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Polyglucosan Body Structure in Lafora Disease

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2

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5

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22

23

24

25

26 **Abbreviations**

27 PGB: polyglucosan body; LD: Lafora disease; LB: Lafora body; GSD: glycogen storage
28 disease; CLD: chain length distribution; TEM: transmission electron microscopy; WAXS:
29 wide-angle X-ray scattering; SAXS: small-angle X-ray scattering; DIC: differential
30 interference contrast; SmLBs: skeletal muscle LBs; BrLBs: brain LBs; HtLBs: heart LBs;
31 PAPS: potato amylopectin starch; SEC: size exclusion chromatography; R_h : hydrodynamic
32 radius; WT: wild-type.

33

34 **Abstract (150 words or less)**

35 Abnormal carbohydrate structures known as polyglucosan bodies (PGBs) are associated with
36 neurodegenerative disorders, glycogen storage diseases (GSDs), and aging. A hallmark of the
37 GSD Lafora disease (LD), a fatal childhood epilepsy caused by recessive mutations in the
38 *EPM2A* or *EPM2B* genes, are cytoplasmic PGBs known as Lafora bodies (LBs). LBs result
39 from aberrant glycogen metabolism and drive disease progression. They are abundant in
40 brain, muscle and heart of LD patients and *Epm2a*^{-/-} and *Epm2b*^{-/-} mice. LBs and PGBs are
41 histologically reminiscent of starch, semicrystalline carbohydrates synthesized for glucose
42 storage in plants. In this study, we define LB architecture, tissue-specific differences, and
43 dynamics. We propose a model for how small polyglucosans aggregate to form LBs. LBs are
44 very similar to PGBs of aging and other neurological disorders, and so these studies have
45 direct relevance to the general understanding of PGB structure and formation.

46

47 **1. Introduction**

48 Polyglucosan bodies (PGBs) are a common feature of glycogen storage diseases (GSDs),
49 neurodegenerative diseases, and physiological aging (Cavanagh, 1999; Duran & Guinovart,
50 2015; Rohn, 2015). PGBs range in size from 2 to 50 μm in diameter and, unlike the
51 proteinaceous inclusion bodies of neurodegenerative diseases, they are primarily made of an
52 aberrant glucose polymer called polyglucosan (Raben et al., 2001). These glucose polymers
53 are considered “abberant” because they differ significantly in structure and appearance from
54 the normal glucose polymers of mammalian tissues, namely glycogen. Subtle chemical and
55 ultrastructural differences distinguish the PGBs of various pathologies (Cavanagh, 1999).
56 What unites them is their chemical resemblance to mammalian glycogen and plant starch, the
57 two major forms of glucose storage in living organisms (Emanuelle, Brewer, Meekins, &
58 Gentry, 2016).

59 Glycogen is a polysaccharide comprised of α -1,4-linked linear chains of glucose with α -
60 1,6-linked branches. Starch contains two types of polysaccharides, amylopectin and amylose.
61 Amylopectin is the major component of starch and, like glycogen, contains both α -1,4 and α -
62 1,6-linkages. Amylose, the minor constituent of starch (typically 15-30% of total starch
63 weight), is comprised almost exclusively of linear α -1,4-linked chains with few branch
64 points. Although glycogen and amylopectin have identical glycosidic bonding, differences in
65 chain length and branching frequency give them distinct properties. The linear chains of
66 glycogen have approximately 13 glucose units and two branch points, making glycogen a
67 water-soluble, continuously branched macromolecule that is designed for rapid glucose
68 release (Melendez-Hevia, Waddell, & Shelton, 1993; Roach, Depaoli-Roach, Hurley, &
69 Tagliabracci, 2012). In contrast, the linear glucose chains of amylopectin contain on average
70 20-30 glucose units and branch points are clustered, although the arrangement of the clusters
71 is still under investigation (Bertoft, 2017; Jane et al., 1999). The long, linear regions of the

72 glucan chains intertwine to form double helices, producing crystalline layers (called lamellae)
73 interleaved with more hydrated, amorphous regions containing the branch points. The
74 crystalline and amorphous lamellae radiate from a central origin, and as a result, starch
75 granules are a densely packed, semicrystalline glucose cache that can reach up to 100 μm in
76 diameter in some plant tissues (Emanuelle et al., 2016; Lourdin et al., 2015). Amylose is
77 interspersed among the amylopectin chains (Bertoft, 2017). Starch and glycogen both contain
78 covalently bound phosphate, located at the 3- and 6-hydroxyls in amylopectin and 2-, 3-, and
79 6-hydroxyls in glycogen (DePaoli-Roach et al., 2014; Nitschke et al., 2013; Ritte et al., 2006;
80 Young et al., 2019). Amylopectin contains higher levels of phosphate than glycogen: 0.1-
81 0.5% versus 0.064-0.25% by weight, depending on the plant or tissue source (Gentry, Dixon,
82 & Worby, 2009).

83 Starch-like structures in the brains of elderly patients were first described in 1836 by J.
84 E. Purkinje, who named them *corpora amylacea* (Latin for "starch-like bodies"). In 1854,
85 Rudolf Virchow observed a substance in the diseased nervous system that stained with iodine
86 in a manner similar to plant starch, coining the term "amyloid" (Virchow, 1854).
87 Subsequently, others also described "amyloid" deposits that were later discovered to be
88 proteinaceous, so the term now typically refers to the inclusions of Alzheimer's and other
89 amyloidoses (Kyle, 2001; Sipe & Cohen, 2000). But *corpora amylacea* and PGBs are, in fact,
90 true amyloid: in addition to their similarity in size and shape and staining characteristics to
91 starch, they are comprised primarily of insoluble glucose polymers with chain lengths longer
92 than normal glycogen (Cafferty et al., 1991; Cavanagh, 1999; Sakai, Austin, Witmer, &
93 Trueb, 1969). In some cases, these PGBs also contain elevated phosphate (Sullivan et al.,
94 2019). Since glycogen synthase is the only known enzyme able to catalyze glucose
95 polymerization *in vivo* in mammals, PGBs are considered a pathological result of aberrant
96 glycogen metabolism (Duran & Guinovart, 2015; Raben et al., 2001; Roach et al., 2012).

97 Lafora disease (LD) is a progressive myoclonus epilepsy and a GSD (OMIM: 254780)
98 that manifests during the teen years and leads to early death (Akman, Oldfors, & DiMauro,
99 2015; Gentry et al., 2020; Gentry, Guinovart, Minassian, Roach, & Serratos, 2018;
100 Minassian, 2001). LD is characterized by cytoplasmic PGBs known as Lafora bodies (LBs)
101 that are found in neurons, astrocytes, skeletal and cardiac myocytes, and other cell types
102 (Augé, Pelegrí, et al., 2018; Criado et al., 2012; Rubio-Villena et al., 2018; Van Heycop Ten
103 Ham, 1975). Like other PGBs, LBs from human tissues were described as starch-like in the
104 early- and mid-1900s based on their chemical and structural characteristics (Lafora, 1911;
105 Yokoi, Austin, Witmer, & Sakai, 1968). Recessive mutations in either of the *Epilepsy*,
106 *progressive myoclonus 2* genes (*EPM2A* or *EPM2B*) cause LD in humans, and *Epm2a^{-/-}* and
107 *Epm2b^{-/-}* mice recapitulate the disease with LB accumulation, neurodegeneration, and
108 seizures (Criado et al., 2012; DePaoli-Roach et al., 2010; Ganesh et al., 2002; Tiberia et al.,
109 2012; Valles-Ortega et al., 2011). *EPM2A* encodes laforin, the mammalian glycogen
110 phosphatase, and *EPM2B* encodes malin, an E3 ubiquitin ligase (Gentry et al., 2007; Gentry,
111 Worby, & Dixon, 2005; Worby, Gentry, & Dixon, 2006). The three-dimensional structure of
112 laforin has been determined, elucidating the mechanism of its glycogen phosphatase activity
113 (Raththagala et al., 2015). Both laforin and malin are believed to regulate the architecture of
114 glycogen molecules and prevent the formation of LBs, although this mechanism has not yet
115 been elucidated (Sullivan, Nitschke, Steup, Minassian, & Nitschke, 2017). Multiple groups
116 have established that LBs drive neurodegeneration and epilepsy using these LD mouse
117 models and complementary fly models (reviewed by (Gentry et al., 2018).

118 The polysaccharides from *Epm2a^{-/-}* and *Epm2b^{-/-}* mice that have been characterized were
119 purified based on a method first described in 1909 by Pflüger for purifying glycogen, in
120 which the tissue is boiled in KOH and the polysaccharides are precipitated with ethanol
121 (Good, 1933; Pflüger, 1909). This method does not separate glycogen from the LBs.

122 Additionally, the Pflüger-purified polysaccharides differ in morphology from LBs observed
123 via microscopy in LD tissue sections. When analyzed by transmission electron microscopy
124 (TEM) the Pflüger-purified particles appear more aggregated than wild-type glycogen
125 (Tagliabracci et al., 2008), but they are dramatically smaller (15-65 nm) than the micron-
126 sized LBs observed in tissue sections (Minassian, 2001). Purification of LBs by the Pflüger
127 method likely disrupts LB morphology and also does not separate glycogen from the LB.
128 More recently, the “soluble” and “insoluble” fractions of glycogen were separated after tissue
129 homogenization and analyzed individually, revealing that only the “insoluble” fraction had an
130 altered chain length (Sullivan et al., 2019). The “insoluble” fraction is likely to correspond to
131 the LBs and the “soluble” fraction to normal glycogen, but this was not clearly demonstrated.
132 It is conceivable that LBs are aggregates of polyglucosan (i.e. abnormal polysaccharide)
133 molecules, and that the Pflüger method breaks apart the LBs and intermixes the polyglucosan
134 molecules comprising LBs with normal, soluble glycogen particles. In the case of starch,
135 many studies have shown that the application of heat and moisture results in solubilization
136 and irreversible disruption of the starch granular structure (Ratnayake & Jackson, 2009).

