

# Stability studies of starch aerogel formulations for biomedical applications

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16 ABSTRACT: Starch aerogels are attractive materials for biomedical applications due to their low  
17 density and high open porosity coupled with high surface areas. However, the lack of  
18 macropores in conventionally-manufactured polysaccharide aerogels is a limitation to their use  
19 as scaffolds for regenerative medicine. Moreover, the stability under storage of polysaccharide  
20 aerogels is critical for biomedical purposes and scarcely studied so far. In this work, the  
21 induction of a new macropore population (1-2  $\mu\text{m}$ ) well-integrated in the starch aerogel  
22 backbone was successfully achieved by the incorporation of zein as a porogen. The obtained  
23 dual-porous aerogels were evaluated in terms of composition as well as morphological, textural  
24 and mechanical properties. Stability of aerogels upon storage mimicking the zone II (25 °C, 65 %  
25 relative humidity) according to International Conference on Harmonization (ICH) guideline of  
26 climatic conditions was checked after 1 and 3 months from morphological, physicochemical and  
27 mechanical perspectives. Zein incorporation induced remarkable changes in the mechanical  
28 performance of the end aerogel products and showed a preventive effect on the morphological  
29 changes during the storage period.

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31

32 INTRODUCTION

33 The development of innovative synthetic grafts, known as scaffolds, offers a promising  
34 response to regenerate damaged tissues encouraging the self-healing capacity of the patients.  
35 Depending on the anatomical target, scaffolds must display a particular 3D interconnected and  
36 hierarchical porous structure for an appropriate performance once implanted<sup>1-3</sup>. Moreover, the  
37 mechanical behavior of the grafts is of particular relevance since they should temporarily  
38 surrogate the requirements of the natural tissue.

39 Aerogels are solid mesoporous materials characterized by extremely low densities and high  
40 open porosities of tailored size and distribution<sup>5,6</sup>. These properties of aerogels have been widely  
41 exploited in several fields, particularly silica and carbon aerogels in the building industries as  
42 thermal insulation materials<sup>7-9</sup>. Nevertheless, bio-based aerogels (i.e. from polysaccharides and  
43 proteins) are the mainstream choice for biomedical applications. In particular, starch aerogels  
44 emerge as an attractive alternative for bone scaffolds, where the advanced properties of aerogels  
45 are supplemented by the biocompatibility, the complete physiological degradation and the  
46 abundance of starch in nature<sup>10-12</sup>. In addition, starch-based blends promote cell adhesion and  
47 proliferation using human osteoblasts<sup>13,14</sup>.

48 Starch aerogels are formed by a network of intermingled fibers of amylose and amylopectin  
49 with a defined micro/mesoporous architecture that can mimic the extracellular matrix.  
50 Nevertheless, the usual absence of pores in the macroscale (1  $\mu\text{m}$  and above) hampers the  
51 interaction of the scaffold with the biological tissue. The addition of sacrificial porogens (e.g.,  
52 salts, sugar or paraffin wax) of defined shapes and dimensions has been explored to confer  
53 macroporosity to different aerogel sources<sup>15-18</sup>. However, these approaches result in tedious and

54 cumbersome protocols for aerogel processing requiring additional leaching steps to remove the  
55 porogen.

56 Stability studies are mandatory for conventional drug products and medical devices to verify  
57 that raw materials and end products meet the legal requirements in terms of identity, output,  
58 quality and purity over time <sup>19</sup>. Stability in terms of chemical identity, physical form and  
59 biological activity, is a critical parameter that could prevent the clinical use and that gives  
60 practical information to decide on the need and choice of primary and secondary packaging for  
61 the product. However, there is a paucity of information focused on the effect of the storage  
62 period on the performance of nanostructured scaffolds, although those with intricate geometries  
63 are particularly affected by environmental conditions.

64 In this work, starch-based aerogels endowed with macroporosity were obtained through an  
65 innovative processing approach involving the use of porogens without extra-leaching steps. Zein,  
66 the major protein of storage of corn, was tested as porogen to induce the formation of well-  
67 integrated macropores in the mesoporous starch aerogel network. The effect of the use of zein  
68 was evaluated on the resulting aerogel composition, textural and mechanical properties. In  
69 addition, quantitative determinations of zein residues in the aerogels were performed, since its  
70 presence favor the *in vivo* promotion of mesenchymal stem cells adhesion and proliferation <sup>20,21</sup>.  
71 The stability upon storage was studied on a mid-term (1 and 3 months) mimicking the zone II  
72 International Conference on Harmonization (ICH) guideline of climatic conditions (25 °C, 60 %  
73 relative humidity) <sup>22</sup>, which corresponds to the worst case storage scenario for the regions of  
74 Europe, Japan and USA. Scaffolds were monitored in terms of morphological, physicochemical  
75 and mechanical stability.

76

77 MATERIALS AND METHODS

78 **Materials.** Native corn starch (52.6 % amylose content,  $\rho_{\text{skel}} = 1.4562 \pm 0.012$  g/mL) was  
 79 provided by Roquette Frères S.A. (Lestrem, France). Zein (m.p. 266-283 °C, size of dry  
 80 agglomerates by the sieving method:  $557 \pm 208$   $\mu\text{m}$ ;  $\rho_{\text{skel}} = 1.167 \pm 0.025$  g/mL) was purchased  
 81 from Sigma-Aldrich, Inc. (Madrid, Spain). CO<sub>2</sub> (purity > 99.9 %) was supplied by Praxair, Inc.  
 82 (Madrid, Spain). Absolute ethanol (EtOH) was provided by VWR (Radnor, PA, USA).

83 **Corn starch aerogels preparation.** Cylindrical aerogel specimens were obtained by adapting  
 84 a previously reported procedure<sup>23</sup>. Briefly, starch-aqueous dispersions (10 % w/w) containing  
 85 varying ratios of zein as porogen (Table 1) were subjected to a thermal treatment for starch  
 86 gelatinization (121 °C, 20 min) and dosed in cylindrical polypropylene molds (length: 14 mm,  
 87 diameter: 12 mm). After storage at 4 °C for 48 h, the resulting gels were immersed in absolute  
 88 ethanol for solvent exchange (gel-alcogel transition) and zein leaching. Solvent was replaced  
 89 with fresh ethanol six times at an exchange frequency of 48 h. Starch alcogels were then loaded  
 90 in a 100 mL autoclave (Thar Process, Pittsburg, PA, USA) containing 45 mL of absolute ethanol.  
 91 A continuous flow of 6 g/min of supercritical CO<sub>2</sub> (40 °C, 130 bar) through the autoclave during  
 92 4 h was employed for ethanol extraction. Subsequently, a controlled depressurization of 2  
 93 bar/min until atmospheric pressure was performed. Aerogel cylindrical probes (length: ca. 11  
 94 mm, diameter: ca. 8.5 mm) were collected from the autoclave for further characterization.

