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1 Low-energy electron beam sterilization of solid alginate and chitosan, and their
2 polyelectrolyte complexes
3

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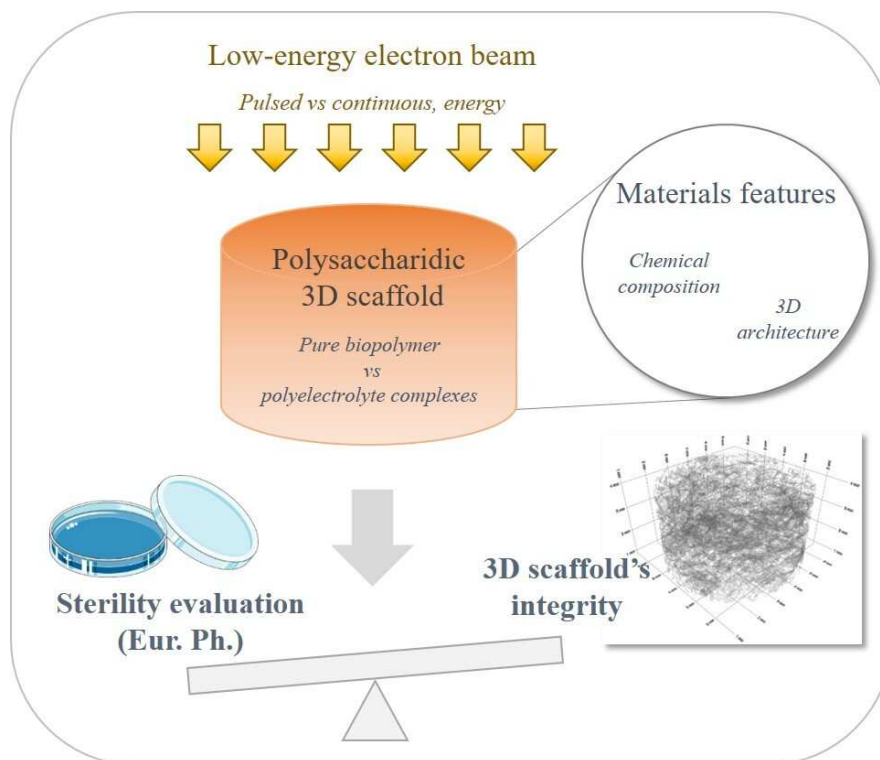
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14

15 **Graphical abstract**



16

17 **Highlights**

- 18 • Low-energy electron beams are valuable sterilization techniques for porous polysaccharidic
19 networks
20 • Continuous beam irradiation lead to less depolymerization of alginate or chitosan alone
21 • PEC formation has a protective effect against degradation under irradiation
22 • Optimal settings of pulsed irradiation were found to sterilize PEC scaffolds
23 • Low-dose (<25 kGy) may be sufficient to sterilize 3D architected polysaccharidic materials

24 **Key-words:** *low-energy electron beams, sterilization, polysaccharides, alginate, chitosan,*
25 *polyelectrolyte complexes, scaffolds*

26 **Abstract**

27 Polysaccharidic scaffolds hold great hope in regenerative medicine, however their sterilization still
28 remains challenging since conventional methods are deleterious. Recently, electron beams (EB) have
29 raised interest as emerging sterilization techniques. In this context, the aim of this work was to study
30 the impact of EB irradiations on polysaccharidic macroporous scaffolds. The effects of continuous and
31 pulsed low energy EB were examined on polysaccharidic or on polyelectrolyte complexes (PEC)
32 scaffolds by SEC-MALLS, FTIR and EPR. Then the scaffolds' physicochemical properties: swelling,
33 architecture and compressive modulus were investigated. Finally, sterility and in vitro
34 biocompatibility of irradiated scaffolds were evaluated to validate the effectiveness of our approach.
35 Continuous beam irradiations appear less deleterious on alginate and chitosan chains, but the use of a
36 pulsed beam limits the time of irradiation and better preserve the architecture of PEC scaffolds. This
37 work paves the way for low energy EB tailor-made sterilization of sensitive porous scaffolds.

38 **1. Introduction**

39

40 During the past decades, polysaccharides-based scaffolds have been widely investigated in tissue
41 engineering. The structural similarity of their network with the human extracellular matrix gives them
42 the advantage of being highly biocompatible (Dai et al., 2016), with a good biodegradability (Shelke,
43 James, Laurencin, & Kumbar, 2014). In this domain, alginate and chitosan are particularly of interest
44 (Catoira, Fusaro, Di Francesco, Ramella, & Boccafocchi, 2019; Jose, Shalumon, & Chen, 2019). Their
45 functional groups, carboxyl (-COOH) and amine (-NH₂) respectively, allow ionic gelation,
46 functionalization (to enhance solubility or promote cell adhesion) and their combination as
47 polyelectrolyte complexes of opposite charges (PECs) (Croisier & Jérôme, 2013; Lee & Mooney,
48 2012; Sæther, Holme, Maurstad, Smidsrød, & Stokke, 2008; J. Sun & Tan, 2013; Xu et al., 2017).
49 This last possibility improves their network mechanical properties (Li, Ramay, Hauch, Xiao, & Zhang,
50 2005) while maintaining their biocompatibility (Meka et al., 2017; Wang, Khor, Wee, & Lim, 2002).

51

52 Alginate-chitosan PECs formation may differ upon biopolymers characteristics (molecular weight,
53 density of charges, degree of ionization, distribution of ionic groups) and conditions in which the
54 polymers are brought together (concentration of polyelectrolytes, mixing ratio, order of reacting
55 polyelectrolytes, pH of reaction medium, temperature, optional drying process, etc.), leading to
56 multilayers, micro- or nano-particles or bulks (hydrogels, sponges, cryogels, aerogels..) (Luo & Wang,
57 2014) with potential applications in drug delivery and bone, cartilage, heart, or skin repair (Deka,
58 Deka, Moni, Kumar, & Kumar, 2016; Florczyk et al., 2013; Kuznetsova, Andryukov, Besednova,
59 Zaporozhets, & Kalinin, 2020; Li et al., 2005; Reed & Wu, 2015).

60 Our team developed some alginate-chitosan PEC sponges formulations, and demonstrated their
61 interest as macroporous 3D-scaffolds for soft cell therapy purposes. These scaffolds exhibit controlled
62 porosity and mechanical properties allowing in-depth cell seeding, optimization of mesenchymal stem
63 cells (MSCs) survival and beneficial modification of their secretion profiles (Bushkalova et al., 2019;
64 Ceccaldi et al., 2014). It is widely acknowledged that scaffold 3D architecture and seeded cells' fate
65 can be correlated (Gómez, Vlad, López, & Fernández, 2016; Pennesi, Scaglione, Giannoni, & Quarto,
66 2011; Santos, Hernández, Pedraz, & Orive, 2012), thus finding an efficient sterilization technique
67 preserving 3D scaffolds physicochemical features is a current challenge, all the more difficult to
68 achieve when the material is of low density.

69

70 In this domain there is no gold standard, each method having its own advantages and drawbacks. Due
71 to their organic nature, polysaccharides-based scaffolds may be exposed during sterilization to
72 chemical and physical alterations as they share structural features with the vital components of
73 pathogens (Munarin, Bozzini, Visai, Tanzi, & Petrini, 2013). These macromolecules tend to degrade
74 when exposed to conventional sterilizing methods such as autoclaving or dry heating sterilization
75 (França et al., 2013; Hu et al., 2014; Leo, Mcloughlin, & Malone, 1990; Rao & Sharma, 1995; San
76 Juan et al., 2012; Vandenbossche, 1993). Other chemical treatments can be considered such as
77 ethylene oxide or hydrogen peroxide exposition. However, besides their hazardous nature for users,
78 they result in the formation of toxic by-products that can remain in the scaffold (Mendes, Brandão, &
79 Da Silva, 2004; Rosiak, Ulanski, Kucharska, Dutkiewicz, & Judkiewicz, 1992). Ionizing radiations
80 appear more environment-friendly; among them, gamma rays and beta radiations, *i.e.* electron beams,
81 are the most frequently used for sterilization purposes of medical devices.