137 A significant body of work has been performed to define the chemical, physical and
138 structural properties of different types of starch, which vary depending on plant species and
139 tissue type (reviewed by (Lourdin et al., 2015)). One of the major features that distinguishes
140 starch from glycogen is its semicrystalline, lamellar organization that can be detected with
141 diffraction techniques such as wide- and small-angle X-ray scattering (WAXS and SAXS,
142 respectively). Treatment of starch granules with mild hydrochloric acid, a process known as
143 lintnerization, preferentially hydrolyzes the amorphous regions, leaving crystalline regions
144 intact and providing valuable information about granule architecture (Gerard, Planchot,
145 Colonna, & Bertoft, 2000; Srichuwong, Isono, Mishima, & Hisamatsu, 2005; Wikman,

146 Blennow, & Bertoft, 2013). Despite the similarities between PGBs and starch, comparable
147 methods have not yet been applied to investigate the structure of native PGBs.

148 In prior studies, variations of the Pflüger method were used to purify LD polyglucosan for
149 biochemical analysis. NMR and enzymatic analyses demonstrated that LD polyglucosan
150 contains elevated phosphate linked to the C2-, C3- and C6- hydroxyls of the glucose moieties
151 in relatively equivalent ratios (DePaoli-Roach et al., 2015; Nitschke et al., 2013; Tagliabracci
152 et al., 2011). Chain length distribution analysis showed an increase in longer chains in
153 polyglucosan from muscle and brain, which causes the polyglucosan to precipitate (Irimia et
154 al., 2015; Nitschke et al., 2017; Sullivan et al., 2019). These samples were always denatured;
155 and native LBs were not analyzed. Furthermore, no biochemical study of isolated
156 polyglucosan from cardiac tissue was performed. We recently described a novel method for
157 isolating native LBs from *Epm2a*^{-/-} and *Epm2b*^{-/-} mice using a protocol that does not rely on
158 the Pflüger method, separates LBs from glycogen, and preserves the endogenous size and
159 structure of the LBs (Brewer et al., 2019). Using light microscopy and scanning electron
160 microscopy, we showed that native LBs from brain, heart, and skeletal muscle have distinct
161 sizes and morphologies, typically ranging from 2 to 10 µm in diameter, consistent with their
162 distinct appearances in fixed tissue sections. We hypothesized that LBs possess starch-like
163 biophysical and chemical properties, beyond just histochemical similarities, that distinguish
164 them from normal glycogen. In the present study, we define the physiochemical and
165 structural characteristics of native LBs isolated from different tissues. Combining these
166 methods with thermal, mechanical and chemical treatments to disassemble LBs allows for a
167 model of how polyglucosan molecules aggregate to form LBs in LD. Although not all PGBs
168 are identical, the *corpora amylacea* of aging and neurodegeneration and the PGBs of multiple
169 GSDs bear striking similarities to LBs. We propose that PGBs may represent a common

170 pathological phenomenon resulting from misregulated glycogen metabolism, which has
171 particularly detrimental consequences in the brain.

172

173 **2. Methods and Materials**

174 *2.1. Purification of native LBs from Epm2a^{-/-} mice*

175 All animal handling and procedures were approved by the University of Kentucky
176 Institutional Animal Care and Use Committee (IACUC). *Epm2a^{-/-}* mice have been previously
177 described (DePaoli-Roach et al., 2012; Ganesh et al., 2002). Native LBs from 19-24 month
178 old *Epm2a^{-/-}* mice were purified as previously described (Brewer et al., 2019).

179

180 *2.2. Confocal microscopy*

181 20× Lugol's iodine was prepared as described previously (Brewer et al., 2019). For
182 confocal microscopy, LBs or corn starch (Sigma) were stained with 20× Lugol's iodine,
183 embedded in Mowiol 4-88 (Sigma cat #81381) on glass slides, and visualized using the
184 TRITC channel (561 nm excitation laser) and differential interference contrast (DIC) using a
185 Nikon AR+ Scope. Z-stack images of LBs were captured in 0.1 μm steps, starch granules in
186 0.25 μm steps, and 3D deconvolution was performed using the Nikon Elements Advanced
187 Research Software. The Richardson-Lucy deconvolution algorithm was utilized with 10
188 iterations per stack.

189

190 *2.3. Small- and wide-angle X-ray scattering (SAXS and WAXS)*

191 Purified SmLBs and BrLBs were centrifuged at 14,000 rpm, and the wet pellets were
192 poured into 1 mm (outer diameter) glass capillaries. For comparison purpose, capillaries
193 containing potato amylopectin starch (PAPS) granules (a gift from E. Bertoft, Turku
194 University) and rabbit liver glycogen (Fluka) were prepared as well. All specimens were

195 equilibrated for 5 days in a closed chamber maintaining a 93% relative humidity. The
196 capillaries were flamed-sealed and X-rayed in air by a Ni-filtered CuK α radiation ($\lambda = 0.1542$
197 nm) using a Philips PW3830 generator operating at 30 kV and 20 mA. Two-dimensional
198 scattering diagrams were recorded on Fujifilm imaging plates placed at a distance of about 5
199 cm from the capillary and read offline with a Fujifilm BAS 1800-II bioanalyzer. Diffraction
200 profiles were calculated by rotationally averaging the 2D diffraction patterns after subtraction
201 of the scattering signal from air. Capillaries containing SmLBs and BrLBs were also heated
202 at 95 °C and analyzed immediately after heating and after 12 and 36 h at 4 °C. Considering
203 the small amount of material, the specimens of acid-hydrolyzed LBs were centrifuged inside
204 the capillaries and X-rayed in excess water. SAXS patterns were recorded from wet
205 specimens (i.e. kept in excess water), with the imaging plates placed at a distance of 30 cm
206 from the capillaries, and scattering profiles were calculated using the same rotational
207 averaging procedure. SAXS data is usually presented as a function of the scattering vector, q
208 (Blazek & Gilbert, 2011). WAXS profiles are presented as a function of the diffraction angle
209 (2θ). The scattering vector (q) is geometrically related to the wavelength of the incident
210 radiation (λ) and the scattering angle (2θ) (Figure S2). Therefore, q can be converted to
211 distance of the repeating unit (d) in real space using Bragg's law:

$$q = \frac{4\pi}{\lambda} \sin \theta = \frac{2\pi}{d} \quad \text{Equation (1)}$$

213

214 2.4. LB treatments and light microscopy

215 SmLBs and BrLBs were aliquoted (100 μ g per tube) and washed in water by
216 centrifugation at 16,000 g for 1 min. LB pellets were resuspended in 100 μ L water or 100 μ L
217 30% KOH for a final concentration of 1 mg/ml. For sonication treatment, five pulses of 5
218 seconds each were applied at 20 kHz with 25% amplitude, with one minute on ice between
219 each pulse to prevent sample heating. For heat treatments at 95 °C, samples were heated for

220 30 min or 2 h as indicated. After treatment, 5 μ L of samples were stained with 2 μ L 20 \times
221 Lugol's iodine, mounted on glass slides with a glass coverslip, and visualized using a Nikon
222 Eclipse E600 using DIC/Nomarski contrast and an AxioCam MRm or Zeiss 512 camera at
223 100 \times . LBs had a tendency to flocculate, so after staining, samples were carefully triturated
224 with a pipet, added to glass slides, and the coverslip was laid on top. The coverslip was very
225 gently prodded and pressed to disperse the LB clumps and reduce the number of focal planes.
226 Image thresholding was performed using the ImageJ software (National Institutes of Health,
227 USA).

228

229 *2.5. Hydrothermal and lintnerization treatments for transmission electron microscopy (TEM)*

230 Two different treatments were performed to disrupt the LB structure and the resulting
231 specimens were observed by TEM. In the first one, dilute aqueous LB suspensions were
232 heated at 95 $^{\circ}$ C for 30 min, and TEM specimens were prepared after cooling down at room
233 temperature and after keeping the suspensions for 3 days at 4 $^{\circ}$ C. The second treatment was
234 carried out by disrupting LBs in 2.2 N HCl and incubating the suspensions at 36 $^{\circ}$ C. This
235 gradual hydrolysis of glucose polymers in mild acid, a process known as lintnerization, is
236 used to study crystalline structure (Bertoft, 2017; Jacobs, Eerlingen, Rouseu, Colonna, &
237 Delcour, 1998). After 5 days, the suspensions were centrifuged; the pellets were repeatedly
238 washed to neutrality by centrifugation in water and finally re-dispersed in water. Droplets of
239 the various dilute suspensions were deposited onto glow-discharged carbon-coated copper
240 grids. After blotting of the excess liquid, the preparations were negatively stained with 2 wt%
241 uranyl acetate, allowed to dry and observed with a JEOL JEM 2100-Plus microscope
242 operating at 200 kV and equipped with a Gatan Rio 16 camera.

243

244 *2.6. Size Exclusion Chromatography (SEC)*

245 Polyglucosan bodies were extracted/dissociated by boiling in 30% KOH for 1 h,
246 followed by ethanol precipitation. This involved adding 4 volumes of ethanol with 15 mM
247 LiCl and storing at -30 °C for 1 h. Samples were then centrifuged at 16,000 g for 20 min at 4
248 °C. Pellets were resuspended in 200 µL of milli-Q water and the ethanol precipitation step
249 was repeated for a total of 3 times. Samples were freeze-dried overnight and then dissolved
250 directly in SEC eluent (50 mM NH₄NO₃ with 0.02% sodium azide) at 80 °C and 350 rpm
251 overnight. Samples were then analyzed using SEC as previously described (Sullivan et al.,
252 2019). Briefly, dissolved polyglucosan (~2.5 mg.mL⁻¹) was injected into SHIMADZU LC-
253 20AD system coupled with differential refractive index detector (Wyatt Technology, USA)
254 using the following columns: SUPREMA preColumn, 1000 and 10,000 (PSS). The column
255 oven was set at 80 °C, and the flow rate was set at 0.3 mL.min⁻¹. The differential refractive
256 index detector was used to obtain the weight distributions, as previously reported (Sullivan et
257 al., 2010). A universal calibration curve was constructed using pullulan standards (ranging
258 from 108 to 1.22 × 10⁶ Da), also directly dissolved into the SEC eluent. Using the Mark-
259 Houwink relationship the elution volumes were converted to hydrodynamic radius, as
260 previously described in detail (Sullivan et al., 2010). Rat muscle glycogen was used as a
261 reference for normal glycogen particles and was extracted using the same KOH method, as
262 previously described (Sullivan et al., 2010).

