500 - 4000 cm^{-1} spectral range with a resolution of 4 cm^{-1} and an average of 8 scans.
339

340 *Cell culture and cell viability and release of calcium and magnesium from hydrogels into cell culture medium*

341 The mouse osteoblast MC3T3-E1 cell line (ATCC® CRL-2594™, USA) was routinely cultured in
342 MEM- α medium supplemented with 10% FBS and incubated in a humidified 5% CO_2 environment
343 at 37°C. Once 80% confluent, cells were trypsinized and used for cytotoxicity testing. **Cell tests were**
344 **carried out in accordance with the International Organization for Standardization (ISO) norm ISO**
345 **10993-5.**

346 In 96 well polystyrene plates, 4×10^3 MC3T3-E1 cells were seeded on WPI hydrogels and
347 incubated for 24 hours to allow the cells to adhere. MC3T3-E1 seeded WPI hydrogels were washed
348 with PBS (**pH 7.4**) before AlamarBlue (10% in media) was added and allowed to incubate for 2 hours.
349 Fluorescence analysis was performed using an excitation wavelength of 530 nm and an emission
350 wavelength of 590 nm. Controls lacking cells were used to determine background fluorescence which
351 was subsequently subtracted from cell viability results. Cell viability was measured after 24 h and 72
352 h.

353 To study release of calcium and magnesium, WPI hydrogels were incubated for 24 h or 72 h in
354 cell culture medium under sterile conditions in the absence of cells. Calcium and magnesium
355 concentrations in media were determined using ICP-OES as described above
356

357 *Statistical analyses*

358 Student's t-test was applied to determine statistical significance using Excel software. The results
359 of average weight, dry mass percentage, calcium and magnesium concentration and biological tests

360 were analyzed. A two-tailed unpaired t-test with 95%, 99% and 99.9% confidence interval was
361 considered statistically significant if $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)).

362 5. Conclusions

363 WPI hydrogels were enzymatically mineralized with an inorganic phase consisting of CaCO_3 or
364 magnesium-enriched CaCO_3 . Calcium was incorporated into the mineral formed to a greater extent
365 than magnesium. Increasing the concentration of magnesium in the mineralization medium led to
366 reduction of mineral formed. These observations were confirmed by dry mass percentage and ICP-
367 OES. Moreover, increasing the amount of magnesium in medium resulted in less crystalline structure
368 of mineral formed in hydrogels as shown by XRD and Raman spectroscopy results. The type of the
369 carbonate phase detected in all hydrogels with urease was mainly calcite, which was confirmed by
370 SEM morphology observation, XRD and FTIR analysis. There were also some vaterite deposits
371 detected in all hydrogels mineralized by urease. XRD analysis showed that in hydrogels incubated
372 in mineralization medium MC with equimolar concentrations of calcium and magnesium, some
373 calcium magnesium carbonate was also present. Biological studies revealed that hydrogels were not
374 cytotoxic. Hydrogels mineralized by urease had similar cell viability, which amounted to 74%. The
375 presence of magnesium in mineral formed did not promote or inhibit cell metabolic activity.

376 Further work should focus on biological studies concerning cell differentiation and in vivo
377 implantation in order to determine the influence of CaCO_3 and magnesium-enriched CaCO_3 on bone
378 tissue regeneration.

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