82
83 Gamma rays are photons emitted from the deexcitation of an atom (commonly ^{60}Co) while beta
84 radiations involve particles, electrons, whose ability of penetration in matter is lower. Gamma rays are
85 high-energy radiation, respectively 1.17 and 1.33 MeV, while electron beams can range from 200 keV
86 to 10 MeV depending on the type of device. The inactivation of microorganisms following ionizing
87 radiation has been thoroughly studied (Lamarche & Demol, 2018; Tallentire, Miller, & Helt-hansen,
88 2010; Zhu et al., 2008). Up to now both these radiations were mainly used for polysaccharides
89 treatment to obtain oligosaccharides of low molecular weights with enhanced properties such as
90 antioxidant, antibacterial or plant growth promoter (Feng, Du, Li, Hu, & Kennedy, 2008; Hien et al.,
91 2000; Kume, Nagasawa, & Yoshii, 2002; Matsushashi & Kume, 1997; Sen & Atik, 2012; Yoksan,
92 Akashi, Miyata, & Chirachanchai, 2004). Many of these studies gave a great understanding of the
93 mechanisms of ionizing radiations effects on polysaccharides. The degradative chemistry of irradiation
94 on organic molecules is well described and consists in free radical initiation, propagation and
95 termination events (Ciesla, 2017; Del Mastro, 2016; Gueven, 2004; Lim, Khor, & Koo, 1998; Yoksan
96 et al., 2004). Most frequently ionizing radiations lead to biopolymer degradation through
97 depolymerization mechanisms (Aliste, Vieira, & Del Mastro, 2000; Leo et al., 1990; Nagasawa,
98 Mitomo, Yoshii, & Kume, 2000; Sen, Rendevski, Akkas-Kavaklı, & Sepehrianazar, 2010;
99 Wasikiewicz, Yoshii, Nagasawa, Wach, & Mitomo, 2005; Wenwei, Xiaoguang, Li, Yuefang, &
100 Jiazhen, 1993). Besides materials characteristics (chemical nature, solid state or in solution, thickness,
101 density), ionizing radiation consequences depend on extrinsic parameters such as environmental
102 conditions (temperature, oxygen or anoxic conditions, moisture content) and radiation parameters
103 (energy, dose and dose-rate) (Chmielewski et al., 2007; Ciesla, 2017; Del Mastro, 2016; Lim et al.,
104 1998; Yoksan et al., 2004). Thanks to its low penetration and high dose-rate, electron beam was first
105 developed for material surface treatment but could easily be diverted for effective sterilization of thin
106 and low-density macroporous materials without compromising their integrity. However much less is
107 known about the sterilization of polysaccharides with electron beam: to our knowledge a scarce
108 number of studies deal with alginate and chitosan beta sterilization (Gryczka et al., 2009; Silva, Elvira,
109 Mano, Roma, & Reis, 2004) and none deals with their PECs. Thus, low energy electron beam could be
110 a valuable sterilization technique for macroporous polysaccharidic scaffolds; the validation of this
111 hypothesis is the purpose of this study.

112
113 In this work, an attempt has been made to compare continuous and pulsed low energy electron beam
114 effects on the chemical properties of alginate, chitosan and their PECs, and on the physicochemical
115 properties of alginate-chitosan PEC scaffolds, as well as their overall microbiocidal effectiveness. To
116 that end, alginate or chitosan references scaffolds and PEC scaffolds have been irradiated with 300
117 keV continuous electron beam (CB) or with 280 keV or 430 keV pulsed electron beam (PB). First,

118 size exclusion chromatography (SEC), Attenuated Total Reflectance Fourier Transform InfraRed
119 spectroscopy (ATR-FTIR) and Electron Paramagnetic Resonance (EPR) were achieved with the
120 intention to unveil underlying chemical degradation mechanisms. Then, the effects of radiation
121 sterilization on alginate-chitosan PEC scaffold' performances (swelling behavior, architecture and
122 compressive mechanical properties) were evaluated. Finally, sterility assays according to European
123 Pharmacopeia and *in vitro* biocompatibility tests were performed to determine if a sterilization at low
124 dose is possible without altering the biomaterial's biocompatibility.

125

126 2. *Materials and methods*

127

128 2.1. *Materials*

129

130 Sodium alginate medium viscosity (reference A-2033, batch 051M0054V), chitosan medium
131 molecular weight (reference 448877, batch STBF8484V), HEPES sodium salt, acetic acid, EDTA, L-
132 glutamine, fetal bovine serum (FBS), as well as antibiotics penicillin-streptomycin, were purchased
133 from Sigma-Aldrich, France. Complete medium for cell culture was prepared by supplementing the
134 Dulbecco's Modified Eagle's medium glutamax (reference 31966-021; ThermoFisher, France) and
135 macrophage-colony stimulating factor (M-CSF) was purchased from Peprotech, France. Calcium
136 chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), sodium chloride (NaCl) and sodium hydroxide (NaOH) were
137 supplied from VWR. Sterile water was purchased from Cooper (France).

138

139 2.2. *Polysaccharides characterization*

140

141 Polysaccharides molecular weights were determined with size exclusion chromatography. The G/M
142 units ratio of alginate was estimated by ^1H NMR (Nuclear Magnetic Resonance) spectroscopy (Vilén,
143 Klinger, & Sandström, 2011) to $\text{M/G} = 2.1$. The deacetylation degree (DD) of chitosan was estimated
144 to be 75% by solid ^{13}C NMR (Heux, Brugnerotto, Desbrières, Versali, & Rinaudo, 2000).

145

146 2.3. *Preparation of Alginate/Chitosan macroporous 3D scaffolds*

147

148 Three-dimensional alginate/chitosan PEC scaffolds containing alginate/chitosan weight ratio of 40/60
149 were prepared as reported previously (Bushkalova et al., 2019; Ceccaldi et al., 2014). Briefly, PEC
150 scaffold were obtained by a combination of freeze-drying and gelation with CaCl_2 0.1M. Final
151 polymer concentrations in 40/60 PEC were respectively 1,5% w/w for alginate and 2,25% w/w for
152 chitosan. Scaffolds made of alginate (ratio 100/0) or chitosan (ratio 0/100) 1.5% w/w were used as
153 references. The final dimensions of 40/60 PEC scaffolds, used in all experiments, were 10 mm
154 diameter \times 5 mm thickness.

155

156 2.4. *Low energy electron-beam treatments and dosimetry*

157

158 Electron beam treatment was performed by two distinct low energy electron beam facilities in this
159 study. The "continuous E-beam" (CB) equipment from COMET group (Flamatt, Switzerland) was
160 used for continuous electron beam whereas the "Pulsed E-beam" (PB) equipment from ITHPP
161 (Thégra, France) was used for pulsed radiation. Table 1 summarizes each generator features:

	CB	PB
Energy	300 keV	280 keV or 430 keV
Max beam current	from 1 to 15 mA	7 kA
Max dose-rate	10 ⁵ kGy/s	10 ¹² kGy/s
Pulse repetition frequency	-	5-100 Hz
Distance from extraction window	2 cm	2 cm

Table 1 - Continuous and pulsed generator features

162
 163 Concerning the PB generator, in this study two different energies were studied 280 keV and 430 keV,
 164 with a pulse duration of 10 and 12 ns, respectively. Scaffolds were sealed into Stericlin® pouches as a
 165 sterile barrier packaging system, and were irradiated in order to reach 2.5, 5 and 25 kGy minimum
 166 absorbed doses at the bottom of the scaffold. Dose measurements were achieved by placing
 167 radiochromic dosimeters (Dosimetryfoil 20 µm (Crosslinking®)) below samples and inside the
 168 pouches. Directly after irradiation radiochromic films were incubated at 37°C for 15 minutes and
 169 passed through the “dose-reader DR 020” (Electron crosslinking AB, Sweden) to know the absorbed
 170 dose.

171
 172 *2.5. Chemical study*

173
 174 *2.5.1. Size exclusion chromatography (SEC)*

175
 176 Experimental conditions for alginate and chitosan scaffolds dissolution and processing for SEC are
 177 resumed in Table 2. After scaffold dissolution, solutions were filtered through 0.45 µm nylon filter
 178 membrane. The detection was operated by a differential refractometer (Shodex RI-101) and a 18
 179 angles static light scattering detector (MALLS Wyatt Dawn Heleos, laser = 658 nm; Wyatt
 180 Technology, USA) and a 254 nm UV detector (Varian, Australia). Data were analyzed with ASTRA
 181 VI software (Wyatt Technology, USA).

	Alginate	Chitosan
Dissolution buffer	50 mM EDTA	1M CH ₃ COOH (24h) + 0.2 M CH ₃ COOH / 0.15 M NH ₄ CH ₃ CO ₂
Mobile phase	0.1 M NaNO ₃ / 0.1g/L NaN ₃	0.1M CH ₃ COONa / 0.1 M CH ₃ COOH
Flow rate	1 mL/min	0,8 mL/min
Injection volume	50 µL	50 µL
Columns	Shodex columns : 805, 804 and 802.5 (Showa Denko, Japan)	TSK gl PWXL-CP cationic columns : G5000 and G3000 (Tosoh, Japan)
dn/dc value	0.150 mL/g	0.192 mL/g

Table 2 - Size exclusion chromatography processing parameters for alginate and chitosan elution

182
 183
 184
 185

186

187

2.5.2. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

188

189 ATR-FTIR spectra of 3D scaffolds in solid state were recorded using a Nicolet iS50 Spectrometer
190 (Thermo Fisher Scientific, Waltham, MA) in monoreflexion with an ATR Crystal diamond, with a 2
191 cm^{-1} resolution over 64 scans in the range from 4000 to 400 cm^{-1} . The spectra baselines were
192 normalized using Origin software (OriginLab Corporation, Northampton, MA, USA). For each
193 scaffold spectra were analyzed for peak intensity changes with respect to reference band within the
194 same spectra.