95  
 96 **Table 1.** Starch aerogel notation regarding the initial content of starch and zein (expressed in  
 97 grams and in weight ratios) used in the batches for the hydrogel formation.

Aerogel	Zein-to-starch weight ratio	$c$ (see Eq. 4)
Z0	0 g : 8 g (0:1)	0
Z1	2 g : 8 g (1:4)	0.25

Z2	4 g : 8 g (1:2)	0.5
Z3	6 g : 8 g (3:4)	0.75
Z4	8 g : 8 g (1:1)	1

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98

99 **Analytical, physicochemical, structural and mechanical characterization of starch**

100 **aerogels.** The volume reduction ( $\Delta V$ , in percentage) of the gels after the solvent exchange and  
 101 the supercritical drying steps was evaluated as

$$102 \quad \Delta V = \left( \frac{V_0 - V}{V_0} \right) \times 100 \quad (1)$$

103 where  $V_0$  denotes the initial volume of the hydrogel and  $V$  the end volume of the alcogel or  
 104 aerogel, accordingly.

105 For zein residues quantification in the aerogels, a bottom-up proteomics approach was applied,  
 106 involving proteolytic digestion of zein before high-resolution tandem-mass spectrometry  
 107 analysis. Starch aerogel samples were dissolved at a concentration of 1 mg/mL in buffer solution  
 108 A (10 mM Tris-HCl pH 8.0, 8 M urea) under agitation overnight. Dissolved samples were  
 109 diluted in buffer solution B (50 mM Tris-HCl pH 8.0, 0.5 mM  $\text{CaCl}_2$ ) in order to reach urea  
 110 concentrations below 6 M. For zein proteins digestion, 370  $\mu\text{L}$  of the previous sample solution  
 111 were mixed with 120  $\mu\text{L}$  of thermolysin stock solution prepared in buffer solution B (enzyme-to-  
 112 substrate weight ratio 1:20), and incubated for 1 h at 80 °C in an Eppendorf ThermoMixer  
 113 (Eppendorf AG, Hamburg, Germany). The digestion reaction was stopped by adding 25  $\mu\text{L}$  of  
 114 formic acid (10 vol.%). Digested solutions were filtered through a Microcon-30 kDa Centrifugal  
 115 Filter (Merck KGaA, Darmstadt, Germany) before analysis to remove non-digested proteins.

116 An Agilent 1290 UHPLC system coupled to an Agilent 6540 quadrupole-time-of-flight mass  
 117 spectrometer (q-TOF MS) and equipped with an orthogonal ESI source was employed for the  
 118 determination and quantification of zein residues. Chromatographic separation of digested zein

119 was conducted using a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm particle  
120 diameter, Agilent Technologies, Santa Clara, CA, USA) at 30 °C. The mobile phase was  
121 composed of water (0.1 vol.% formic acid, solvent A) and acetonitrile (0.1 vol.% formic acid,  
122 solvent B). A 5-μL aliquot of the sample was injected at a flow rate of 0.5 mL/min during  
123 gradient elution. The gradient program was as follows: 0 min, 0 % B; 7 min, 30 % B; 9 min, 80  
124 % B; 11 min, 100 % B; 13 min, 100 % B; 14 min, 0 % B. The mass spectrometer was operated in  
125 MS and MS/MS modes. MS parameters were the following: capillary voltage, 4000 V; nebulizer  
126 pressure, 40 psi; drying gas flow rate, 10 L/min; gas temperature, 350 °C; skimmer voltage, 45  
127 V; fragmentor voltage, 110 V. The MS and Auto MS/MS modes were set to acquire m/z values  
128 ranging between 50-1100 and 50-800, respectively, at a scan rate of 5 spectra per second.  
129 Operating the ESI source in positive ionization mode, four proteolytic peptides were monitored:  
130 LQQQ (m/z 516.2776), LQQ (m/z 388.2190), FNQ (m/z 408.1877) and FSQ (m/z 381.1768).

131 Skeletal density of starch aerogels ( $\rho_{skel}$ ) was determined by helium pycnometry  
132 (Quantachrome, Boynton Beach, FL, USA) at room temperature (25 °C) and 1.01 bar. Values  
133 were obtained from five replicates (standard deviation < 4 %). Bulk density of the aerogels ( $\rho_{bulk}$ )  
134 was determined by weighing and measuring their dimensions. The resulting overall porosity ( $\varepsilon$ )  
135 and total pore volume were calculated from Eqs. (2) and (3), respectively.

$$136 \quad \varepsilon = \left(1 - \frac{\rho_{bulk}}{\rho_{skel}}\right) \times 100 \quad (2)$$

$$137 \quad V_p = \left(\frac{1}{\rho_{bulk}} - \frac{1}{\rho_{skel}}\right) \quad (3)$$

138 Textural properties of the aerogels were determined by N<sub>2</sub> adsorption-desorption analyses  
139 (ASAP 2000 Micromeritics Inc, Norcross, GA, USA). Prior to the measurements, aerogels were  
140 outgassed at 80 °C and under vacuum (<1 mPa) for 24 h. Specific surface area ( $A_{BET}$ ) of the  
141 aerogels scaffolds were determined by the Brunauer-Emmett-Teller (BET) method. Specific pore

142 volumes ( $V_{p,BJH}$ ) and mean pore diameter ( $d_{p,BJH}$ ) were evaluated from the desorption branch of  
143 the isotherms using the Barrett-Joyner-Halenda (BJH) method (Figure S1).

144 Based on the BJH-pore volume distribution, the contributions (in percentage) of mesopores (2-  
145 50 nm range,  $V_{p,meso}$ ) to the total pore volume were determined. The contribution of the  
146 macropore population ( $>50$  nm,  $V_{p,macro}$ ) was determined by the difference between the total  
147 specific pore volume and the specific mesopore volume ( $V_{p,meso}$ ).

148 The structure of the aerogels was evaluated by scanning electron microscopy (FESEM,  
149 ULTRA-PLUS, Zeiss, Oberkochen, Germany) running at 3 kV. Prior to imaging, aerogels were  
150 sputtered with a layer of iridium of 10 nm thickness.