263

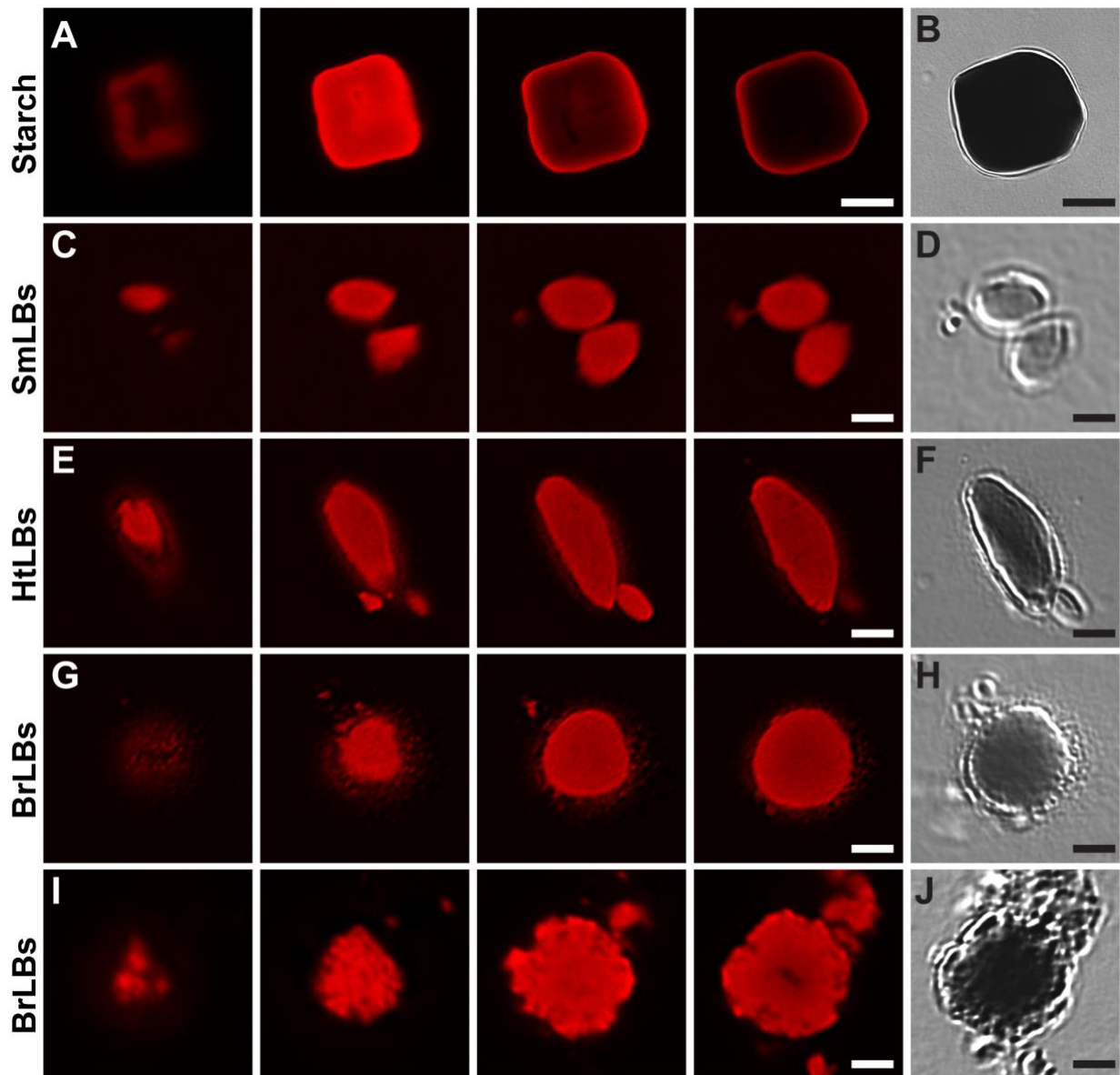
264 **3. Results**

265 *3.1. Confocal micrographs of iodine-stained LBs*

266 Lugol's iodine is a chemical stain that has been used for over a century to detect and
267 differentiate polysaccharides such as glycogen, amylose and amylopectin. Amylose,
268 composed almost entirely of α-1,4 linear chains, produces a deep blue-black color when
269 stained with Lugol's iodine, with a maximum absorption at 650 nm (Rundle, Foster, &

270 Baldwin, 1944; Swanson, 1948). The linear glucan chains of amylose form a complex with
271 iodine in the form of a single chromophoric helix. In contrast, the branch points within
272 amylopectin interfere with the interaction, producing a less stable complex with triiodide.
273 The result is a reddish-purple color and a maximum absorption at 550 nm (Swanson, 1948).
274 Glycogen contains even shorter chains and more frequent branching than amylopectin, and
275 therefore stains poorly with Lugol's iodine. Glycogen yields an absorbance maximum at 440
276 nm, although this varies slightly depending on glycogen source, i.e. glycogen from different
277 tissues and/or organisms (Archibald et al., 1961).

278 Iodine staining of LBs, *corpora amylacea*, and other PGBs indicates a branching degree
279 that is more similar to amylopectin than glycogen (Herrick, Twiss, Vladutiu, Glasscock, &
280 Horoupian, 1994; Reed Jr, Dixon, Neustein, Donnell, & Landing, 1968; Sakai et al., 1969).
281 We previously showed that native LBs isolated from skeletal muscle, heart tissue, and brain
282 of *Epm2a*^{-/-} mice stained intensely reddish-brown in Lugol's iodine with an absorbance
283 maximum at approximately 510-520 nm (Brewer et al., 2019). The iodine-stained LBs not
284 only absorb at this wavelength, but they also produce red fluorescence when excited by a 561
285 nm confocal laser. Confocal microscopy has previously been used as a sensitive method for
286 visualizing starch granules *in vitro* and *in planta* (Bahaji et al., 2011; Ovecká et al., 2012). Z-
287 stack images of iodine-stained starch granules visualized using the 561 nm laser and
288 differential interference contrast (DIC) at high magnification show that only the smooth,
289 outer edge of the granule is stained (Figure 1A and B, Figure S1A through D). This staining
290 pattern indicates that iodine more efficiently penetrates the outer portion than the densely
291 packed semicrystalline interior of the starch granule.



292

293 **Figure 1.** Representative z-stack confocal images via the 561 nm laser line of iodine-stained
 294 starch (A), SmLBs (C), HtLBs (E), and BrLBs (G,I). Differential interference contrast (DIC)
 295 is also shown for each: starch (B), SmLBs (D), HtLBs (F), and BrLBs (H,J). In (A) and (B),
 296 scale bar = 10 μm and TRITC panels represent 2 μm steps. In (C) through (I), scale bar = 2
 297 μm . For SmLBs (C), TRITC panels represent 0.4 μm steps. For HtLBs (E) and BrLBs (G, I)
 298 TRITC panels represent 0.9 μm steps.

299

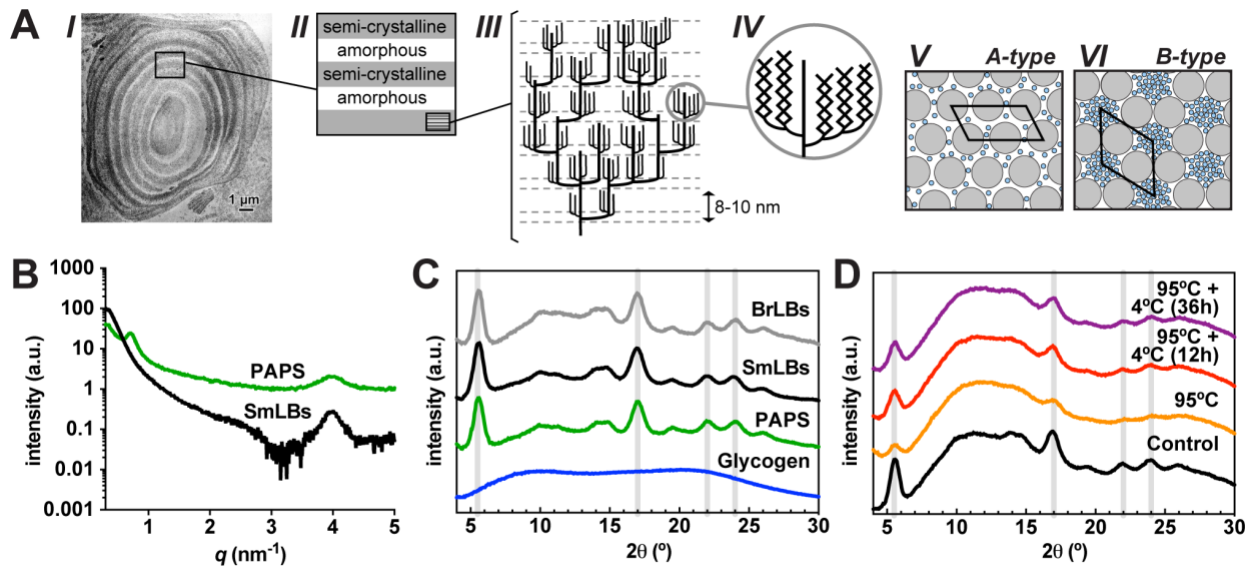
300 Isolated LBs from *Epm2a*^{-/-} skeletal muscle (SmLBs) have a characteristic ovoid
301 morphology and are typically 1-3 μm in length (Brewer et al., 2019). Like starch, they also
302 fluoresce brightly with 561 nm excitation after iodine staining, but they display staining
303 throughout the LB, suggesting their smaller size and less dense interior facilitates greater
304 penetration of the triiodide ions (Figure 1C and 1D, Figure S1E through H). LBs isolated
305 from *Epm2a*^{-/-} hearts (HtLBs) are larger in size (typically 2-4 μm , up to 10 μm in length) and
306 more elongated than SmLBs (Brewer et al., 2019). They were also stained homogeneously
307 with iodine and were comprised of a visible interior texture and a brighter rim (Figure 1E,
308 Figure S1I and S1K). The textured surface of the LBs was evident using DIC and consistent
309 with what we previously observed via scanning electron microscopy (Figure 1F and 1H)
310 (Brewer et al., 2019). Brain LBs (BrLBs) were also larger in size (typically 2-4 μm ,
311 occasionally $>10 \mu\text{m}$ in diameter) and varied in morphology more than SmLBs and HtLBs.
312 Some BrLBs were very round and homogeneously stained, with a bright rim and textured
313 appearance, similar to HtLBs except that they are not typically elongated (Figure 1G and
314 1H). Some BrLBs had a very jagged edge with a dense core that did not stain well with
315 Lugol's iodine (Figure 1I and 1J). Very small iodine-positive granules were also evident
316 around LBs from heart and brain (Figure 1 and Figure S1), consistent with previous
317 observations describing "dust-like particles" (Brewer et al., 2019; Van Hoof & Hageman-Bal,
318 1967). Overall, the fluorescence produced from the interaction of LBs with the triiodide ions
319 suggests they possess crystalline regions like plant starch. In general, the LBs are more
320 permeable to the triiodide ions than starch, indicating less compact and/or less orderly
321 architecture. However, the lack of central iodine fluorescence in some of the BrLBs (Figure
322 1I) suggests that some bodies had a more densely packed center, like that of starch (Figure
323 1A, Figure S1A and S1C).