195

196

2.5.3. Electron paramagnetic resonance (EPR)

197

198 EPR experiments were acquired in X-band with a high-sensitivity cavity at room temperature, using a
199 Bruker Elexsys with the following settings: power of 1 mW and a modulation of 1G. An angular
200 dependence was observed: the spectra provided are the sum of the 8 spectra obtained by rotation of
201 45° with respect to the field.

202

203

2.6. Physicochemical characterization

204

205

2.6.1. Swelling

206

207 The swelling behaviors of irradiated scaffolds and their non-treated (NT) counterparts was studied at
208 room temperature by measuring scaffolds' weight in a dry (W_{dry}) and in a wet (W_{wet}) state using an
209 electronic balance (precision $d=0.0001$ g) as previously described (Bushkalova et al., 2019). The
210 swelling ratio of each scaffold was calculated using the following formula:
211 swelling ratio (%) = $[(W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}}] \times 100$.

212

213

2.6.2. Scanning electron microscopy

214

215 Irradiated and NT scaffolds were coated under vacuum with 10 nm platinum alloy. Images were
216 acquired with an electron microscope QuantaTM 250 FEG (FEI, USA) at an accelerating voltage of 5
217 kV. Both the irradiated surface and cross-section of each sample were examined at magnification 20
218 and 75 times.

219

220

2.6.3. Computed X-ray Micro-tomography (Micro-CT)

221

222 The micro-CT study of samples was carried out on Phoenix Nanotom180 (GE Sensing, Germany)
223 using the following parameters: 60 kV voltage, 240 μA current, no filter material, 0.25° rotation step,
224 5 frames as frame averaging, 1440 tomographic projections over a 360° scan angle, 750 ms exposure
225 time. A binning 1×1 was applied for the slices reconstruction and the resulting voxel size was 11.5
226 μm^3 . Three-dimensional virtual models of scaffolds were obtained using VG StudioMAX 2.1. A
227 region of interest (ROI) was drawn within the reconstructed volume and a threshold was defined to
228 identify the polymeric phase. Then, a morphometric analysis of the ROI was performed to obtain the
229 total porosity and void interconnectivity. Scaffold's pore walls thickness were analyzed on the basis of
230 2D X-ray tomographic slices using ImageJ (NIH, USA). ImageJ tool called "local thickness" was
231 applied on cross-sections defined ROI, and subsequent color gradient allowed us to visualize
232 polymeric thickness differences. Afterwards an ImageJ macro was developed to quantify relative

233 proportions of thick polymeric walls across scaffolds' depth through pixel quantification. For each
234 condition, at least 30 slices were assessed, each slice corresponding to a 100 μm increment.

235

236

237 *2.6.4. Mechanical properties under compression*

238

239 Mechanical behavior of irradiated and NT scaffolds was evaluated by three successive uniaxial
240 compression tests (TA-XT2 Texture Analyzer, Stable Microsystems, UK) in a hydrated state,
241 according to a protocol already described (Ceccaldi et al., 2014). Prior to mechanical testing, the
242 scaffolds were immersed in Milli-Q water for 24 hours at room temperature. The apparatus consisted
243 of a mobile probe (1256.6 mm^2) moving vertically up and down at a constant and predefined velocity
244 ($0.5 \text{ mm}\cdot\text{s}^{-1}$) with a strain target of 50%. The stress area (mm^2) of each scaffold and the force $F_{\text{strain}\%}$
245 (N) were collected. The secant moduli $E_{50\%}$ (kPa) were calculated from at least five independent
246 observations as the slope of a line connecting the point of zero strain to a point at a 50% deformation.

247

248 *2.7. Biological evaluation*

249

250 *2.7.1. Bioburden determination and sterility assay*

251

252 Bioburden determination and sterility evaluation after irradiation were performed according to
253 European standards, respectively ISO 11737-1 and ISO 11737-2. Initial bioburden of 3D scaffolds and
254 bulk polymers were determined. Prior to sterility assays, scaffolds ability to allow microorganism
255 growth was checked. Sterility assay of 40/60 PEC scaffolds was performed by incubating 5 pooled-
256 samples in tryptic-soya broth (for aerobic bacteria) and 5 pooled-samples in thioglycolate broth with
257 rezasurin (for anaerobic bacteria) as recommended in European Pharmacopeia (Ph. Eur. 2.6.1, 2008).
258 Broth were respectively incubated at $22.5 \pm 2.5 \text{ }^\circ\text{C}$ and $32.5 \pm 2.5 \text{ }^\circ\text{C}$, and were checked regularly for
259 up to 14 days. If not stated otherwise all experiments were performed in triplicates (3 replicates of 5
260 pooled-samples for each broth).

261

262 *2.7.2. In vitro biocompatibility after irradiation*

263

264 For in vitro biocompatibility evaluation, primary bone-marrow derived murine macrophages were
265 used. Briefly, cells were isolated from femurs and tibiae of C57BL/6 mice, red blood cells were then
266 lysed with ACK (Ammonium-Chloride-Potassium) lysis buffer. BMDM were selected by adhesion to
267 petri dishes after 4 days of differentiation in DMEM glutamax medium supplemented with 10% FBS,
268 1% penicillin-streptomycin, 1% L-glutamine and 30 ng/mL M-CSF. Cell seeding on scaffolds was
269 performed according to previously described protocol (Bushkalova et al., 2019). After 24 hours
270 Live/Dead assays were performed on seeded scaffolds using the Viability/Cytotoxicity Assay kit
271 (FluoProbes®, Interchim, France). Staining solution were concentrated with 2 μM ethidium
272 homodimer-3 (necrotic marker measuring nucleus membrane integrity) and 1 μM calcein AM
273 (viability marker measuring the intracellular esterase activity). Confocal microscopy was achieved
274 (Zeiss LSM780) by exciting samples with a 488 nm Argon laser and with a 543 nm helium-neon
275 laser, and using 10X objective. Then three-dimensional reconstructions were generated using IMARIS
276 software (Bitplane) from microscopic images where the green and red channels were merged.

277

278 *2.8. Statistical analysis*

279

280 Data in the figures are given as mean \pm standard error of the mean (SEM). Statistical significances
 281 were determined using Graph Pad Prism software by unpaired t-tests if only two groups were in the
 282 study or by two-way analysis of variance (ANOVA) with Tukey post-tests for multiple comparisons
 283 with more than two groups (GraphPad Prism 6, version 6.01). Differences between the groups were
 284 considered as statistically significant at the level of $p < 0.05$ and marked with asterisks (*; **; *** = p
 285 < 0.05 ; 0.01 ; 0.001).
 286

287 3. Results and discussion

288

289

3.1. Dose distribution across 3D scaffolds

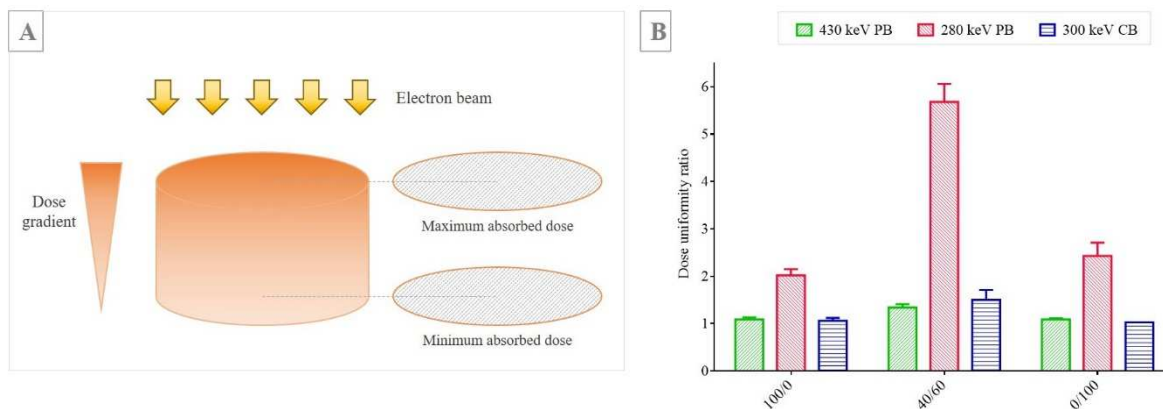


Figure 1 - Dose distribution through PEC scaffolds. A) Diagram showing maximum and minimum dose localization. B) Dose uniformity ratios of 40/60 PEC scaffolds and references scaffolds (100/0 and 0/100) according to the electron beam treatment applied

290

291 In order to compare the effect of pulsed versus continuous electron beam, we decided in a first
 292 approach to work at similar energy level (i.e. to compare 280 keV PB vs 300 keV CB) with equal
 293 minimum absorbed dose, assuming that the minimum absorbed dose was reached at the bottom of
 294 PEC scaffolds (Figure 1). Dose setting was established as 2.5, 5 and 25 kGy, the latter being the
 295 sterilizing dose required in European standards. Although 25 kGy effect on PEC is to date still
 296 unknown, it is likely to be detrimental so we decided to test lower doses such as 2.5 and 5 kGy and to
 297 evaluate their sterilizing properties. Dose uniformity ratios (DUR) were calculated as the ratio of
 298 maximum and minimum absorbed doses. Whatever the irradiation treatment, PB provides a less
 299 homogenous dose deposition. This heterogeneity is more pronounced in the case PEC scaffolds
 300 (Figure 1), because of PEC scaffolds' higher density due to stronger interchain interactions. As a
 301 consequence, we decided to include in our study a third condition corresponding to a higher PB
 302 electron energy of 430 keV, for a similar dose uniformity with 300 keV CB. Indeed, energy is known
 303 to be a key factor concerning the DUR of an irradiated product (Helt-Hansen et al., 2010; Lambert &
 304 Martin, 2013). DUR differences between PB and CB at a same level of energy is a consequence of
 305 voltage signal's shape, which is a bell shape in the case of PB generator. Consequently, a non-
 306 negligible part of electrons have a lower energy than 280 or 430 keV (Lamarche, 2019). For the 430
 307 keV generator, the mean energy of electron beam is 302 keV, a value almost similar to that of CB
 308 generator.
 309

310 This work is a comparative study of pulsed and continuous electron beam at similar energy levels (280
 311 keV PB and 300 keV CB) or at similar DUR ratios (430 keV PB and 300 keV CB). We aimed at
 312 evaluating electron beam irradiation effect on both polysaccharides chemical properties and scaffolds
 313 3D architecture, which are crucial for biomaterials biocompatibility. Due to technical limitations, PEC

314 chemical changes were not pursued as thoroughly as for pure biopolymers, but were assessed by
315 indirect methods.