151 The mechanical behavior of cylindrical aerogel specimens was analyzed by means of uniaxial  
152 quasistatic compression tests using a 10 kN load cell on the universal testing machine Z010  
153 (Zwick/Roell GmbH, Ulm, Germany). The strain rate of 10 %/min was applied for all  
154 compression tests. To characterize the inelastic features of the aerogels, cyclic compression was  
155 conducted, whereby the aerogel specimens were subjected to three sets of loading and unloading  
156 cycles with the strain amplitude increased stepwise by 20 %. All the experiments were  
157 performed at 20 °C, atmospheric pressure and in triplicate.

158  
159 **Stability tests under storage of starch aerogels.** Aerogel cylindrical probes of each  
160 composition were placed inside sterile glass vessels with hermetic closure, containing a solution  
161 of sulfuric acid (37 % v/v) to maintain the relative humidity at 65 %<sup>24</sup>. Containers were stored  
162 for either 1 or 3 months at 25 °C. After the storage time was elapsed, aerogels were collected for  
163 their complete characterization-



164 **Statistical analysis.** All results were expressed as mean  $\pm$  standard deviation. Statistical  
165 analyses of shrinkage values (1-way ANOVA) were performed followed by the post hoc Tukey-  
166 Kramer method test using Statistica v.8.0 software (StatSoft Inc., Tulsa, OK, USA)

167

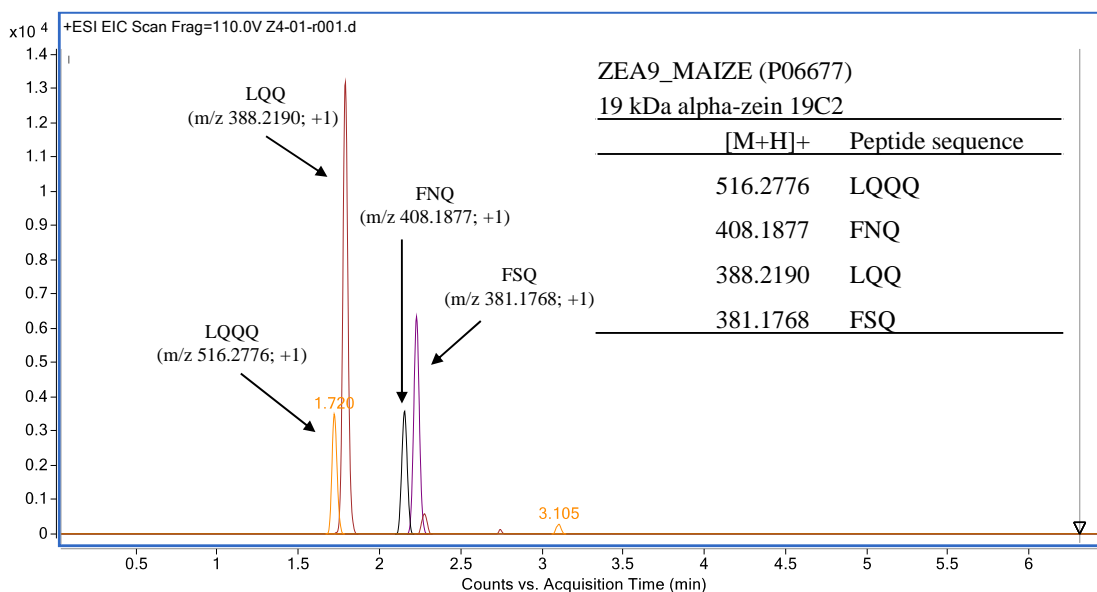
## 168 RESULTS AND DISCUSSION

169 **Morphological and physicochemical characterization of starch-based macroporous**  
170 **aerogels.** Corn starch aerogels were prepared in the form of cylindrical monoliths for a  
171 reproducible determination of their densities and mechanical properties. White solid lightweight  
172 structures were obtained in all cases, although the modified starch aerogels showed a slight  
173 yellow coloration suggesting the presence of zein residues (Figure S2). The use of zein favored  
174 the homogeneous dosing of the aqueous dispersion in the moulds. The reduced content of  
175 amylose in the admixture extended its retrogradation rate since less intermolecular hydrogen-  
176 bondings were formed within the dispersion<sup>11,25</sup>.

177 A determination method based on a bottom-up approach was set up to quantify zein residues in  
178 the aerogel samples. The full sequence of 19 kDa alpha-zein 19C2 (ZEA9 MAIZE – P06677)  
179 protein was obtained from Uniport database, and the whole sequence of peptides was exported to  
180 PeptideMass tool from ExPasy website for *in silico* digestion. Theoretical peptide masses of the  
181 input proteins were generated applying the following stringent criteria: thermolysin was selected  
182 as digestion enzyme, and only one missed cleavage was allowed for thermolysin digestion.

183 Operating the HPLC-ESI-QTOF system in the positive ionization mode (ESI+), a targeted  
184 screening analysis in full MS mode ( $m/z$  100–1100 mass range) was performed to identify the  
185  $m/z$   $[M+H]^+$  peptide masses obtained from *in silico* digestion in a zein standard solution and in  
186 the starch aerogel sample theoretically containing the highest zein content (Z4). Figure 1 shows

187 four selected zein peptides masses ( $m/z = 516.2776$  [LQQQ+H]<sup>+</sup>;  $388.2190$  [LQQ+H]<sup>+</sup>;  $408.1877$   
 188 [FNQ +H]<sup>+</sup>;  $381.1768$  [FSQ +H]<sup>+</sup>) in Z4 sample. These peptides were selected for zein  
 189 determination, exhibiting satisfactory intensity, sensitivity and dynamic range.



190  
 191 **Figure 1.** High-resolution extracted ion chromatograms (HREICs) of Z4 starch aerogel, showing  
 192 the target peptides masses (10 ppm extraction window) for zein residues determination in  
 193 aerogels.