324

325 3.2. *LBs contain B-type crystallites lacking long-range order*

326 Unlike glycogen, starch granules possess both short- and long-range order, which can be
327 measured by X-ray scattering techniques. Long-range order refers to the alternation of
328 crystalline and amorphous layers within the growth rings of the starch granule (i.e. lamellae),
329 with a repeat distance of 8-10 nm (Figure 2A, panels *I* and *II*) (Blazek & Gilbert, 2011;
330 Lourdin et al., 2015). Small-angle X-ray scattering (SAXS) is better suited for measuring
331 these nanometer-sized distances (Figure S2A) (Donald, Kato, Perry, & Waigh, 2001). Short-
332 range order refers to repeating units on the sub-nanometer scale, i.e. the crystalline
333 organization of double helices formed by the short linear segments of amylopectin (Figure
334 2A, panels *III* and *IV*). Wide-angle X-ray scattering (WAXS) is used to probe sub-nanometer-
335 sized distances and has been widely utilized to study crystallinity in starch and other
336 polymers (Figure S2A) (Blazek & Gilbert, 2011; Donald et al., 2001). In starch, amylopectin
337 double helices crystallize in two different allomorphs depending on the starch source (e.g.
338 potato or corn). Typically, A-type is found in cereals while B-type occurs in tubers and high-
339 amylose starch (Bertoft, 2017; Qiao et al., 2017). The allomorphic composition can be
340 determined from X-ray diffraction patterns. The double helices of A-type crystals are densely
341 packed into a monoclinic unit cell that contains little water (Figure 2A, panel *V*) (Popov et al.,
342 2009). In the B-type crystal, the double helices form a hexagonal network with a central
343 channel that contains water molecules, rendering this allomorph more open and hydrated
344 (Figure 2A, panel *VI*) (Imberty & Perez, 1988). We used SAXS and WAXS to characterize
345 the order in native, purified LBs.

346



347
 348 **Figure 2.** (A) Schematic depiction of long- and short-range order within the starch granule. *I*:
 349 An ultrathin section of a waxy maize starch granule visualized by transmission electron
 350 microscopy (TEM) and showing the typical growth rings. *II*: Growth rings are composed of
 351 alternating amorphous and semicrystalline layers (known as lamellae). *III*: Semicrystalline
 352 lamellae are made up of branched amylopectin molecules with amorphous regions containing
 353 the branch points and a crystalline region containing the linear segments forming double
 354 helices. The total lamellar thickness equals the so-called repeat distance which is typically 8-
 355 10 nm (Nakamura, 2015). The arrangement of the crystalline clusters is still debated. *IV*:
 356 Long, linear glucan chains (black lines) form double helices that make up the crystalline
 357 layers of the starch lamellae. The helical packing determines the allomorphic type. *V*: In A-
 358 type, the helices (grey circles) are closely packed, with some water molecules (blue circles)
 359 in-between. *VI*: In B-type, the helices form a hexagonal array surrounding a water-filled
 360 channel (Imberty & Perez, 1988; Nakamura, 2015; Tester, Karkalas, & Qi, 2004). Reprinted
 361 by permission from Springer Nature: Springer, Crystalline Structure in Starch, Lourdin et al.
 362 © 2015. (B) SAXS profiles of LBs and potato amylopectin starch (PAPS). (C) WAXS
 363 profiles of BrLBs and SmLBs compared to those of PAPS and rabbit liver glycogen. (D)
 364 WAXS profiles of SmLBs after heating at 95 °C and cooling down to room temperature,
 365 then with a retrogradation period at 4 °C either 12 or 36 h after heating at 95 °C. Grey lines
 366 denote the location of characteristic B-type reflections in WAXS profiles. The corresponding
 367 2D SAXS and WAXS patterns are shown in Figure S3.

368
 369

370 We first performed SAXS on SmLBs. As a control, we also analyzed a genetically
 371 modified starch engineered to contain exclusively amylopectin and no amylose called potato
 372 amylopectin starch (PAPS) (Svegmark et al., 2002). PAPS produced a characteristic SAXS
 373 profile with two distinct peaks (Figure 2B and Figure S3A). The peak at $q = 4.1 \text{ nm}^{-1}$
 374 corresponds to a d -spacing of 1.53 nm and is equivalent to the innermost reflection of the B-

375 type allomorph in a WAXS profile. It corresponds to the spacing of (100) planes in the unit
 376 cell (Figure S2A). The reflection at $q = 0.76 \text{ nm}^{-1}$ corresponds to a repeat distance of 8.3 nm
 377 (Figure 2B), i.e. the lamellar repeat in the amylopectin structure (Figure 2A, panel III)
 378 (Blazek and Gilbert 2011). While the profile of SmLBs also contained the (100) reflection of
 379 B-type, the peak corresponding to the lamellar repeat was absent (Figure 2B and Figure
 380 S3B). This result indicates that SmLBs do not contain any lamellar units like starch granules.

381 To establish whether LBs are crystalline, we studied them using WAXS with PAPS as a
 382 control. We analyzed both SmLBs and BrLBs to determine whether there are allomorphic
 383 differences between LB types. PAPS, SmLBs and BrLBs all produced a WAXS diffraction
 384 pattern containing characteristic reflections of the B-type allomorph at $2\theta = 5.6, 17, 22$ and
 385 24° (Figure 2C, S3C, S3D and S3E) (Buléon, Bizot, Delage, & Pontoire, 1987). The WAXS
 386 reflection at $2\theta = 5.6^\circ$ corresponds to the 100 reflection, a typical signature of allomorph B
 387 (Svegmark et al., 2002). Using Equation (1), d -spacings were calculated from these values of
 388 2θ and are in agreement with those of B-type potato starch (Table 1, also see Figure S2B)
 389 (Cleven, Van den Berg, & Van Der Plas, 1978). In contrast, rabbit liver glycogen can be
 390 considered amorphous since its WAXS profile does not contain any distinct peak (Figure
 391 2C).

392
 393
 394 **Table 1.** d -Spacings (in nm) calculated from the position (diffraction angle 2θ) of the
 395 diffraction peaks in the WAXS profiles of PAPS and LB samples using Equation (1)
 396 compared to published values (*from Cleven et al. 1978).

Peak #	1	2	3	4
PAPS	1.59	0.52	0.40	0.37
SmLBs	1.59	0.52	0.40	0.37
BrLBs	1.59	0.52	0.40	0.37
Potato*	1.58	0.53	0.40	0.37

398

399

400 When starch is heated in water, it swells and amylose leaches out of the granule. The
401 peak in the SAXS profile disappears, indicating that the lamellae organization is disrupted
402 and no longer present. The peaks in the WAXS profile concomitantly disappear due to the
403 complete loss of crystal structure, i.e. glucan chains are no longer oriented in a repeating
404 helical units (Cameron & Donald, 1992). However, after gelatinization, as the starch cools
405 down to room temperature, amylose and amylopectin regain B-type crystallinity, a process
406 known as retrogradation. Double helices form from intra- and intermolecular interactions,
407 and aggregate into networks (Putaux, Buleon, & Chanzy, 2000). However, in retrograded
408 starch, the lamellar periodicity of the native granule does not return (Cameron & Donald,
409 1991). To test the effect of heat denaturation of LBs, we heated SmLBs at 95 °C, analyzed
410 them by WAXS, and observed that the B-type reflections were significantly reduced (Figure
411 2D). However, when the samples were incubated for 12 or 36 h at 4 °C post-heating, the
412 reflections gradually reappeared (Figure 2D). These data indicate that solubilized LBs
413 structurally behave like retrograding starch: crystallinity is reduced upon heating but
414 reappears spontaneously in a B-type arrangement after incubation at low temperature.

415

416 *3.3. Microscopic effects of mechanical, thermal and chemical treatments on LBs*

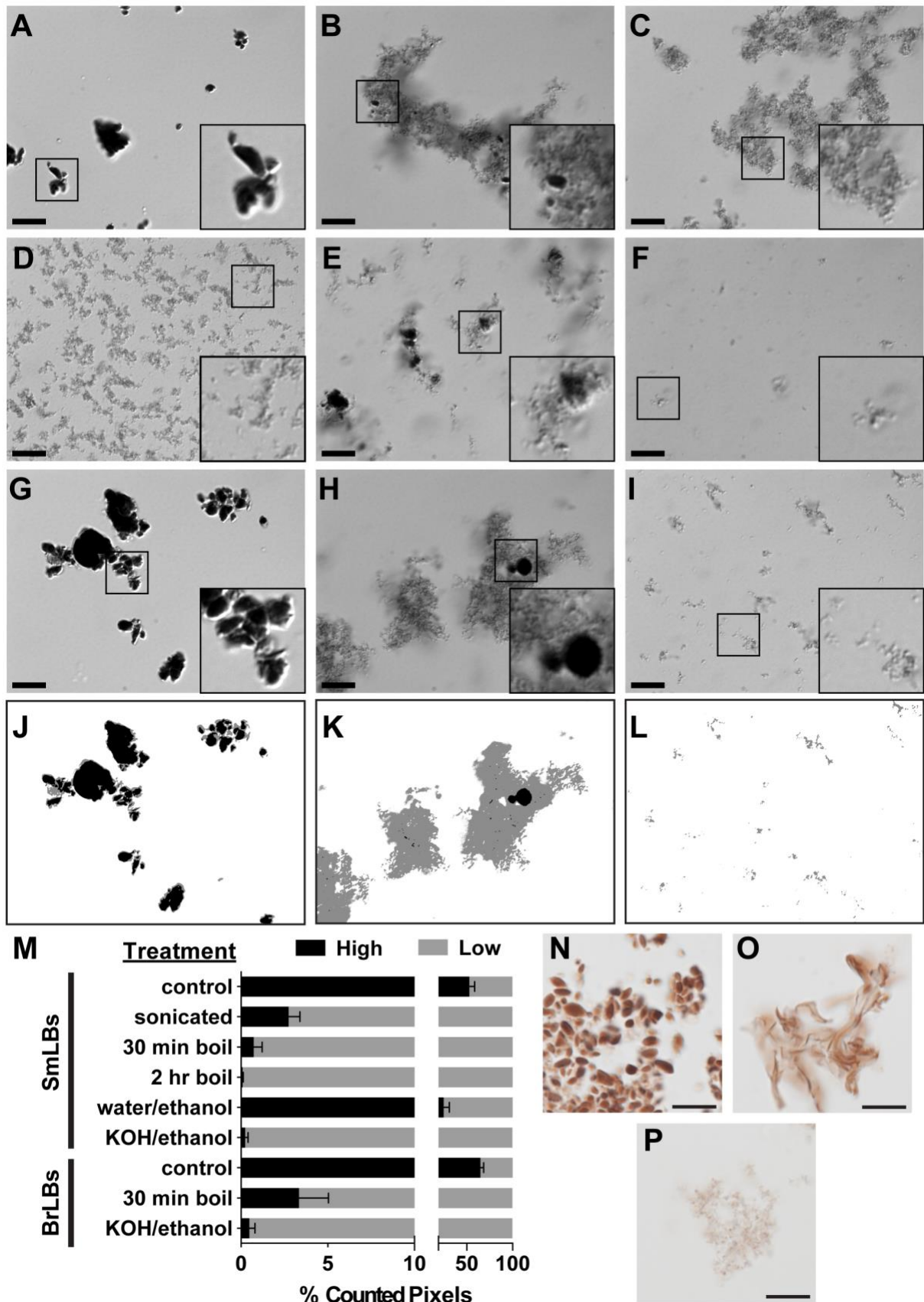
417 Thus far, the data demonstrate that LBs are comprised of B-type crystallized glucan
418 chains. As discussed in the introduction, the Pflüger method involves both thermal and
419 chemical treatment resulting in the conversion of micron-sized LBs to nanometer-sized
420 particles. Since modification of starch and other polysaccharides by mechanical, thermal,
421 chemical, or enzymatic means provides structural insights, we sought to explore the effects of
422 multiple treatments on LB structure by light microscopy and iodine staining.