316

317

318

319 *3.2. Study of chemical changes after irradiation*

320

321 On the contrary of the well-studied degradation effects of gamma irradiation on polysaccharides, and
322 especially on alginate and chitosan, low-energy beta irradiation chemical effects are yet to be
323 thoroughly evaluated. Alginate and chitosan were irradiated in the solid state, which is known to be
324 less sensitive to irradiation effects than the liquid state (Hien et al., 2000; Kume et al., 2002;
325 Nagasawa et al., 2000; San Juan et al., 2012; Wasikiewicz et al., 2005). Biopolymer's sensitivity to
326 irradiation depends on some intrinsic properties of starting material such as the M/G ratio of alginate
327 and degree of deacetylation (DDA) for chitosan, although those values are not expected to change in
328 themselves upon irradiation. Sen and coworkers have shown that alginate degradation increased with a
329 higher mannuronate content (Sen et al., 2010). Others have shown that even if irradiation does not
330 induce any changes with regards to DDA (Lim et al., 1998; Zainol, Akil, & Mastor, 2009), it is mostly
331 effective on acetylated parts of chitosan, implying a higher degradation susceptibility with higher
332 DDA (Taskin, Canisag, & Sen, 2014; Wenwei et al., 1993).

333 The weight average molecular weight M_w and the polydispersity index \mathcal{D} of the polymers constituting
334 the scaffolds were evaluated by SEC-MALLS (Figure 2). This technique was not applicable on PEC
335 scaffolds as PEC can hardly be dissociated.

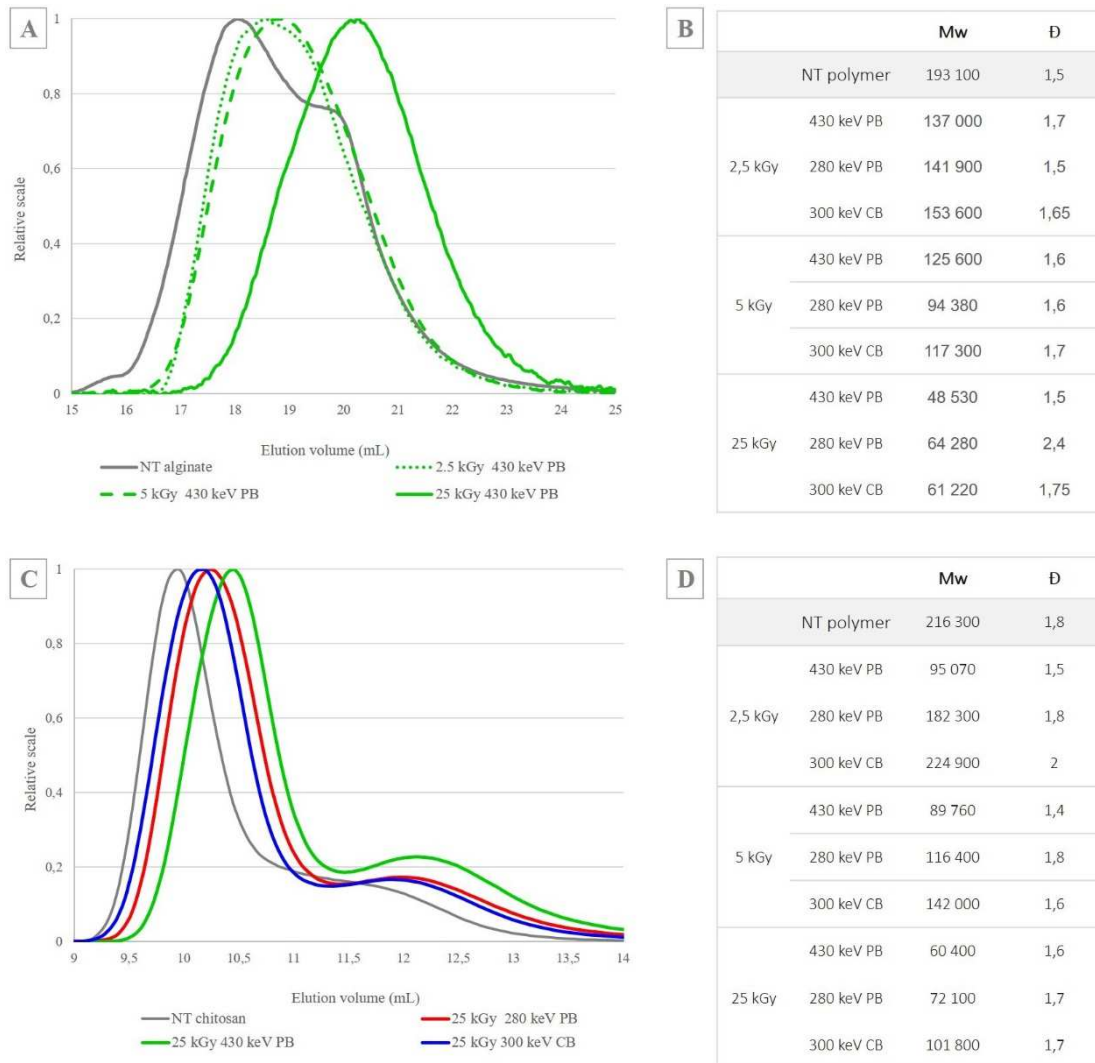


Figure 2 - Irradiation impact on alginate and chitosan molecular weight. Graph A shows elution curves obtained with light scattering detector of 430 keV PB treated alginate references scaffolds (100/0) after different doses. Graph C shows elution curves of 25 kGy irradiated chitosan references scaffold (0/100) after different irradiation treatments. Tables B and D indicate the corresponding molecular weight values Mw and polydispersity D index D.

336

337 In the case of alginate, the Mw distribution evolves from slightly bimodal to monomodal after
 338 irradiation. In the case of chitosan it remains bimodal. Such bimodal shape is typical of chitosan
 339 samples (Thevarajah, Bulanadi, Wagner, Gaborieau, & Castignolles, 2016; Yanagisawa, Kato,
 340 Yoshida, & Isogai, 2006), and is related to high mass aggregates which were not taken into account for
 341 Mw determination. As expected, Mw decreases with increasing irradiation dose (Figure 2, Graph A).
 342 Whatever the irradiation technique, the effect of 2.5 kGy is limited for both polymers, whereas 25
 343 kGy, which is the suggested sterilizing dose in norms (NF EN ISO 11137-2, 2006), appears clearly
 344 deleterious on Mw. Such a decrease testifies for main chain scission. By direct energy absorption, the
 345 main carbon chain depolymerizes as a consequence of glycosidic bonds cleavages. Both polymers
 346 appeared depolymerized upon irradiation, however chitosan chains appeared more sensitive than
 347 alginate chains to an energy increase from 280 keV to 430 keV (when irradiated at 2.5kGy chitosan
 348 depolymerizes 4-fold more at 430 keV than 280 keV). Some differences are observable according to
 349 irradiation treatment (Figure 2, Graph C), especially at 25 kGy: 300 keV CB seems to induce less
 350 polymer chain degradation than PB. This could be due to lower beam current associated with the CB

351 generator, which implies a lower electron flow and thus leads to less damaging effect (Table 1). An
352 irradiation dose of 2.5 kGy do not prevent alginate nor chitosan polymers from scission events but
353 they are limited, particularly in the case of CB irradiation.