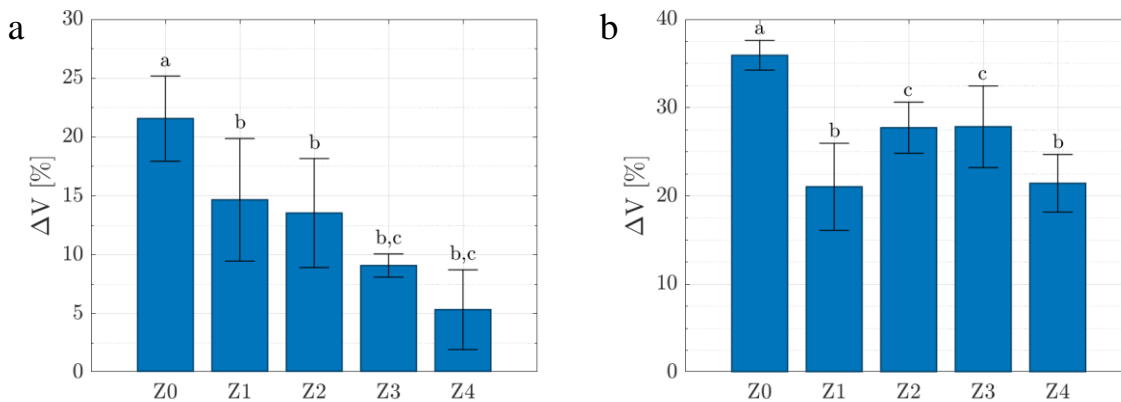
194 Table 2 shows the main LC-HRMS parameters for the target peptides, including  
 195 chromatographic retention time, monoisotopic mass, protonated molecular ion and calculated  
 196 mass error ( $\Delta m/z$ ). The identification of zein peptides was based on identity of the exact mass,  
 197 monoisotopic profile and MS/MS fragmentation spectra (Figure S3). Zein content in Z0-Z4  
 198 starch aerogels was determined by external standard calibration using a zein standard solution  
 199 submitted to the same digestion process as the starch samples (see Table 2).

200  
 201 **Table 2.** HPLC-HRMS parameters of target zein peptide fragment. Concentration values (%  
 202 w/w) for zein residues in different starch aerogels.

RT (min)	Peptide sequence	Formula	Monoisot opic mass	[M+H] <sup>+</sup> (m/z)	Error (ppm)	Concentration in starch (% w/w ± std)				
						Z0	Z1	Z2	Z3	Z4
1.719	LQQ Q	C <sub>21</sub> H <sub>37</sub> N <sub>7</sub> O <sub>8</sub>	515.2704	516.2776	1.0	nd	4.0±0.1	13.5±0.1	12.8±1.1	27.4±0.9
1.795	LQQ	C <sub>16</sub> H <sub>29</sub> N <sub>5</sub> O <sub>6</sub>	387.2118	388.2190	3.6	nd	3.3±0.1	9.9±0.2	10.4±0.3	21.2±0.2
2.153	FNQ	C <sub>18</sub> H <sub>25</sub> N <sub>5</sub> O <sub>6</sub>	407.1805	408.1877	1.5	nd	3.8±0.1	11.3±0.7	12.3±1.0	25.5±0.2
2.229	FSQ	C <sub>17</sub> H <sub>24</sub> N <sub>4</sub> O <sub>6</sub>	380.1696	381.1768	4.2	nd	3.7±0.4	7.2 ±0.5	11.1±1.1	21.1±0.9
Average zein concentration						nd	3.7±0.3	10.5± 2.6	11.6±1.1	23.8±3.1

203

204 All the manufactured starch aerogels had a certain volume shrinkage mainly during the solvent  
205 exchange step and, in a lesser extent, during the supercritical drying step (Figure 2). The addition  
206 of the zein in the aerogels strongly reduced the shrinkage values, particularly during gel-alcogel  
207 transition. For instance, a 4-fold reduction in these values was observed for Z4 aerogels.  
208 However, this effect was not linear and aerogels with similar residual zein content (Z2, Z3, in  
209 Table 2) behaved differently. On the other hand, the volume reduction detected during the  
210 supercritical drying was severe, although the overall shrinkage values are in accordance with  
211 those reported for starch aerogels with similar amylose contents (30-40 %) <sup>26,27</sup>. Interestingly, Z1  
212 aerogels presented similar values to Z4, despite of the fact that zein residues were much higher in  
213 the latter formulation.



214 **Figure 2.** Volume shrinkage of starch-based gels after (a) the solvent exchange and (b)  
 215 supercritical drying. Equal letters denote statistically homogeneous groups.

216 Bulk densities of the obtained aerogels ( $\rho_{\text{bulk}}$ ) strongly depended on the initial hydrogel  
 217 composition (Table 3). Aerogel formulations prepared from hydrogels with lower zein contents  
 218 (Z1, Z2) were significantly lighter (*ca.* 30%) than pure starch aerogels (Z0) ( $p > 0.05$ ).  
 219 Conversely, denser structures were proportionally obtained with zein content when the 1:2 zein-  
 220 to-starch weight ratio was exceeded for the Z2-Z3-Z4 aerogel sequence. The remaining zein  
 221 residues are responsible for this effect as depicted in Table 2. For example, Z3 presented almost  
 222 identical density values to the unmodified formulations (Z0), whereas Z4 aerogels were the  
 223 heaviest structures. Accordingly, the overall porosity of the aerogels ( $\varepsilon$ ) followed a reverse trend  
 224 with respect to the bulk density, but falling in the 85-91 % range in all cases, which is  
 225 advantageous for regenerative medicine applications as scaffold matrices<sup>28</sup>.

226 The technical feasibility of the processing strategy to induce a larger pore population in starch  
 227 aerogels was confirmed from the textural analysis (Table 3). The zein addition in the aerogel  
 228 formulations resulted in increased specific surface areas ( $A_{\text{BET}} = 183\text{-}228 \text{ m}^2/\text{g}$ ) with values in  
 229 the range of those reported for high amylose corn starch aerogels<sup>12,26,29</sup>. Similarly, the specific  
 230 pore volume ( $V_{\text{p,BJH}}$ ) was higher as the porogen content increased while the mean pore diameter

231 ( $d_{p,BJH}$ ) remained constant at 18-20 nm. The formation of dual porous aerogels was confirmed  
232 from the macropore contribution to the overall porous values ( $V_{p,macro}$  in Table 3). The total pore  
233 volume and macropore contribution were determined by the combination of  $N_2$  adsorption-  
234 desorption and helium pycnometry analyses, since the contribution of macropores in the pore  
235 volume (over 80 % of the overall porosity for other bio-aerogels<sup>30,31</sup>), may not be taken into  
236 account in the characterization of aerogels through the BJH method. The zein effect in  
237 macropore formation is clearly appreciated from Z0 to Z2 aerogels by an increase in  $V_{p,macro}$  of  
238 up to 4 %. Conversely,  $V_{p,macro}$  values for Z3 and Z4 aerogels were similar and 10 % lower than  
239 that ones for unmodified aerogels (Z0). Although most of the porogen was leached during the  
240 solvent exchange step, the zein residue of 10 to 20 wt.% quantified in the abovementioned  
241 formulations is responsible for their densification and thus directly decreasing the pore volume  
242  $V_p$  since it is a specific parameter (i.e. expressed in a mass basis). (Table 3).