423 Untreated SmLBs are uniformly small and ovoid and stain intensely with Lugol's iodine
424 (Figure 3A). We found that mild sonication (5 x 5 s at 20 kHz with 25% amplitude) disrupted

425 SmLB structure almost entirely (Figure 3B). After this treatment, most of the LBs were
426 fragmented into clumped, lace-like networks of polyglucosan material that lightly stained
427 with Lugol's. Only a few intact SmLBs were left associated with the lace-like networks
428 (Figure 3B, inset). This result is quite different from what has been observed with starch
429 granules: treatment of starch granules with ultrasonication for minutes or longer produces
430 only mild disruption of granular structure (Majzoobi, Seifzadeh, Farahnaky, & Mesbahi,
431 2015).

432 We next examined the effect of heat denaturation on LB structure. SmLBs were heated
433 in water for 30 min at 95 °C and were completely converted to lacy networks (Figure 3C).
434 The resulting material after heating appeared very similar to the material produced by
435 sonication, except that no intact SmLBs were observed after the heat treatment. The clumps
436 of material were even more dispersed with longer heating (2 h at 95 °C), and very small,
437 freely floating particles with diameters less than 1 µm were more abundant (Figure 3D).

438 Since the Pflüger method is the standard technique used to precipitate glycogen and has
439 been often employed to purify polysaccharides from LD mice (Sullivan et al., 2019;
440 Tagliabracci et al., 2008), we next analyzed the effects of this treatment on SmLB structure.
441 In this method, the tissue is boiled in 30% KOH for 2 h, the polysaccharide is precipitated
442 three times with 2 volumes of cold ethanol, and then it is re-dissolved in water. LiCl is added
443 with the ethanol to ensure complete precipitation (Tagliabracci et al., 2008). The successive
444 precipitation steps remove impurities and residual KOH, and the resulting individual particles
445 can be analyzed by TEM or SEM (Sullivan et al., 2019; Tagliabracci et al., 2008). To test the
446 contribution of the different portions of the Pflüger method on LB structure, we performed 2
447 h heat treatments in either water (i.e. hydrothermal) or 30% KOH, followed by ethanol
448 precipitation. The SmLBs that were precipitated with ethanol and LiCl three times following



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Figure 3. Effects of multiple treatments on the structure of LBs. LBs were stained with Lugol's iodine after each treatment and visualized by light microscopy. Control SmLBs (A); mildly sonicated SmLBs (B); SmLBs heated in water at 95 °C for 30 min (C); SmLBs heated

453 in water at 95 °C for 2 h (D); SmLBs heated in water at 95 °C for 2 h, then precipitated 3
454 times with 2 volumes cold ethanol and LiCl (E); SmLBs treated with the Pflüger method (2 h
455 at 95 °C in 30% KOH) (F). Control BrLBs (G); BrLBs heated in water for 30 min (H);
456 BrLBs treated with the Pflüger method (I). Thresholding was used to quantitate LBs (with
457 high intensity staining) and polyglucosan (with low intensity staining) in micrographs.
458 Examples of applied thresholds for (G) through (I) are shown in (J) through (L). Pixels with
459 high intensity (grey values 0-50) and low intensity (grey values 51-140) were counted and
460 shown as a percentage of total counted per micrograph. Pixels with grey values above 141
461 were considered background and not counted. The values shown are the averages of 9
462 micrographs \pm standard error. (N, O, P) Structural transition of SmLBs during heating.
463 Control SmLBs (N), SmLBs heated for 1 minute and flash frozen (O), and SmLBs heated for
464 5 min and flash frozen (P). After freezing, samples were thawed, stained with Lugol's iodine,
465 and visualized using a light microscope with a Zeiss 512 color camera. All scale bars = 10
466 μ m. Each inset is a 2.5x magnification of the boxed region.

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469 a 2-h boil in water were more difficult to resuspend in water: the white pellet did not easily
470 go into solution, and flakes of polysaccharide were visible. Using light microscopy, this
471 material contained intensely stained aggregates, filamentous structures and granular particles
472 (Figure 3E). The aggregates were more amorphous and less compact than control SmLBs, but
473 stained with a similar intensity, suggesting that the polyglucosan became re-crystallized with
474 precipitation. However, the LBs that underwent precipitation after a 2-h hydrothermal
475 treatment did not reform into the compact, ellipsoidal shape of native SmLBs (Figure 3F). In
476 contrast, LBs that were boiled in 30% KOH for 2 h and then precipitated three times with
477 ethanol and LiCl were very easily re-suspended in water and produced no visible precipitate.
478 This material did not contain intensely-staining aggregates with Lugol's solution and only a
479 few small clumps of polyglucosan material were visible (Figure 3F). Thus, boiling in KOH
480 instead of water fully dissociates the LBs into polyglucosan particles rather than networks,
481 and in these alkaline conditions, the polyglucosan is much less prone to re-aggregate upon
482 ethanol/LiCl precipitation.

483 Hydrothermal and Pflüger treatments were also applied to BrLBs and the products were
484 visualized via light microscopy. As expected, in the absence of treatment BrLBs were larger

485 and more irregularly shaped than SmLBs and stained with Lugol's iodine (Figure 3G). A 30-
486 min hydrothermal treatment partially converted BrLBs to polyglucosan networks, though
487 some BrLBs remained intact, surrounded by the lacy material (Figure 3H). As with the
488 SmLBs, Pflüger treatment released a large amount of small granular particles, but more lacy
489 polyglucosan was still present in these samples compared to the Pflüger-treated SmLBs
490 (Figure 3I versus 3F). These samples were also easy to re-suspend after precipitation.

491 Image thresholding was used to quantitate LBs, LB-like aggregates and the lace-like
492 polyglucosan in treated samples. In the recorded micrographs, intact LBs were very dark grey
493 or black, with grey values of 50 or less (Figure 3J and K). Polyglucosan networks or particles
494 appeared in lighter shades of grey, with values between 51 and 140 (Figure 3K and L). Pixel
495 counts from multiple micrographs showed that >95% high intensity staining (i.e. intact LBs)
496 were disrupted by sonication and heating in water for 30 min (Figure 3M). No intact LBs
497 were found in the SmLB samples that were heated in water for 2 h or the Pflüger-treated
498 samples. Interestingly, the high intensity observed in control samples (53% of counted pixels)
499 could be partially restored by ethanol treatment after heating for 2-h in water (25% of
500 counted pixels). These results demonstrate that crystalline LBs undergo a structural transition
501 to polyglucosan networks in the presence of heat, and they can be fully dissociated with the
502 Pflüger method. Without KOH, ethanol precipitation causes polyglucosan to re-aggregate and
503 form dense, LB-like structures.