354
355 The study of irradiation effects on alginate and chitosan scaffolds was supplemented by FTIR analyses
356 to assess any functionality changes of alginate, chitosan or their PEC formation, caused by irradiation.
357 ATR-FTIR spectroscopy was performed on 25 kGy irradiated scaffolds in order to identify at a surface
358 level potential changes in functional groups or new bonds formation (Figure 3). For each biopolymer,
359 the most representative signals were followed. Concerning alginate spectra (Figure 3), peaks at 3290
360 cm^{-1} , 1590 cm^{-1} , 1410 cm^{-1} and 1025 cm^{-1} can be respectively ascribed to hydroxyl O-H stretching,
361 asymmetric and symmetric carboxylate salts COO^- stretching and finally glycosidic C-O-C bonds
362 (Daemi & Barikani, 2012; Sartori, Finch, & Ralph, 1997; Yu, Cauchois, Schmitt, Louvet, & Six,
363 2017). For chitosan spectra (Figure 3, Graph B), the most representative peaks were at 3290 cm^{-1} ,
364 1578 cm^{-1} and 1148 cm^{-1} which can be respectively attributed to hydroxyl, N-H bending from amine
365 and amide II and finally C-O-C groups (Ji & Shi, 2013; Lawrie et al., 2007; Pawlak & Mucha, 2003).
366 Relative peak intensity were calculated using carboxylate group and amine/amide group as references
367 band for alginate and chitosan within each spectra as they are not supposed to change under irradiation
368 (Wasikiewicz et al., 2005; Wenwei et al., 1993). Concerning chitosan spectra, an increase of hydroxyl
369 groups, associated with a decrease of C-O-C groups, is in accordance with the hypothesized glycosidic
370 bonds (C-O-C) cleavages, leading to hydroxyl group formation (Wenwei et al., 1993). In the case of
371 alginate, differences between irradiated scaffolds at 25 kGy are more tenuous to detect and no new
372 band appeared. PEC spectra (Figure 3, Graph C) are more similar to alginate ones but they display
373 band shifts from 1590 cm^{-1} to 1595 cm^{-1} and 1410 cm^{-1} to 1414 cm^{-1} . These shifts have been attributed
374 to an overlap of the amide signal of chitosan and alginate carboxylate groups, confirming polymers
375 interaction (Lawrie et al., 2007; W. Sun et al., 2018) and thus PEC presence. No change in intensity of
376 the peaks of PEC spectra was observed after irradiation treatments (Figure 3, Graph C); this suggests
377 that PECs are not affected by beta irradiation, whatever the beam type and the energy tested in our
378 study.

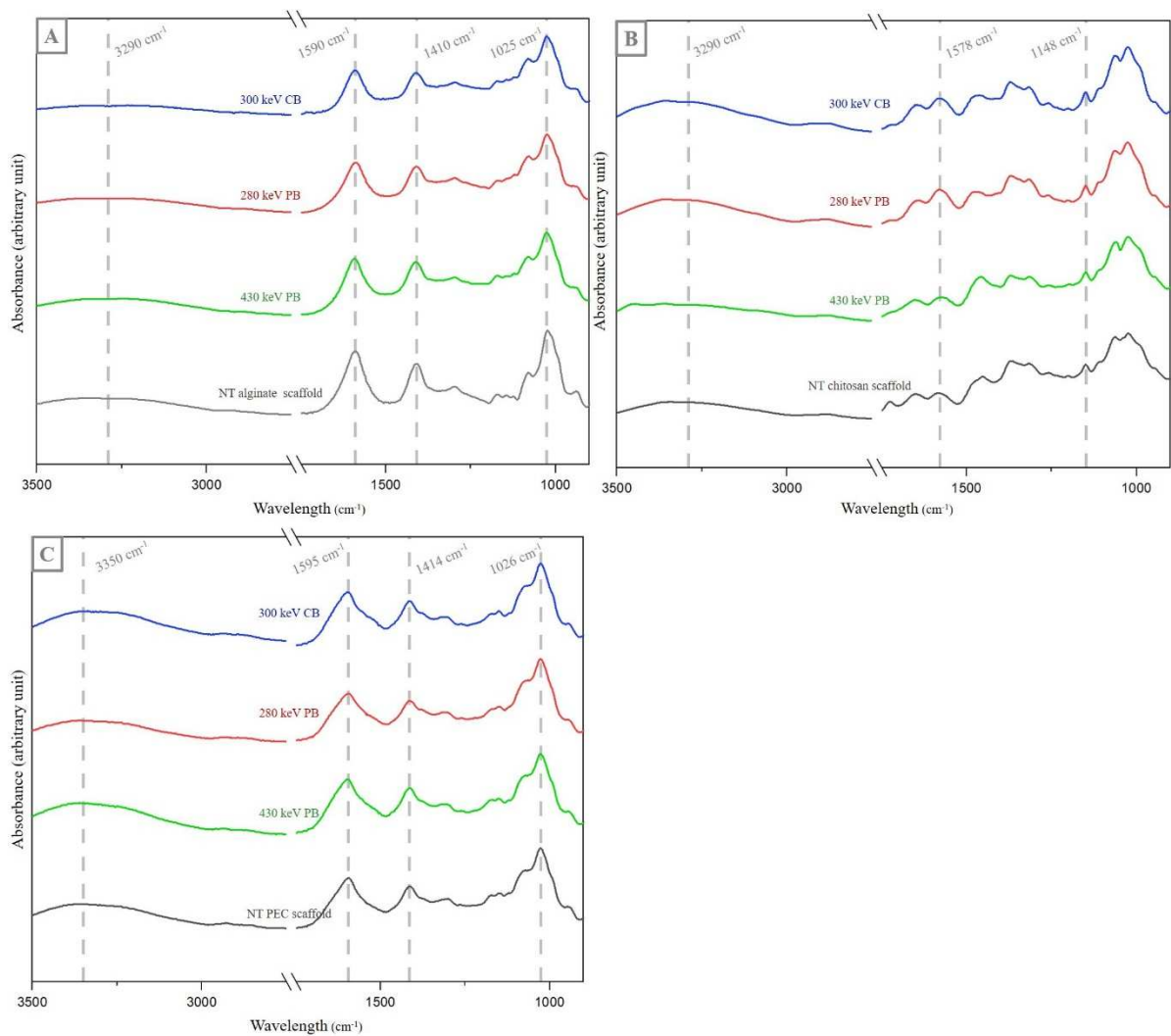


Figure 3 - FTIR analysis of non-irradiated and 25 kGy irradiated scaffolds. FTIR spectra with ATR reflection mode of alginate (Graph A), chitosan (Graph B) and PEC scaffolds (Graph C).

379
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384

385 EPR is described as a useful tool to detect free radicals formation after irradiation of biodegradable
386 polymers (Gryczka et al., 2009; Mäder, Domb, & Swartz, 1996). The presence of such radicals may
387 cause cell oxidative stress, which could hence impact further scaffold biocompatibility and interest for
388 regenerative applications. Because PB revealed a higher damaging effect on polysaccharides, we
389 evaluated the amount of organic radicals in PEC scaffold over time after 280 keV and 430 keV PB
390 irradiation by EPR. For both PB conditions, there is an obvious dissipation of such radicals (Figure 4).
391 The presence of organic radicals confirms carbon backbone scission mechanism (Ershov, 1987;
392 Rosiak et al., 1992). However, it is interesting to note that their presence remains negligible at 2.5
393 kGy, and moderate at 5 kGy whatever the PB energy tested. In both cases, the level of radicals goes
394 back to normal within a week, with a RPE signal similar to the NT reference scaffolds. At higher
395 irradiation energy (25 kGy), the presence of free radicals is significantly higher, even if after a week it
396 appears considerably decreased.

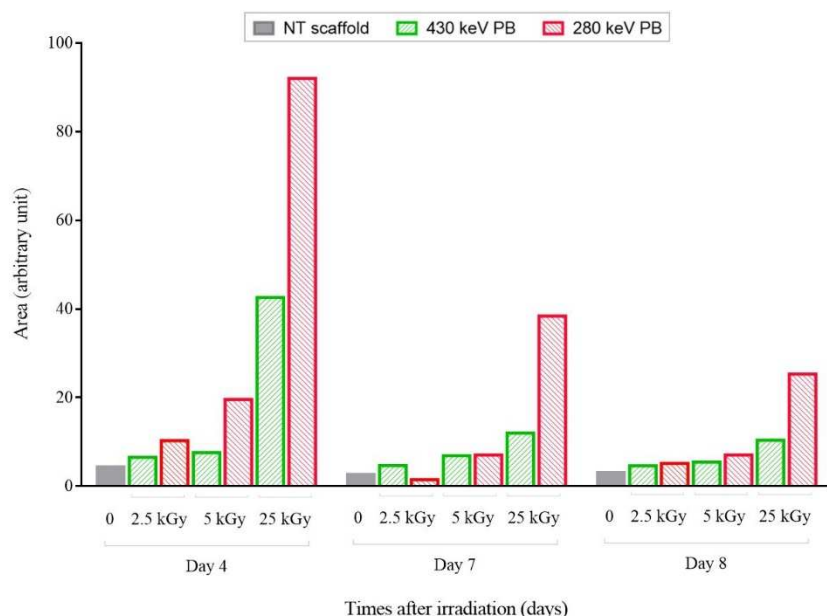


Figure 4 - Evolution of the amount of organic radicals present in PEC scaffolds at 4, 7 and 8 days after pulsed beam irradiation treatment.