243 Scanning electron microscopy (SEM) images of starch aerogels confirmed that their  
244 morphology and texture were dramatically influenced by the presence of zein porogen in the  
245 aerogel processing (Figure 3). The unmodified aerogel (Z0) presented an interconnected fiber  
246 network in the 30-60 nm diameter range (Figures 3a,b) typical for starch aerogels<sup>32</sup>. The  
247 incorporation of zein during the aerogel processing induced remarkable morphological changes  
248 to the aerogel architectures with the presence of spherical macropores (*ca.* 2  $\mu$ m) even in the  
249 formulation with lower zein content (Z1 in Figures 3c,d). This new pore family presented inner  
250 rough surfaces, but the presence of a thin film in certain pores (Figure 3d) suggested an  
251 incomplete zein removal during the solvent exchange step. The observed morphology was thus  
252 coherent with the zein quantifications (Table 2). The thermal treatment for the starch  
253 gelatinization disrupts the close-packed tertiary globular structure of zein, increasing its water

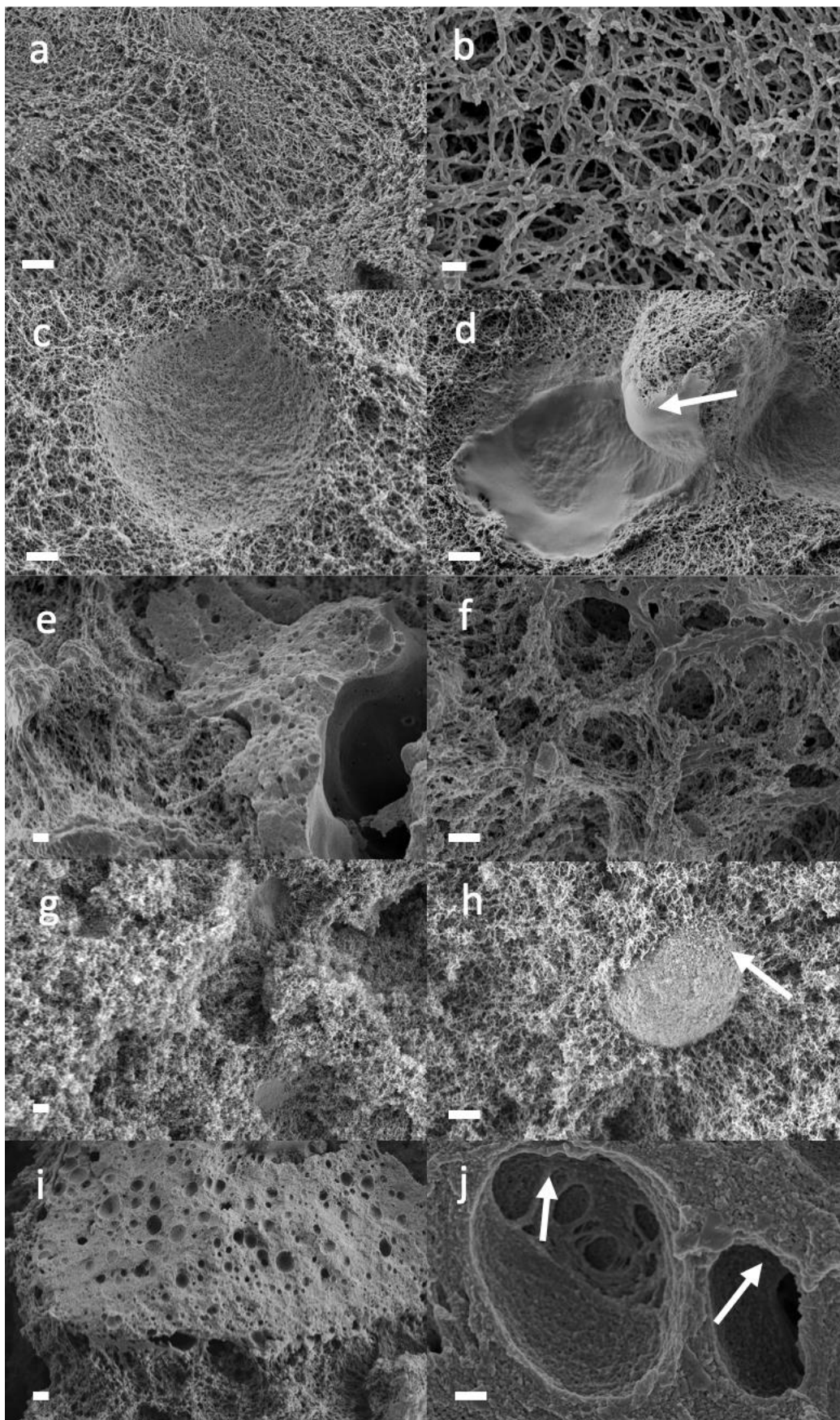
254 soluble fraction and promoting the formation of disulphide bonds<sup>33-35</sup>. Therefore, the formation  
255 of zein agglomerates are favored due to the higher interactions between polypeptide chains<sup>36,37</sup>.  
256 Z2 aerogels presented regions of large protein aggregates (>10 μm) and also regions of perfectly  
257 integrated dual and interconnected porosity (Figures 3e,f). Aerogels prepared with higher  
258 contents of porogen led to more irregular structures (Z3 in Figures 3g,h, and Z4 in Figures 3i,j),  
259 supporting the increased specific surface areas values ( $A_{BET}$  in Table 3). The formation of larger  
260 pores in Z3 aerogels was clearly identified as the footprint of zein particles after the leaching  
261 (Figure 3g). The remaining globular zein residues embedded in the starch mesoporous backbone  
262 were also observed (Figure 3h). The presence of porous zein films was more abundant in Z4  
263 formulation (Figure 3i). Overall, the formation of a family of large (1-3 μm) and interconnected  
264 macropores was achieved through the use of zein as porogen (Figure 3j).

265

266

**Table 3.** Morphological and textural properties of the obtained aerogels. Values expressed as mean values and standard deviation.