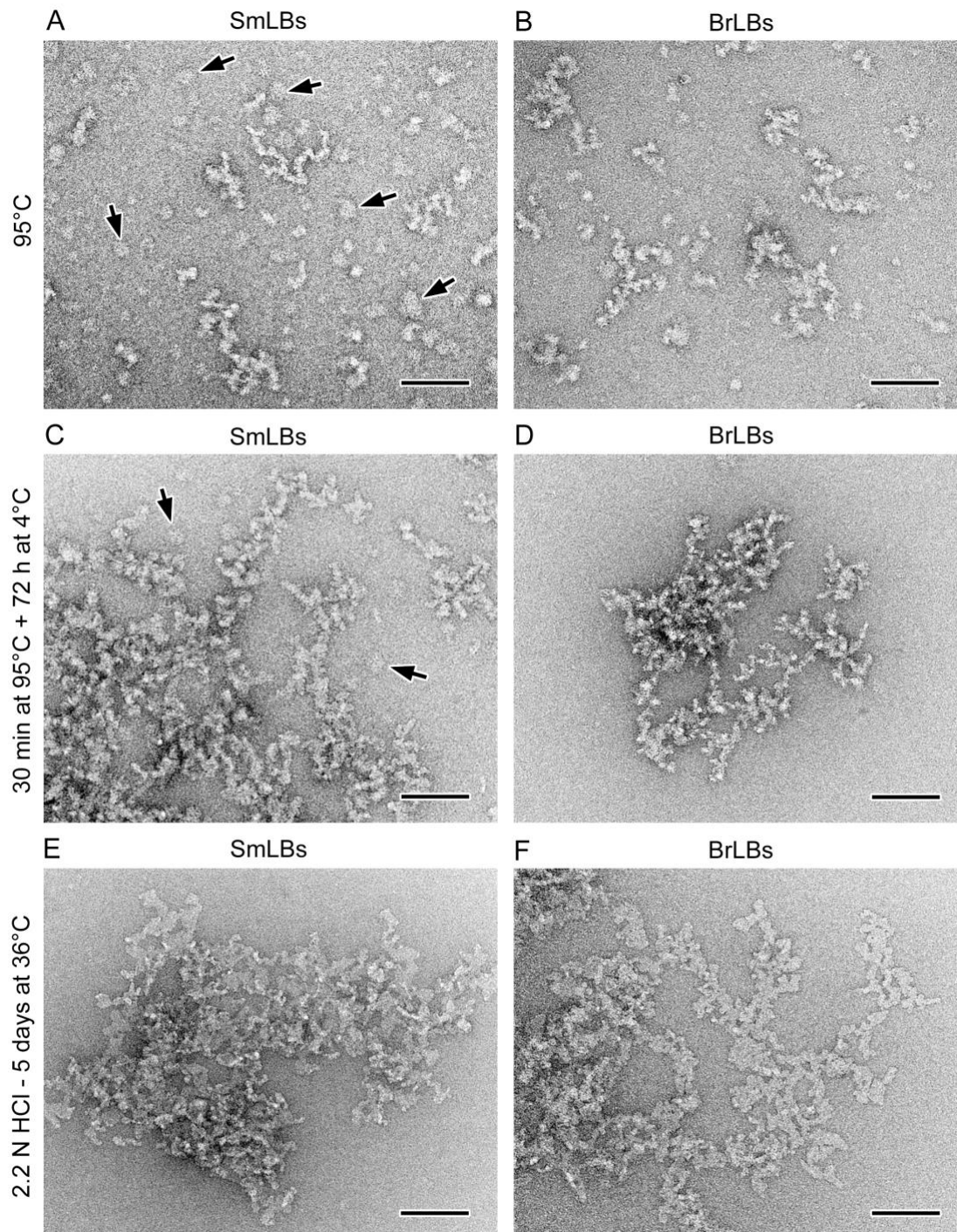
504 To visualize the transition from native LB to lacy polyglucosan networks, SmLBs were
505 heated at 95 °C for 1 or 5 min in water, immediately flash frozen in liquid nitrogen, thawed,
506 and stained with Lugol's iodine. Control, native LBs stained bright reddish-brown in Lugol's
507 iodine (Figure 3N). Samples that were heated for 1 min displayed numerous amorphous, red-
508 brown gelatinous structures in addition to small fragments of lightly stained lace-like
509 polyglucosan (Figure 3O). By 5 min, only lace-like polyglucosans were visible, and no

510 gelatinous structures were observed (Figure 3P). These results are strongly reminiscent of
511 gelatinization, the irreversible order-disorder transition that starch undergoes when heated in
512 water characterized by swelling, increased viscosity and a loss of crystallinity (Ratnayake &
513 Jackson, 2006). These results strongly suggest that like starch, LBs gelatinize at high
514 temperatures.

515

516 *3.4. TEM observation of hydrothermally-treated and lintnerized LBs*

517 Normal glycogen particles can easily be visualized using transmission electron
518 microscopy (TEM) (Revel, Napolitano, & Fawcett, 1960; Ryu et al., 2009). To study the
519 structure of disrupted LBs with higher resolution than can be achieved by the light
520 microscope, the hydrothermally-treated SmLBs were analyzed by TEM. After heating
521 samples for 30 min at 95 °C, they were allowed to cool to room temperature, stained with
522 uranyl acetate, and visualized. Two types of species were observed: i) individual spheroidal
523 particles with diameters ranging from 15 to 25 nm and appearing similar to typical glycogen
524 granules (Ryu et al., 2009) and ii) knobby worm-like objects (Figure 4A). The worm-like
525 objects had widths varying between 5 and 15 nm, bearing a strong resemblance to
526 retrograded amylopectin (Putaux et al., 2000). BrLBs were also visualized by TEM after a
527 30-min hydrothermal treatment (Figure 4B). The individual sponge-like particles were
528 slightly smaller in size (12 to 23 nm), irregular, and difficult to distinguish; the worm-like
529 objects were also present, with a similar width to those of the SmLBs. After heating, the
530 SmLB and BrLB samples were also incubated at 4 °C to determine whether the particles
531 would aggregate into networks, i.e. retrograde, in a manner similar to amylopectin. Indeed,
532 after 72 h, larger networks formed that appeared to be aggregates of the worm-like elements
533 observed after the initial solubilization at 95 °C (Figure 4C and 4D). However, individual
534 glycogen-like particles that did not aggregate in the SmLBs samples were still observed



535

536 **Figure 4.** Transmission electron microscopy (TEM) observation of LB structure. (A, B, C, D)
 537 TEM images of negatively stained specimens prepared by heating dilute aqueous SmLB (A,
 538 C) and BrLBs (B, D) suspensions at 95 °C for 30 min. The specimens were observed
 539 immediately after cooling them to room temperature (A, B) and after 3 days (72 h) at 4 °C (C,
 540 D). (E, F) Images of negatively stained specimens prepared by hydrolyzing LBs in 2.2 N HCl
 541 for 5 days at 36 °C: (E) SmLBs; (F) BrLBs. Scale bars = 100 nm.

542

543 (Figure S4). Interestingly, retrograded amylopectin takes on a very similar necklace-like
544 appearance where over time the molecules associate to form networks (Putaux et al., 2000).
545 These data are consistent with the re-formation of B-type crystallinity after a cooling period
546 post-heating (Figure 2D). Like amylopectin, polyglucosan particles of LBs can retrograde,
547 i.e. spontaneously form crystalline associations via intermolecular helical interactions to form
548 a network or necklace-like pattern.

549 Lintnerization is the process of treating starch granules with mild HCl at sub-
550 gelatinization temperatures for long periods of time (Bertoft, 2017; Jacobs et al., 1998). The
551 amorphous regions of the starch granule hydrolyze early during lintnerization, while
552 crystalline regions are left intact. This effect is due to the protection of glucose residues and
553 glycosidic linkages within the crystalline lamellae containing the double helices, which are
554 less easily penetrated by the hydrogen ions (Jacobs et al., 1998; Putaux, Molina-Boisseau,
555 Momaur, & Dufresne, 2003). The morphology and properties of the lintners, the insoluble
556 residues after hydrolysis, vary with species, amylose content, and starch allomorph
557 (Goldstein et al., 2016; Putaux et al., 2003; Wikman et al., 2014). This process allows the
558 crystalline residues of the starch to be analyzed in isolation.

559 BrLBs and SmLBs were treated with 2.2 N HCl for 5 days at 36°C, washed with water
560 and imaged by TEM after negative staining. The BrLB and SmLB lintners were very similar
561 in morphology, both appearing as lacy networks of flat elements (Figure 4E and 4F). Similar
562 lacy networks have been reported for lintnerized potato starch, particularly waxy potato
563 starch, which exhibit B-type crystallinity (Wikman et al., 2014). This result is in contrast to
564 A-type waxy maize starch or barley starch lintners that form well-defined polyhedral platelet
565 nanocrystals, reflective of A-type helix packing (Goldstein et al., 2016; Putaux et al., 2003).

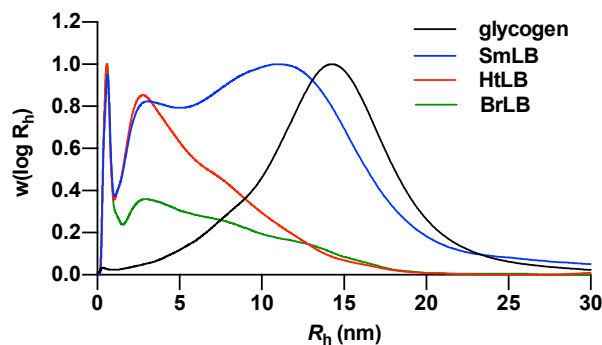
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567 *3.5 Size distribution of the polyglucosan particles in LBs*

568 When visualized by TEM, normal glycogen particles typically appear as individual
569 granules ranging from 20 to 50 nm in diameter, but can form large α rosettes in the liver (Ryu
570 et al., 2009). Heated SmLBs contained individual particles from 15 to 25 nm in diameter,
571 which appeared similar to normal glycogen (Figure 4A and 4C). However, heated SmLBs
572 and BrLBs both contained worm-like elements with a diameter of 5-15 nm after heating and
573 unambiguous B-type lintners after treatment with mild acid. Such structures are quite distinct
574 from the normal structure of glycogen and may result from an aggregation of very small or
575 very long polysaccharide particles.

576 To determine the distribution of particle sizes within the LB preparations, native SmLBs,
577 BrLBs and HtLBs were dissociated into polyglucosan particles via the Pflüger method and
578 then analyzed using size exclusion chromatography (SEC). Rat muscle glycogen was used as
579 a reference for normal glycogen particle distribution, with a single peak corresponding to
580 particles with hydrodynamic radius (R_h) ~10-20 nm (Figure 5). All LB samples displayed a
581 very narrow peak at R_h ~1 nm, likely too small to be a polyglucan (estimated to be the
582 approximately the same size as an oligosaccharide or peptide). Beyond this peak, SEC data
583 for SmLB particles were consistent with size distributions of polysaccharide from *Epm2a*^{-/-}
584 skeletal muscle as previously published (Sullivan et al., 2019): a primarily bimodal
585 distribution with the first major peak at hydrodynamic radius (R_h) ~3 nm and the second at R_h
586 ~10-15 nm. The second peak is consistent with the size of rat muscle glycogen particles and
587 previous reports of glycogen from various sources (Sullivan et al., 2019; Sullivan et al.,
588 2014). The polyglucosan particles from BrLBs and HtLBs, however, lacked the peak at R_h
589 ~10-15 nm, and contained only a major peak at R_h ~3 nm (Figure 5). These striking results
590 indicate that pure SmLBs contain a variety of particle sizes, with an enrichment of particles
591 comparable in size to glycogen and very small particles. In contrast, dissociated BrLBs and
592 HtLBs contain exclusively very small particles, which are likely to form early during

593 glycogen synthesis (Sullivan et al., 2019). The worm-like aggregates and networks that were
594 observed after heating via TEM (Figure 4A, B, C and D) are likely to be aggregates of the
595 small particles with $R_h \sim 3$ nm (i.e. roughly 6 nm in diameter), since retrogradation and re-
596 aggregation can rapidly start upon cooling the suspension (Michen et al., 2015). The absence
597 of the individual glycogen-like particles in the BrLB samples in Figure 4 is consistent with
598 the SEC data.