397

398 FTIR and SEC results on pure polymer scaffolds show that both biopolymers depolymerize under low-
 399 energy electron beam irradiation as doses increase; this could have been limited by adapting the
 400 features of the starting polymers, i.e. with a lower M/G ratio for alginate or a lower DDA for chitosan.
 401 In this study, chitosan scaffolds seem to be more sensitive to irradiation-induced degradation than
 402 alginate ones. Whatever the type of electron beam, the lower the dose, the lower the deleterious
 403 effects. Concerning pure polymers, CB appears more adapted to scaffolds sterilization as its
 404 depolymerizing effects are lower than those observed at the tested PB doses. The low doses of 2.5 and
 405 5 kGy clearly appear less deleterious than 25 kGy. Concerning the PEC scaffolds, they appear more
 406 resistant to irradiation thanks to their strong chain-to-chain interactions and consecutive higher
 407 density. In their case, both PB and EB seem applicable, particularly at low doses.

408

409 Anyway, a simple study on the chemical effects of electron beam irradiations on biopolymers is
 410 insufficient to predict the deleterious effects of these irradiations on 3D materials intended to be
 411 seeded with cells. Indeed, in this case, the specifications of the material go well beyond its simple
 412 composition: its retention in rehydration, its 3D architecture, its porosity, its mechanical resistance are
 413 all essential characteristics influencing the fate of cells at their contact. Therefore, a complete study of
 414 the effects of irradiation on this type of material must take into account the macroscopic effects of
 415 irradiations on 3D structures. With the aim to find radiosterilization operating conditions respecting
 416 scaffold's specifications, while ensuring its sterility, the main physico-chemical characteristics of
 417 irradiated PEC scaffolds were studied and compared to reference non irradiated scaffold.

418

419 *3.3. Effect of various irradiation treatments on 3D scaffolds physicochemical properties*

420

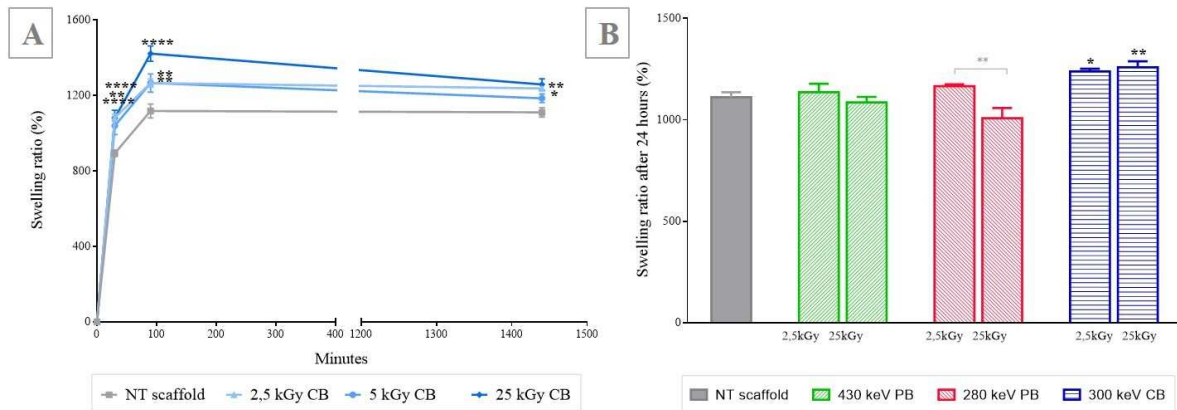


Figure 5 - Swelling behavior of irradiated and non-irradiated 40-60 PEC scaffolds. Graph A shows continuous beam swelling kinetic, and graph B shows swelling ratio after 24 hours for all irradiation conditions. Five replicates were used for each condition ($n=5$; two-way ANOVA; $*p<0.05$; $**p<0.01$; $****p<0.0001$; significant differences with NT scaffold and within the same irradiation treatment are respectively shown with black bold asterisks and grey asterisks)

421

422 First, a particular attention was paid to scaffolds swelling properties, as stability under rehydration
 423 means that chemical bonds remain sufficient in number to ensure macroscopic cohesion. The swelling
 424 curves of all the scaffolds, irradiated or not, exhibited the same shape (Figure 5). In the first 30
 425 minutes, scaffolds absorb 75% of total absorbed volume and reach a plateau within 4 hours, with for
 426 some samples a decrease in slope which can be attributed to sample sensitivity to successive handling.
 427 Water uptake was significantly higher after 300 keV CB treatment (Figure 5), suggesting a loosening
 428 of the scaffold network in this case. These differences in swelling ratio after a terminal sterilization
 429 treatment testify modification of the network architecture (Stoppel et al., 2013).

430

431 In order to gain insight on scaffolds architecture after irradiation, SEM images of 40-60 PEC scaffolds
 432 cross-sections (Figure 6) and surfaces (see supplementary data for surface SEM images) were
 433 acquired. All images displayed an interconnected macroporous structure which is mandatory for in-
 434 depth cell seeding, and therefore essential to preserve upon irradiation. At a 2.5 kGy dose no particular
 435 differences can be mentioned about scaffolds architecture. For doses higher than 2.5 kGy, pores walls
 436 seem more friable and the whole structure appears more fragile with increasing doses. Qualitatively,
 437 no differences can be pointed out between CB and PB.

438

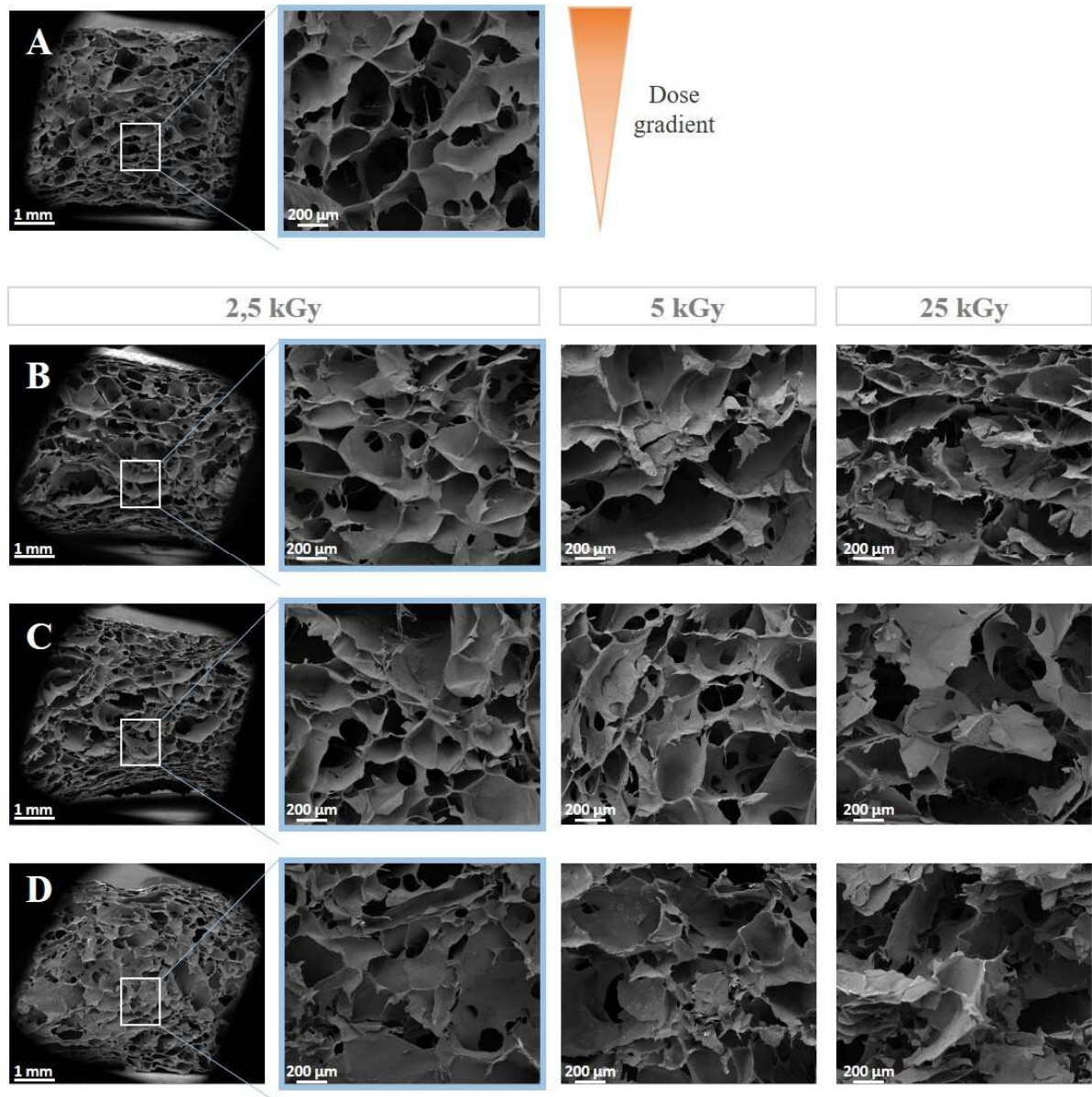


Figure 6 - Representative images of 40-60 PEC scaffold cross-section acquired by scanning electron microscopy (A) non irradiated scaffold (B) 430 keV pulsed beam (C) 280 keV pulsed beam and (D) 300 keV continuous beam (magnifications X20 and X75, corresponding scale bars are respectively 1 mm and 200 μm)

439

440 Scaffolds architecture analysis was expanded with micro-CT scans. From a qualitative point of view,
 441 scaffolds showed a similar airy foam structure before and after irradiation treatment (Figure 7, Graph
 442 A). Porosity quantification was estimated to 96% and confirmed a highly porous and entirely
 443 interconnected structure, allowing optimal cell seeding as previously demonstrated by our group
 444 (Bushkalova et al., 2019). Considering the higher DUR (Figure 1) observed when irradiating PEC
 445 scaffolds with PB at 280 keV, it seemed important to assess degradation degree depending on
 446 scaffold's depth or distance to the beam source. To that end, pore walls thickness was measured to
 447 study its distribution across scaffold's depth. Thicker walls might be related to PEC, whereas thinner
 448 ones might be imputable to single polymers network (alginate or chitosan). The limit of detection of
 449 thinner walls with thresholding didn't permit to study the latter, but was adapted for the former.