Aerogel	$\rho_{bulk}$ (g/mL)	$\rho_{skel}$ (g/mL)	$\varepsilon$ (%)	ABET (m <sup>2</sup> /g)	V <sub>p,BJH</sub> (cm <sup>3</sup> /g)	d <sub>p,BJH</sub> (nm)	V <sub>p</sub> (cm <sup>3</sup> /g)	V <sub>p,meso</sub> (%)	V <sub>p,macro</sub> ( %)	
Z0	<i>No storage</i>	0.175 ± 0.004	1.478 ± 0.05	88.1 ± 0.4	183 ± 9	1.01 ± 0.05	19.1 ± 1.0	5.03	15.1	84.9
	<i>1 month</i>	0.200 ± 0.005	1.495 ± 0.03	86.6 ± 0.4	217 ± 11	1.30 ± 0.07	21.6 ± 1.1	4.33	20.8	79.2
	<i>3 months</i>	0.184 ± 0.006	1.467 ± 0.03	87.5 ± 0.4	213 ± 11	1.30 ± 0.06	23.3 ± 1.2	4.76	18.6	81.4
Z1	<i>No storage</i>	0.120 ± 0.013	1.349 ± 0.02	91.1 ± 1.0	228 ± 11	1.29 ± 0.06	18.9 ± 0.9	7.57	12.7	87.3
	<i>1 month</i>	0.158 ± 0.005	1.389 ± 0.04	88.6 ± 0.5	226 ± 11	1.07 ± 0.05	16.9 ± 0.8	5.59	13.8	86.2
	<i>3 months</i>	0.150 ± 0.006	1.414 ± 0.05	89.4 ± 0.5	85 ± 4	0.43 ± 0.02	18.4 ± 0.9	5.94	4.9	95.1
Z2	<i>No storage</i>	0.120 ± 0.006	1.465 ± 0.01	91.8 ± 0.4	226 ± 11	1.25 ± 0.06	19.0 ± 1.0	7.62	11.0	89.0
	<i>1 month</i>	0.134 ± 0.009	1.394 ± 0.03	90.4 ± 0.7	164 ± 8	0.87 ± 0.04	18.0 ± 0.9	6.76	8.6	91.4
	<i>3 months</i>	0.135 ± 0.006	1.433 ± 0.04	90.6 ± 0.5	120 ± 6	0.60 ± 0.03	16.5 ± 0.8	6.69	6.4	93.6
Z3	<i>No storage</i>	0.172 ± 0.006	1.385 ± 0.02	87.6 ± 0.5	204 ± 10	1.18 ± 0.06	19.9 ± 1.0	5.08	16.0	84.0
	<i>1 month</i>	0.182 ± 0.006	1.369 ± 0.03	86.7 ± 0.5	184 ± 9	0.97 ± 0.05	17.3 ± 0.9	4.78	14.5	85.5
	<i>3 months</i>	0.176 ± 0.007	1.360 ± 0.01	87.0 ± 0.5	178 ± 9	0.98 ± 0.05	17.8 ± 0.9	4.93	15.1	84.9
Z4	<i>No storage</i>	0.192 ± 0.016	1.353 ± 0.02	85.8 ± 1.2	226 ± 11	1.35 ± 0.07	19.0 ± 0.9	4.46	22.5	77.5
	<i>1 month</i>	0.197 ± 0.002	1.350 ± 0.03	85.4 ± 0.4	207 ± 10	1.30 ± 0.07	21.2 ± 1.1	4.35	20.1	79.9
	<i>3 months</i>	0.187 ± 0.008	1.303 ± 0.03	85.6 ± 0.7	157 ± 8	0.91 ± 0.05	19.0 ± 0.9	4.58	15.1	84.9





268 **Figure 3.** SEM images of horizontal cross-sections of the obtained starch-based aerogels. (a,b)  
269 Characteristic microstructure of unmodified aerogels (Z0). (c,d) The addition of low contents of  
270 the porogen (Z1) and later leaching induced the formation of larger pores with rough inner  
271 surfaces, although thin films of zein residues could be observed along the aerogel (d, arrow).  
272 (e,f) More residues were detected for Z2 in certain areas, but an interconnected porous network  
273 was obtained. (g,h) The incorporation of higher zein amounts (Z3) leads to more irregular  
274 surfaces and entire spherical zein particles were identified (h, arrow), highlighting the  
275 uncompleted porogen leaching. (i,j) Z4 aerogel formulation presented numerous porous zein  
276 plates well-integrated with the starch network backbone. (j) In addition, larger and  
277 interconnected pores (arrows) with noticeable roughness were obtained. Scale bars: 300 nm (b, j)  
278 and 2  $\mu\text{m}$  (a, c-i).

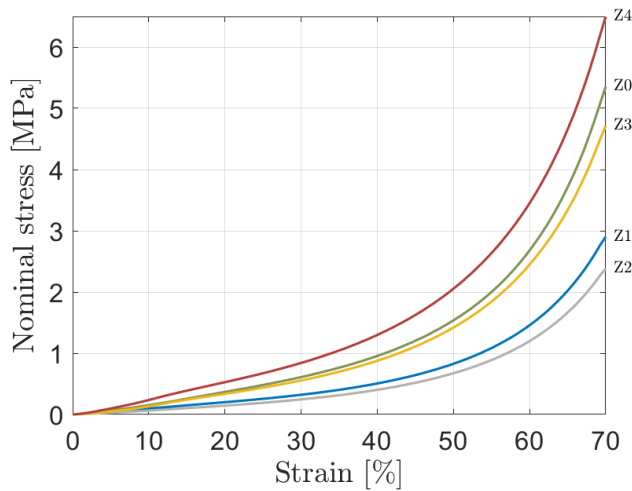
279 **Mechanical characterization of starch aerogels.** All starch aerogel formulations were  
280 subjected to uniaxial quasistatic compressions of up to 70 % strain (Figure 4). The mechanical  
281 response of the aerogels showed an irregular nature subject to addition of the zein component.  
282 Considering the pure starch aerogel (Z0) as the reference, the curves corresponding to Z1 and Z2  
283 showed that the addition of zein strongly softened their stress-strain response. This behavior is  
284 clearly related to the formation of hollow spaces in the starch aerogel backbone (Figure 3).  
285 However, this softening trend was reversed for the case of the aerogels with higher zein residues  
286 (Z3, Z4 in Table 2) and its stiffness was strongly enhanced. For instance, the stiffness of the  
287 starch aerogel processed with the highest zein content (Z4) was even stronger than the reference  
288 aerogel Z0. The compression moduli of the five aerogel formulations are illustrated in Figure 4  
289 to quantitatively show this effect. A polynomial fit expressing the relation between Young's