599

600 **Figure 5.** SEC weight distributions, $w(\log R_h)$ of KOH-dissociated polyglucosan particles
601 from BrLBs (green), HtLBs (red) and SmLBs (blue), as a function of hydrodynamic radius,
602 R_h . Rat muscle glycogen was used as a reference (black). Normalization is arbitrary with SEC
603 weight distributions and was chosen to be at the peak with an R_h of ~ 1 nm.

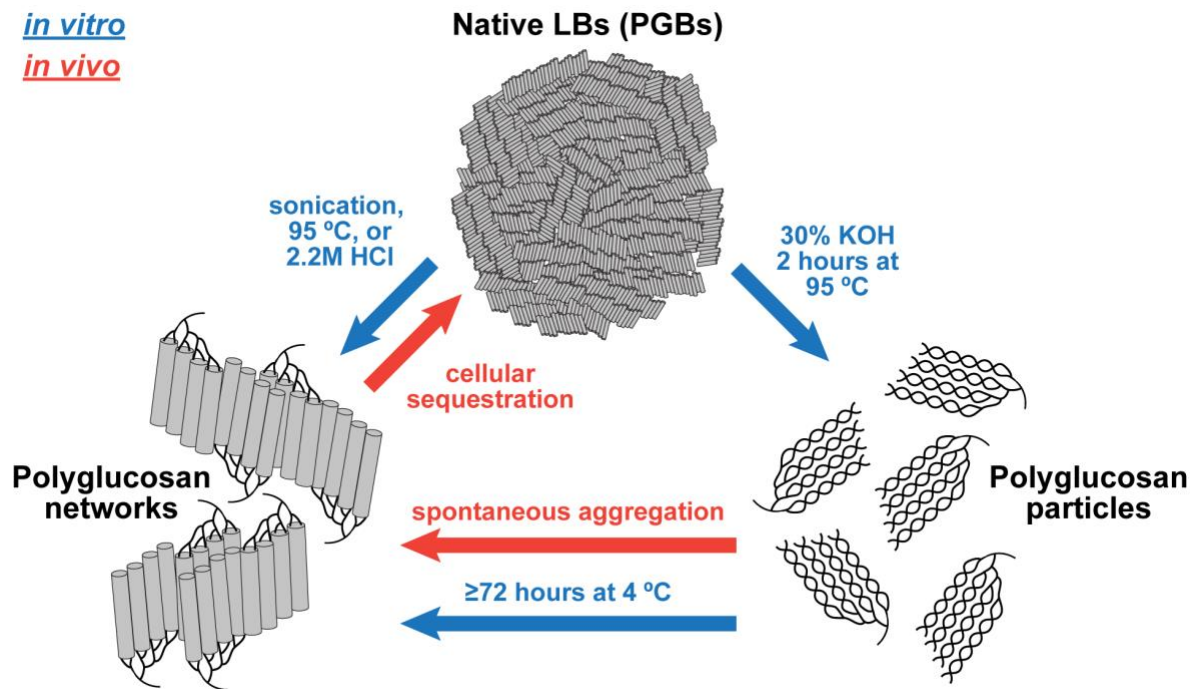
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606 4. Discussion

607 4.1. Proposed model for LB architecture and formation

608 We describe three levels of polyglucosan architecture in our study: the native LB, the
609 polyglucosan network, and the polyglucosan particle. The largest entity is the native LB,
610 typically ranging in diameter from 2-10 μm (Figure 6). In heart and brain, LBs consist only of
611 very small polyglucosan particles, while in skeletal muscle, LBs contain a mixture of
612 glycogen-like particles and very small polyglucosan particles. Minimal branching favors B-
613 type crystal packing, where double helices form a hexagonal unit with a water-filled interior
614 channel. It is possible that some double helices are formed from adjacent chains within the
615 same polyglucosan molecule (intramolecular interactions), while others may be formed from



617

618 **Figure 6.** A model for LB structure and formation, as in heart and brain. Blue arrows and text
 619 illustrate what was observed *in vitro*, and red arrows and text illustrate an *in vivo* model. LBs
 620 are primarily comprised of very small polyglucosan particles with abnormally long glucan
 621 chains that can crystallize into the B-type allomorph. LBs (i.e. intact PGBs) can be disrupted
 622 into networks by sonication, heat, or acid, and the individual particles can be completely
 623 dissociated via the Pflüger method. Aggregation of these polyglucosan particles into
 624 networks is likely to occur spontaneously *in vivo* since we observed spontaneous
 625 retrogradation *in vitro* after 72 hours at 4 °C. Since LBs have a very defined structure, active
 626 cellular sequestration is likely responsible for the distinct tissue-specific morphologies. LBs
 627 in skeletal muscle also contain glycogen-like particles (not shown).

628

629

630 chains from two different polyglucosans (intermolecular interactions). For most LB types,

631 crystalline units are randomly arranged, in stark contrast to the lamellar structure of starch

632 granules. The only exception is BrLBs where in some granules iodine did not penetrate to the

633 center. These results are consistent with previous TEM studies suggesting some LBs are

634 composed of radially arranged fibrils and a dense core (Berard-Badier et al., 1980; Cavanagh,

635 1999; Ishihara et al., 1987).

636 The second structural entity is the polyglucosan network that emerges when intact LBs
637 are heated, mechanically agitated, or lintnerized (Figure 6). These networks resemble potato
638 lintners and retrograded (i.e. re-precipitated) amylopectin, both of which exhibit B-type
639 crystallinity (Putaux et al., 2000; Wikman et al., 2014). Since amylopectin and LD
640 polysaccharide are both infrequently branched polysaccharides with long glucan chains
641 capable of crystallizing, it is not surprising that the two polysaccharides would associate in a
642 similar network formation. The networks are composed of knobby fibers between 5 and 20
643 nm in diameter. The morphology has been described as resembling a necklace, where the
644 "pearls" are laterally packed helical bundles connected by longer amylopectin chains (Putaux
645 et al., 2000). In the case of LD, it is likely that molecules associate via intermolecular helical
646 interactions to form these necklace-like fibers (Figure 6).

647 The third structural entity is the polyglucosan particle (Figure 6). This particle is
648 presumably an aberrantly branched glycogen β -particle with such long chains that it
649 crystallizes. We have shown that polyglucosan particles are best dissociated with Pflüger
650 treatment, due to heating in KOH. Indeed, alkaline treatment increases the solubility of both
651 glycogen and starch (Kerly, 1930; Wang et al., 2014), and certain forms of these
652 polysaccharides (desmoglucosan or 'fixed' glycogen and resistant starch) can only be
653 solubilized with alkali (Perera, Meda, & Tyler, 2010; Stetten, Katzen, & Stetten, 1958). This
654 phenomenon is likely simply because alkali solutions disrupt hydrogen bonding between
655 hydroxyl groups of neighboring glucosyl units (Thys et al., 2008). Once dissociated, SEC
656 distributions show an enrichment of molecules smaller than typical glycogen particles, most
657 prominently in the brain and heart (Figure 5). Dissociated polyglucosan particles from
658 skeletal muscle are comprised of small molecules of $R_h \sim 3$ nm (i.e. ~ 6 nm in diameter) and
659 also contain molecules similar in size to regular muscle glycogen with a peak R_h of ~ 10 - 15
660 nm (i.e. ~ 20 - 30 nm in diameter). These data are in agreement with previous reports while

661 providing additional insights (Sullivan et al., 2019). These results indicate that glycogen from
662 LD mice may be particularly vulnerable to precipitate early during synthesis of the glycogen
663 granule, before it reaches its normal size (~25 nm). Dissociated polyglucosan bodies from the
664 skeletal muscle, heart and brain all also contain a peak at an R_h of ~1 nm (estimated to be the
665 size of a short oligosaccharide or peptide). Whether this is small carbohydrate or protein that
666 is associated with LBs is unknown and is being further explored.

667 In starch, the crystalline, double helical conformation of glucan chains retards their
668 access by degrading enzymes (Emanuelle et al., 2016). Presumably, the long chains of
669 polyglucosan particles take on a similar conformation and prevent them from being degraded
670 by the glycogenolytic machinery (Sullivan et al., 2017). As a result, polyglucosan particles
671 accumulate, potentially associating into networks inside of cells like the ones we observed *in*
672 *vitro* (Figure 6). Since we observed that LBs undergo retrogradation (Figure 2D, 5C and 5D),
673 crystallization and network formation likely occur spontaneously, similar to the natural
674 retrogradation of amylopectin after it is cooked. It is likely that the cell, detecting an
675 unknown and potentially toxic species, sequesters the spontaneously forming polyglucosan
676 networks into non-membrane-bound inclusions that become LBs. LBs have been shown to be
677 decorated with a variety of proteins involved in glycogen metabolism, ER stress, ubiquitin,
678 and autophagy and delineated by cytoskeletal elements *in vivo* (Criado et al., 2012; Machado-
679 Salas et al., 2012). Thus, the formation of this structural entity may not be entirely
680 spontaneous; cellular mechanisms may assist in polyglucosan sequestration. We showed that
681 ethanol precipitation of LBs after boiling in water led to some polyglucosan clumps that
682 stained as intensely as native LBs. This treatment may in some way resemble the process of
683 active polyglucosan sequestration that occurs in cells. The shape and size of the LB is likely
684 dictated by the internal constraints of the cell type where it forms, but it may also be

685 influenced by the CLD of the constituent polyglucosan and/or effects of surrounding
686 molecules.

687 The lack of higher order in LBs is not surprising, given that they are an aggregate that
688 forms in response to an aberrant cellular mechanism. B-type crystallization is favored by
689 longer chains and lower temperatures (Cai & Shi, 2013; Gidley & Bulpin, 1987). Even native
690 A-type amylopectin can take on a B-type conformation after retrogradation (Qiao et al.,
691 2017). A previous study showed that the *in vitro* enzymatic chain elongation of glycogen
692 produced dendritic particles with B-type crystallinity (Putaux, Potocki-Véronese, Remaud-
693 Simeon, & Buleon, 2006). TEM micrographs suggest that over time, double helical segments
694 formed as a result of intra- and intermolecular entanglement of the extended glucan chains. It
695 has been proposed that in LD, the lack of laforin or malin leads to an imbalance in glycogen
696 synthase and branching enzyme activities, causing aberrant chain elongation (Sullivan et al.,
697 2017). Thus, the entanglement and crystallization of artificially elongated glycogen as
698 described by Putaux et al. (2006) may resemble what occurs *in vivo* in LD.