450 Graph B and C from Figure 7 shows 280 keV PB, 2.5 kGy, data as the irradiation treatment giving the
 451 highest DUR and likely to show a degradation gradient. No changes were observed across scaffolds'
 452 depth, confirming indirectly that PEC were not impacted by irradiation.
 453

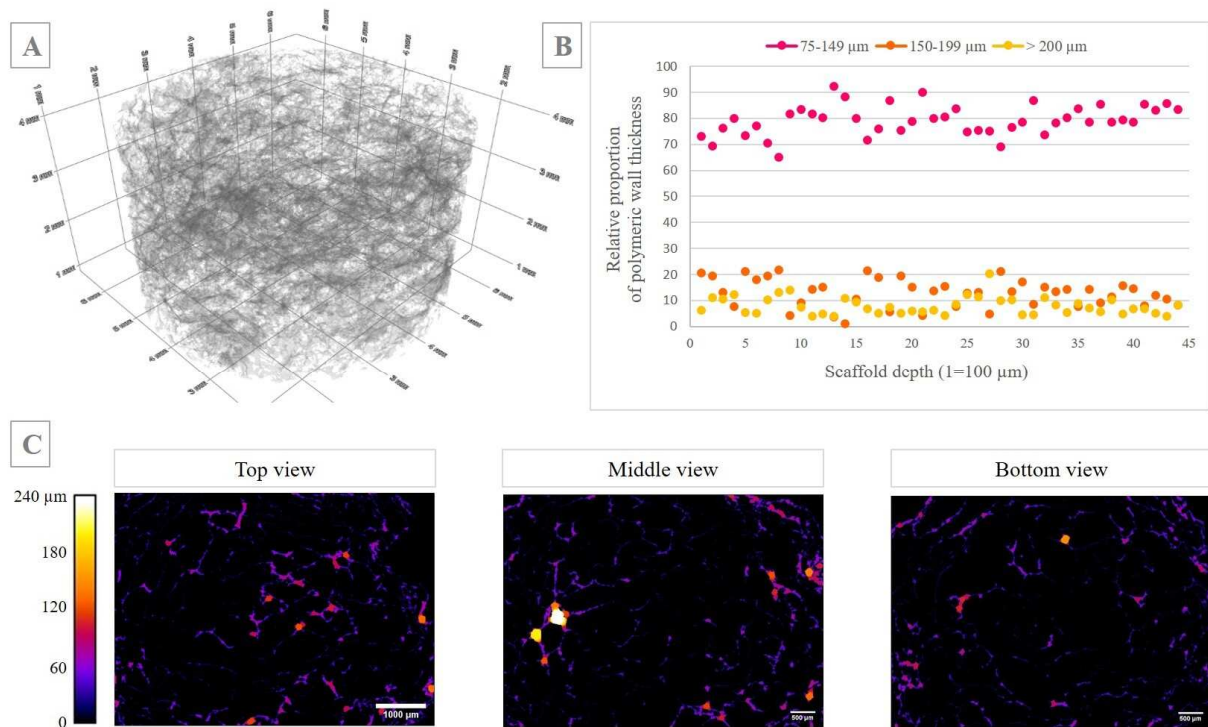


Figure 7 - Micro-CT analysis of 40-60 PEC scaffold. Volume reconstruction of 2.5 kGy irradiated 40-60 PEC scaffold with 280 keV PB (Image A). Relative proportions of polymeric wall thickness among walls thicker than 75 μm (Graph B) and corresponding cross sections ROI after ImageJ local thickness analysis (Image C).

454
 455 Finally, compression tests give additional understanding on how scaffolds react to irradiation and how
 456 these modifications impact the scaffolds mechanical behavior. Overall compressive moduli increased
 457 with successive compression, meaning a stiffness increase due to water elimination during scaffolds
 458 compression. This behavior remained unchanged after irradiation (Figure 8, Graph B). First, PEC
 459 scaffolds showed a decrease of their mechanical resistance after irradiation (at least 28% and 37%
 460 decrease at 2.5 kGy for 280 keV PB and 300 keV CB, respectively) on their first compression (Figure
 461 8, Graph A) but still fit in the range of magnitude of soft tissues elastic moduli (Guimarães, Gasperini,
 462 Marques, & Reis, 2020). However, 25 kGy is clearly detrimental for PEC scaffolds whatever the
 463 irradiation treatment. Finally, 430 keV PB treatment revealed only slight compressive moduli changes
 464 and offers the best scaffolds mechanical properties preservation. A major difference between
 465 continuous and pulsed electron beam technologies is the dose rate they offer (Chalise, Hotta, Matak, &
 466 Jaczynski, 2007; Lamarche, 2019). Electron beam technologies offer much higher dose rate than
 467 gamma radiations especially when electron beam is pulsed (Gotzmann et al., 2018; Silindir & Özer,
 468 2009; Ziaie, Anvari, Ghaffari, & Borhani, 2005). Higher dose rate implies shorter treatment times and
 469 usually results in less damaging effect on the materials. This assumption is confirmed here as 430 keV
 470 PB irradiation is less damaging than 300 keV CB even if its energy is higher.

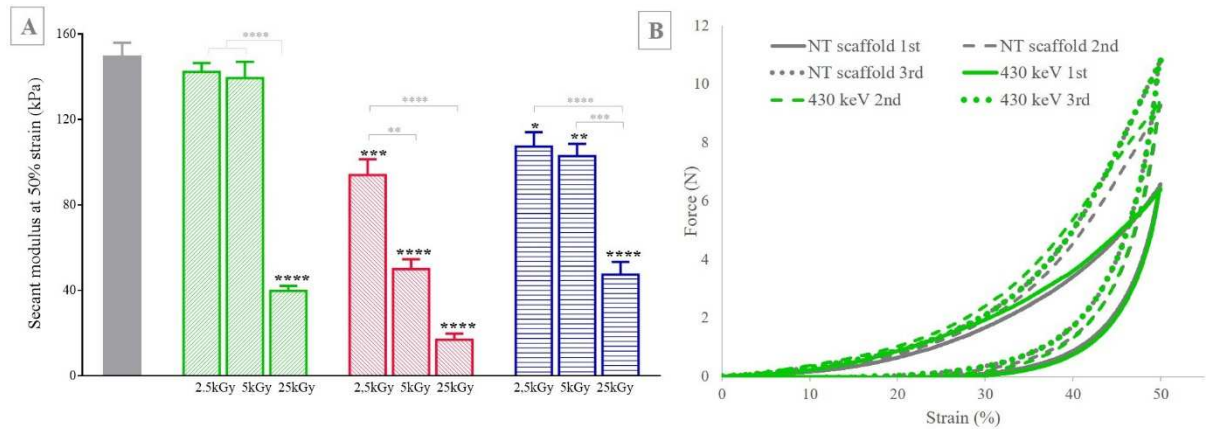


Figure 8 – Secant modulus of irradiated and non-irradiated 40-60 PEC scaffolds measured at 50% compressive strain (Graph A). Graph B shows the force needed to reach 50% strain after three successive compression of NT and 2.5 kGy 430 keV irradiated scaffolds. Five replicates were used for each condition (n=5; two-way ANOVA; **p<0.001; **** p<0.0001; significant differences with NT scaffold and within the same irradiation treatment are respectively shown with black bold asterisks and grey asterisks).

471

472 The results of this physico-chemical study on irradiated PEC scaffolds complete and confirm the
 473 results of the chemical study. Whatever the type of electron beam, the doses at 25kGy are deleterious
 474 for the scaffolds whose porous architecture appears weakened and mechanical properties strongly
 475 reduced, even if the scaffolds generally resist rehydration. PEC scaffolds were affected by irradiation
 476 on a dose-dependent way, but to an acceptable extent at low dose such as 2.5 kGy. While continuous
 477 beam appeared more suitable for scaffolds made of alginate or chitosan, the pulsed electron beam at
 478 low dose has given the best results for PEC scaffolds, with preserved rehydration, porous structure and
 479 wall thickness. Scaffolds' mechanical integrity was even preserved when irradiated with 430 keV PB.
 480 This can be explained by the higher dose rate and subsequently shorter treatment time permitted by
 481 pulsed beam compared to continuous beam, and by the better dose uniformity at 430 keV.