290 modulus under compression (given in MPa) and the zein-to-starch ratio is expressed as follows  
291 (Eq. 4)

$$292 \quad E(c) = -4.2157c^3 + 12.1715c^2 - 7.1856c + 2.0639 \quad (4)$$

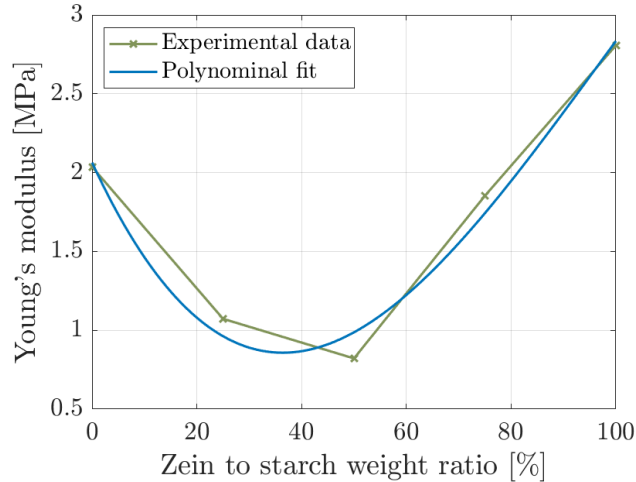
293

294 where  $c$  varies from 0 to 1 and denotes the zein-to-starch weight ratio (Table 1). For the aerogels  
295 in consideration, an explanation to the trend seen in Figure 5 can be deduced from the bulk  
296 density measurements in Table 3. Porous materials, such as aerogels, exhibit a power-law scaling  
297 relation between Young's modulus  $E$  and the bulk density ( $\rho_{bulk}$ )<sup>38-40</sup>. Such scaling behavior is  
298 also specifically observed in other polysaccharide-based aerogels<sup>41-45</sup>. Table 3 shows the effect  
299 of zein on the bulk densities of the aerogels, where a decreasing trend from Z0→Z1→Z2 and an  
300 increasing trend from Z2→Z3→Z4 were observed. This explains the trend of Young's modulus  
301 vs. the zein-to-starch weight ratio (density) curve. The addition of zein as a porogen induced the  
302 formation of macropores, which also influenced the overall macroscopic mechanical behavior of  
303 the aerogels. Such influence of the hierarchical porous structure on the mechanical behavior was  
304 previously reported for cellulose aerogels<sup>46</sup>.



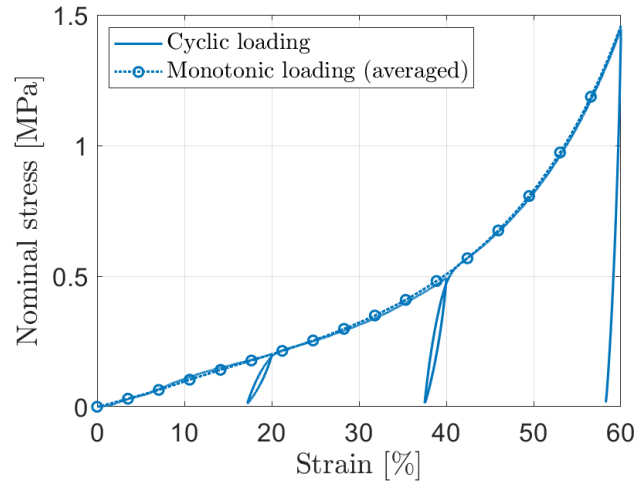
305

306 **Figure 4.** Stress-strain curves of starch aerogels processed with different zein contents (Z0 to Z4  
307 with increasing zein content) tested under compression.



308  
309 **Figure 5.** Effect of the zein content used during the starch aerogel processing on Young's moduli  
310 of the aerogels. A polynomial fit is generated to show the relation between the zein-to-starch  
311 weight ratio (in percentage, adhering to Eq. (4)) to Young's modulus.

312 Under cyclic loading, all the tested aerogels show typical elastoplastic behavior, with very  
313 large permanent set (Figures 6 and S4). This behavior is typical of other biopolymer-based  
314 aerogels<sup>43</sup>. The very small hysteresis (area between the unloading curve of a cycle and the  
315 reloading curve of the subsequent cycle) along with the permanent set indicate severe  
316 irreversible damage within the microstructure of the aerogel network. However, the aerogels  
317 exhibit a good strain memory as the reloading curve comes back to the point of the maximal  
318 strain of the previous loading cycle and continues the path as if it were the monotonic loading  
319 (Figure 6).



320

321 **Figure 6.** Stress-strain response of Z1 aerogels under cyclic loading-unloading quasistatic  
 322 compression. The specimen was subjected to three sets of loading cycles with the strain  
 323 amplitude increased stepwise by 20 %. The monotonic loading curve is illustrated as a dotted  
 324 line, demonstrating a memory of the aerogels. Curves of the other tested formulations (Z0, Z2,  
 325 Z3 and Z4) showed a similar behavior and can be found as supplementary material (Figure S4).

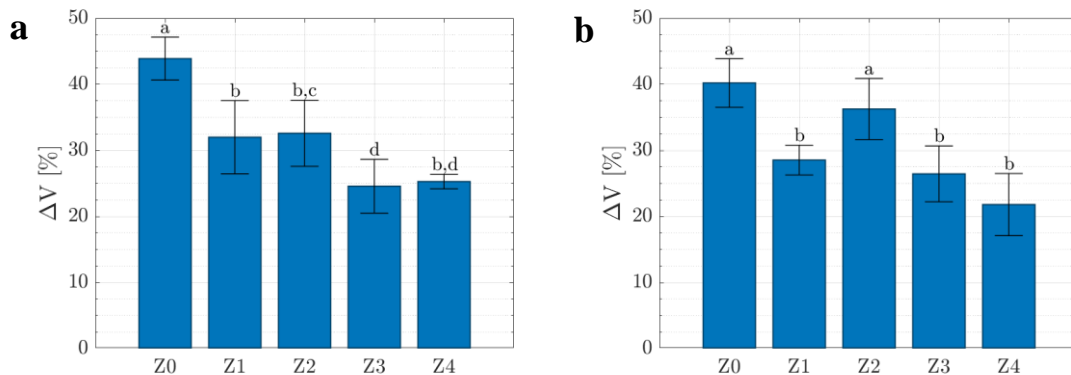
326 **Effect of storage time.** The stability under storage of drug products, medical devices and  
 327 combination products (i.e. products comprising a drug and a medical device, or a biological  
 328 product and a medical device) is a critical quality parameter within a well-established legal  
 329 framework, since the variety of degradation processes (chemical, physical, biopharmaceutical)  
 330 that may occur could render products ineffective or unsafe before patient use <sup>22</sup>. Nevertheless,  
 331 there is paucity of information on research regarding the stability of complex porous  
 332 architectures conceived as scaffolds <sup>47</sup>.