699

700 4.2. Nomenclature: polysaccharides, glycogen, polyglucosan, PGBs and LBs

701 Terminology choices have been carefully considered in this study. The term
702 'polysaccharide' denotes any polymer of sugar molecules, and thus glycogen, amylopectin,
703 amylose, and polyglucosan are all polysaccharides. The term 'polyglucan' refers only to
704 polysaccharides containing glucose units attached by glycosidic bonds, and more specifically,
705 α -polyglucans are those with α -1,4 and α -1,6 glycosidic bonds. Thus, all of the
706 polysaccharides discussed in this paper are also α -polyglucans. However, we abstained from
707 using the term 'polyglucan' due to its similarity to 'polyglucosan' and also since no other types
708 of polysaccharides are discussed.

709 The polysaccharides purified from LD mice via the Pflüger method are frequently called
710 'glycogen', and LD mice are said to have increased glycogen in their tissues. However,
711 studies of the polysaccharide content of 'insoluble' vs. 'soluble' glycogen fractions in LD
712 mice show that LD mice display an increase in the insoluble fraction, but the soluble fraction
713 (i.e. normal glycogen) is equivalent to wild-type (WT) mice (DePaoli-Roach et al., 2010;
714 Sullivan et al., 2019). The Pflüger purified polysaccharides are actually a mixture of both
715 normal glycogen and abnormal polysaccharides that we refer to herein as polyglucosan. LD
716 tissues have approximately the same amount of soluble glycogen as WT mice, and the total
717 increase in polysaccharide, is exclusively due to insoluble polyglucosan (Sullivan et al.,
718 2019; Sullivan et al., 2017). The polyglucosan of LD is a precipitation-prone polysaccharide
719 with long chains and a molecular size much smaller than the typical glycogen particle that
720 can form B-type crystalline units. This material is quite divergent from the typical definition
721 of glycogen, i.e. a soluble glucose polymer with ~13 glucose units per chain, regular
722 branching, and an average diameter of about 25 nm (i.e. 10,000 glucose units) (Brewer &
723 Gentry, 2019; Roach, 2002). Therefore, the abnormal polysaccharide should be called
724 polyglucosan rather than glycogen.

725 One should also recognize the difference between 'polyglucosan', 'polyglucosan bodies'
726 (PGBs), and 'LBs'. Polyglucosan refers to any abnormal glucose polymer containing α -
727 glycosidic linkages found in mammalian tissues, but its exact chemical characteristics vary
728 with its pathological origin. 'PGBs' refer to the compacted inclusions of polyglucosan
729 observed *in vivo*, which can be isolated as intact entities (Brewer et al., 2019; Sakai et al.,
730 1969; Yokoi et al., 1968). *Corpora amylacea* and LBs are considered PGBs, but not all PGBs
731 are exactly equivalent. 'LBs' are the PGBs found in LD, which we have shown are
732 morphologically distinct based on tissue type. Additionally, in this study, we specifically
733 used the term 'disrupt' to refer to any modification of the PGB structure, such as by

734 sonication or heating, while ‘dissociate’ referred to the complete separation of individual
735 polyglucosan particles via the Pflüger method.

736

737 *4.3. Similarities of LBs with other PGBs and amyloid and their relevance to neurological* 738 *disease*

739 PGBs of different pathologies are overall very similar but distinguishable by subtle
740 ultrastructural and histochemical qualities. LBs are the only PGB to display a darkly staining
741 central core with radiating fibrils visible by electron microscopy (Cavanagh, 1999). The
742 polyglucosan fibrils in LBs are somewhat thicker and more electron dense than those in the
743 PGBs of GSD IV, a disease caused by a deficiency in glycogen branching enzyme (GBE)
744 (Ishihara et al., 1987). With Lugol's iodine, LBs are brown, *corpora amylacea* are purplish-
745 brown, and the GSD IV PGBs are distinctly purple, reflecting their abnormally long glucan
746 chains (Reed Jr et al., 1968; Sakai et al., 1969; Sullivan et al., 2019). The PGBs of GSD IV
747 do not stain with toluidine blue, in contrast to *corpora amylacea* and LBs, suggesting they
748 have a much lower phosphate content (Reed Jr et al., 1968). Furthermore, a recent study
749 reported that polyglucosan from GBE mutant mice (a model of Adult Polyglucosan Body
750 Disease) had normal levels of phosphorylation at the 6-hydroxyls and even longer chains than
751 LD polyglucosan (Sullivan et al., 2019). According to another study, *corpora amylacea* had
752 even more phosphate than LBs (Sakai, Austin, Witmer, & Trueb, 1970). The data presented
753 in the present study on LBs are likely to be very relevant to understanding the architecture
754 and formation of other PGB types. It is likely that the aggregation of polyglucosan into
755 networks and B-type crystallization are common features of PGBs. It will be very interesting
756 to compare their CLD profiles and particle size distribution and chemical compositions to
757 understand their architectures and *in vitro* properties.

758 The different levels of polyglucosan association (PGB, network, and particle) are
759 analogous to the levels of protein aggregation in amyloid disorders. Polyglucosan particles
760 aggregate into networks much like misfolded proteins aggregate into soluble amyloid
761 oligomers. Eventually the polyglucosan compacts into PGBs, and soluble oligomers
762 polymerize into fibrils and plaques, although these higher-order structures are distinctly
763 different. For example, PGBs contain crystalline α -helices, while amyloid contains β -sheets;
764 polyglucosan fibrils are branched, but amyloid fibrils are not; PGBs are typically not
765 birefringent, in contrast to amyloid. It is possible that for both types of inclusion, the
766 intermediate aggregates are toxic, and the higher-order structures are reactionary, or even
767 protective. There is a significant amount of evidence supporting the toxicity of soluble
768 oligomers rather than amyloid fibrils (Fändrich, 2012; Glabe, 2006). Since LBs are often
769 found in apparently healthy neurons, and degenerating neurons usually lack LBs, some have
770 suggested that the sequestration of polyglucosan into LBs may be a protective mechanism in
771 neuronal cells (Ganesh et al., 2002; Machado-Salas et al., 2012). *Corpora amylacea*, which
772 appear with aging and various neurological conditions, are also considered a protective
773 response to age-related or pathological degeneration (Rohn, 2015). It has been suggested that
774 the *corpora amylacea* are a repository for hazardous cellular waste that can be cleared by the
775 immune system (Augé, Duran, Guinovart, Pelegrí, & Vilaplana, 2018). Both LBs and
776 *corpora amylacea* are decorated with ubiquitin and p62, a cargo receptor for ubiquitinated
777 proteins that has important roles in autophagy and cellular waste disposal (Augé, Duran, et
778 al., 2018; Criado et al., 2012). Misfolded protein aggregates are also rich in ubiquitin and
779 p62 (Donaldson et al., 2003). Active cellular sequestration of misfolded proteins has been
780 shown to enhance their response to stress (Escusa-Toret, Vonk, & Frydman, 2013). Also,
781 conversion of low molecular weight amyloid oligomers to high molecular weight aggregates
782 reduces their toxicity within cells (Cohen, Bieschke, Perciavalle, Kelly, & Dillin, 2006). It is

783 possible that when polyglucosan accumulates, cells activate autophagic pathways to sequester
784 the potentially toxic species into compact bodies, analogous to the sequestration of toxic
785 misfolded proteins and amyloid.

786 While PGB formation may be a cellular adaptation mechanism, data from multiple
787 diseases indicate that polyglucosan accumulation is pathogenic. Genetic ablation of glycogen
788 synthase in the brain rescues the neurological phenotype in LD mouse models, and
789 overexpression of glycogen synthase induces polyglucosan accumulation and
790 neurodegeneration in WT flies and mice (Duran, Gruart, Garcia-Rocha, Delgado-Garcia, &
791 Guinovart, 2014; Duran et al., 2012). Similarly, inhibition of glycogen synthase eliminates
792 PGBs and reverses neurotoxicity in a cell model of Adult Polyglucosan Body Disorder
793 (Kakhlon et al., 2013). Glycogen synthase ablation in WT mice also eliminates the formation
794 of *corpora amylacea* with aging, and a reduction in glycogen synthesis eliminates
795 polyglucosan deposits and increases lifespan in *Drosophila* (Sinadinos et al., 2014). Further
796 investigation will be necessary to understand how polyglucosan is sequestered as PGBs *in*
797 *vivo*, why various forms of polyglucosan are pathological, and what can be done to alleviate
798 their toxicity.

799

800 **5. Conclusions**

801 In this study, we defined the architecture of LBs, the hallmark PGBs of a fatal childhood
802 epilepsy. The hypothesis that LBs contain starch-like properties was confirmed: LBs possess
803 B-type crystallinity like starch, but they lack a lamellar arrangement and contain randomly
804 arrayed helical chains. We show that multiple treatments drastically alter the appearance of
805 LBs, breaking them into particles and/or networks. Like starch, the polyglucosan of LBs
806 undergoes retrogradation, acquiring crystallinity spontaneously over time. We propose a
807 model of LB hierarchical structure and formation based on these results, hypothesizing that *in*

808 *vivo*, aggregation into networks occurs spontaneously, while sequestration into compact LBs
809 is assisted by proteins. Future work will be required to define how LBs are sequestered, why
810 morphology differs between tissues, and whether the polyglucosan particles, networks or
811 intact PGBs are most toxic. We also present clear nomenclature for describing the various
812 levels of LB structure. These results are also relevant to understanding PGBs found in other
813 diseases and pathologies.

814

815 **Author Contributions**

816 MKB, AR and AU purified LBs, performed light microscopy experiments and analyzed data.
817 JLP performed SAXS, WAXS, and TEM experiments and analyzed data. MAS performed
818 SEC and analyzed data. MKB, JLP, MAS and MSG wrote the paper.

819

820 **Conflict of Interest Statement**

821 All authors declare no competing interests.

822

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838

839 **Supplementary Files:**

840 **Figure S1.** Additional confocal images of starch and LBs.

841 **Figure S2.** Schematic diagram of SAXS and WAXS experimental setup and theory.

842 **Figure S3.** 2D SAXS and WAXS diffraction patterns.

843 **Figure S4.** Additional TEM image of heated SmLBs.

844

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