482

483 Critical comparison of the results obtained in this study with other electron beam
 484 sterilization/irradiation studies dealing with similar materials is quite challenging. To our knowledge,
 485 electron beam sterilization/irradiation of alginate has never been reported in the literature. Whereas
 486 several studies highlight chitosan depolymerization after electron beam irradiation, most of them
 487 where achieved at 10 MeV and concern chitosan under various physical conditions (Chmielewski et
 488 al., 2007; Gryczka et al., 2009; Matsushashi & Kume, 1997; San Juan et al., 2012; Silva et al., 2004;
 489 Stöbel et al., 2018). Thus, direct comparison of our results, obtained with alginate and/or chitosan in
 490 solid state, with other works is not self-evident since the starting carbohydrate polymers present
 491 different features (physical form, thickness and density if solid state) which impact their response to
 492 irradiation. However, our results are in accordance with other teams findings concerning the
 493 degradation mechanism of polysaccharides upon ionizing radiations.

494

495 3.4. Biological evaluation of sterilized scaffolds

496

497 Surprisingly, there are few studies in the literature that actually assess the sterility of materials after
 498 irradiation (Asasutjarit et al., 2017; Galante, Pinto, & Serro, 2017; Hartman, Nesbitt, Smith, &
 499 Nuessle, 1975; Hu et al., 2014; Rao & Sharma, 1995). In our case, sterility assessment was essential to
 500 validate irradiations at low doses. As specified in European standard 11137-2, sterilizing dose
 501 establishment can be obtained through two alternative methods to ensure a predetermined sterility
 502 assurance level (SAL). The most common method, called VDmax method, consists in the

503 substantiation of 15 or 25 kGy as sterilization dose. The other method relies on a dose setting to obtain
 504 a product-specific dose. The latter method was the one applied in this study because of the sensitivity
 505 of alginate and chitosan to high doses such as 15 or 25 kGy. Thus, bioburden determination is required
 506 and was obtained in accordance with 2.6.1 European Pharmacopeia chapter (Ph. Eur. 2.6.1, 2008).
 507 Bioburden is the result of microbial contributions from raw materials, manufacturing steps and product
 508 packaging. As Table 3 shows, bioburdens were very low whatever the scaffolds chemical composition,
 509 which permits the use of low doses for sterilization (Table 3).

510
 511 At 2.5 kGy, only 280 keV PB irradiation ensured PEC scaffolds' sterility. It is likely that the high
 512 DUR in these conditions is responsible of this result. It allows to reach the sterilizing dose that is not
 513 reached at 430 keV PB and 300 keV CB, without exceeding the tolerance of the biomaterial as
 514 scaffolds' integrity over rehydration and porous architecture were preserved, with acceptable
 515 (although diminished) mechanical properties for soft tissue applications.

516

A		Alginate/chitosan ratio		Bioburden average quantification		
				Alginate 100/0	< 6 CFU / scaffold	
		PEC 40/60	< 6 CFU / scaffold			
		Chitosan 0/100	< 6 CFU / scaffold			

B		PEC 40/60		Pulsed		Continuous
				430 keV	280 keV	300 keV
Minimum absorbed dose	2.5 kGy	-	+	-		

Table 3 – Biological evaluation. Table A indicate bioburden determination according to alginate/chitosan ratio. Duplicates of 5-pooled samples were used. Table B shows sterility results in both aerobic and anaerobic conditions according to 2.6.1 European Pharmacopeia chapter. For each broth type, sterility assays was performed with three replicates (n=3) or a single replicate (n=1) of 5-pooled samples respectively for pulsed irradiation and continuous irradiation.

517

518 In this study, the feasibility of using low-energy pulsed electron beam for the sterilization of porous
 519 scaffolds of polysaccharidic nature was demonstrated (at 280 keV PB). Despite validated sterility
 520 assays, as recommended in European Pharmacopoeia, those conditions do not fulfill the requirement
 521 of the current European irradiation sterilization standards, since they do not address emerging
 522 techniques like low-energy irradiations. Other works also demonstrated the establishment of sterilizing
 523 doses for sensitive polysaccharides or complex medical devices at lower doses than required in the
 524 norms (Alcaraz et al., 2016; Farag Zaied, Mohamed Youssef, Desouky, & Salah El Dien, 2007). In
 525 the years to come, European standards will have to take into account the use of low-energy irradiation
 526 technologies able to answer to the sterilization needs of new materials such as porous polysaccharidic
 527 scaffolds. In that sense, the lack of standards for low-energy electron dosimetry has been underlined in
 528 recent study (Helt-Hansen et al., 2010); this observation can be extended to the sterilizing dose
 529 establishment after low-energy electron beam irradiation.

530

531

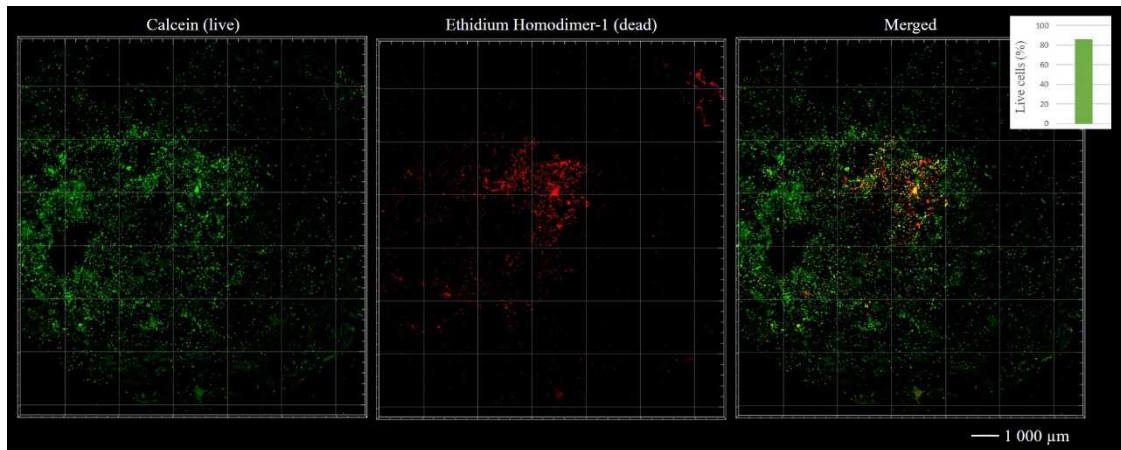


Figure 9 - 3D reconstruction of confocal microscopy images after Live/Dead staining of BMDM macrophages seeded 40-60 PEC scaffold after 2.5 kGy irradiation with 280 keV PB generator. Scale bar corresponds to 1000 μm .

532

533

534 In order to definitively validate the operating conditions for sterilization of PEC scaffolds, an in vitro
 535 biocompatibility study was carried out on sterilized scaffolds. Scaffolds were irradiated with 2.5 kGy
 536 PB with an energy of 280 keV, before BMDM seeding. After 24 hours, in vitro constructs were
 537 stained fluorescent viability markers which stain dead cells in red and live cells in green. Scaffolds
 538 were imaged in depth with a confocal microscope. Finally, 3D reconstructions were obtained (Figure
 539 9) and cell viability was estimated to 86% (number of green cells over total number of green and red
 540 cells). As previously demonstrated with other cell types such as mesenchymal stem cells (Bushkalova
 541 et al., 2019; Ceccaldi et al., 2014), 40-60 PEC scaffolds biocompatibility was high and maintained
 542 even after low-energy electron beam sterilization.

543

544 4. Conclusions

545

546 To our knowledge, this is the first study concerning low-energy electron beam use for sterilization of
 547 polysaccharidic scaffolds, and more particularly those made of alginate, chitosan or their complexes.
 548 Sterilization of polysaccharides materials is still an unmet challenge for tissue engineering. Irradiation
 549 technologies remain very promising in that sense that they are environmentally friendly and do not
 550 produce toxic residues that could threaten scaffolds biocompatibility. In this work we have compared
 551 continuous and pulsed low-energy electron beam technologies and their impact on polysaccharides-
 552 based biomaterial properties for sterilization purposes. If irradiation-induced degradation cannot be
 553 denied on single alginate and chitosan polymers, it is highly limited when alginate and chitosan form
 554 polyelectrolyte complexes. An optimal sterilizing dose setting was found without compromising
 555 scaffolds properties when irradiated with 280 keV PB. This work paves the way for low-energy
 556 electron-beam sterilization of porous natural biopolymer materials. However low penetration ability
 557 limits the size of the constructs that can be sterilized and an adaptation of the beam energy is needed to
 558 achieve dose uniformity within the considered scaffold. In this way, European norms should consider
 559 low-energy irradiation suitability in the particular case of thin and low-dense materials such as 3D
 560 scaffolds developed for tissue engineering purposes.

561

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563

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575

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577

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