333 The exposition to the storage conditions induced certain volume shrinkage of the starch  
 334 aerogels. Formulations containing higher zein residues (Z3, Z4) presented values identical to  
 335 their non-stored counterparts, thus preserving their initial structure. On the other hand, higher

336 volume shrinkages close to 5 % were observed for unmodified aerogels (Z0) after 3 months of  
337 storage (Figure 7).

338 After 1 month of storage at 25 °C and 65 % relative humidity, aerogels experienced a  
339 densification in the 3-32 % range, depending on the formulation (Table 3). The highest  
340 densification was reached for Z1 aerogel, whereas this effect was very low in formulations with  
341 higher initial zein-to-starch weight ratio (Z2-Z4). This preventive effect can be directly attributed  
342 to the zein residues (Table 2).

343 Interestingly, bulk densities of aerogels after 3 months were lower than after 1 month,  
344 regardless the aerogel composition. The incorporation of higher amounts of zein reduced the  
345 storage impact, obtaining slightly lighter structures for Z4 after 3 months of storage. Overall, all  
346 manufactured aerogels experienced a densification and a mild reduction in the overall porosity  
347 after the storage period (Table 3).

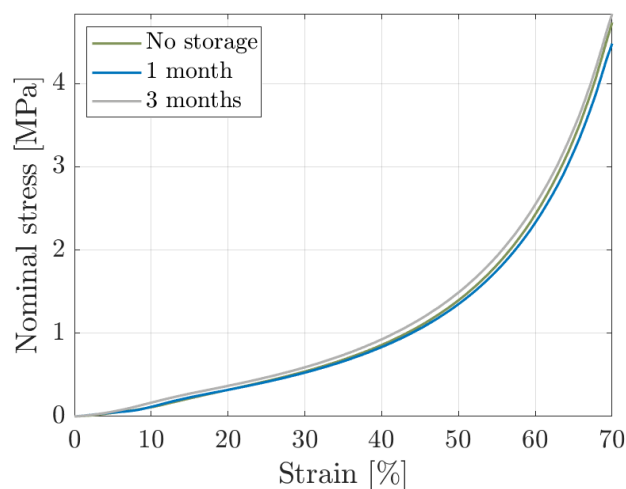


348  
349 **Figure 7.** Overall shrinkage values of starch aerogels after the storage periods of (a) 1 month and  
350 (b) 3 months. Equal letters denote statistically homogeneous groups.

351 The densification of the aerogels after the storage had a parallel impact on the textural  
352 properties, with consistent decreases in the specific surface areas ( $A_{BET}$  in Table 3). Aerogel  
353 formulations containing zein presented a reduction in the  $V_{p,BJH}$  values. This is attributed to the

354 swelling ability of amylopectin in humid environment that would cause the pore collapse, mainly  
355 affecting the smaller pore population <sup>48</sup>. In general, the impact of storage on the aerogel  
356 formulations depended on the remaining porogen traces. In Z1 and Z2, the major part of the zein  
357 was leached during the aerogel processing, leading to a more open structure and thus favoring  
358 the water intake, as suggested by both the remarkable decrease in  $V_{p, BJH}$  and the increase in the  
359  $V_{p, macro}$  values. The presence of hydrophobic zein residues along the aerogel monoliths may  
360 hinder the starch interaction with the moisture <sup>49</sup>. Accordingly, Z3 and Z4 aerogels had less  
361 drastic variations in the textural properties. For instance,  $V_{p, macro}$  of Z3 aerogels after 3 months of  
362 storage was nearly identical to its non-stored counterpart.

363 After the storage period (1 and 3 months), aerogels were tested again under quasistatic  
364 compression. Despite the abovementioned morphological changes mainly in the smaller pore  
365 population (micropores), their mechanical performance was virtually unaffected after the storage  
366 period under 25 °C and 65 % relative humidity (Figure 8). In previous theoretical studies on  
367 modeling of biopolymer aerogels <sup>40,50</sup>, it was proposed that pores (cellular fiber-network) within  
368 the microporous region and lower mesoporous region do not play a significant role in the overall  
369 mechanical performance of the aerogels. This could explain the absence of an effect on the  
370 stress-strain response due to a reduction in the amount of micropores and lower mesopores. The  
371 result illustrated in Figure 8 opens up questions that need further investigations by theoretical  
372 and experimental approaches. The stored aerogels were further subjected to cyclic loading and  
373 showed similar elastoplastic behavior as that of the non-stored aerogels.



374

375 **Figure 8.** Uniaxial quasistatic compression curves of starch aerogel (Z3) specimens subject to  
 376 different storage duration (0, 1 and 3 months) at 25 °C and 65 % relative humidity. Curves for  
 377 the rest of aerogels can be found in the supplementary information (Figure S5).

378

### 379 CONCLUSIONS

380 Starch aerogels displaying a new macropore population (1-2  $\mu\text{m}$ ) were successfully  
 381 manufactured by the incorporation of zein as a porogen. Highly porous aerogels (85-92 %) were  
 382 obtained with well-integrated macropores in the mesoporous starch aerogel backbone,  
 383 encouraging its use as scaffolds for tissue engineering applications. Zein incorporation induced  
 384 remarkable changes in the mechanical performance of the end aerogel products with an enhanced  
 385 stiffness. The storage period mimicking the ICH-climatic conditions of Europe, USA and Japan  
 386 induced morphological modifications in the aerogels whilst the mechanical behavior was  
 387 virtually unaffected. The presence of zein residues along the aerogel scaffolds had a preventive  
 388 effect on the morphological changes during the storage period. Overall, zein appears as an  
 389 advantageous biocompatible porogen for the processing of dual-porous starch aerogels from the

390 technological (integration in classical aerogel processing pathway without extra-leaching steps)  
391 and materials performance (enhanced stiffness and stability) points of view.

392

## 393 ASSOCIATED CONTENT

394 **Supporting Information.** Pore size distributions from BJH-desorption, physical appearance of  
395 aerogel, HPLC-QTOF-MS/MS fragmentation spectra of target peptides, compressive behavior of  
396 starch aerogel composites under cyclic loading and different storage time periods.

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### 401 **Author Contributions**

402 The manuscript was written through contributions of all authors. All authors have given approval  
403 to the final version of the manuscript.

### 404 **Notes**

405 The authors declare no competing financial interest.

406

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418

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