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1 **A review of paramylon processing routes from microalga biomass to non-derivatized**
2 **and chemically modified products.**

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8 **Abstract**

9 Paramylon is a linear β -1,3-glucan, similar to curdlan, produced as intracellular granules by the
10 microalga *Euglena gracilis*, a highly versatile and robust strain, able to grow under various
11 trophic conditions, with valorization of CO₂, wastewaters, or food byproducts as nutrients. This
12 review focuses in particular on the various processing routes leading to new potential
13 paramylon based products. Due to its crystalline structure, involving triple helices stabilized by
14 internal intermolecular hydrogen bonds, paramylon is neither water-soluble nor thermoplastic.
15 The few solvents able to disrupt the triple helices, and to fully solubilize the polymer as random
16 coils, allow non derivatizing shaping into films, fibers, and even nanofibers by a specific self-
17 assembly mechanism. Chemical modification in homogeneous or heterogeneous conditions is
18 also possible. The non-selective or regioselective substitution of the hydroxyl groups of
19 glucosidic units leads to water-soluble ionic derivatives and thermoplastic paramylon esters
20 with foreseen applications ranging from health to bioplastics.

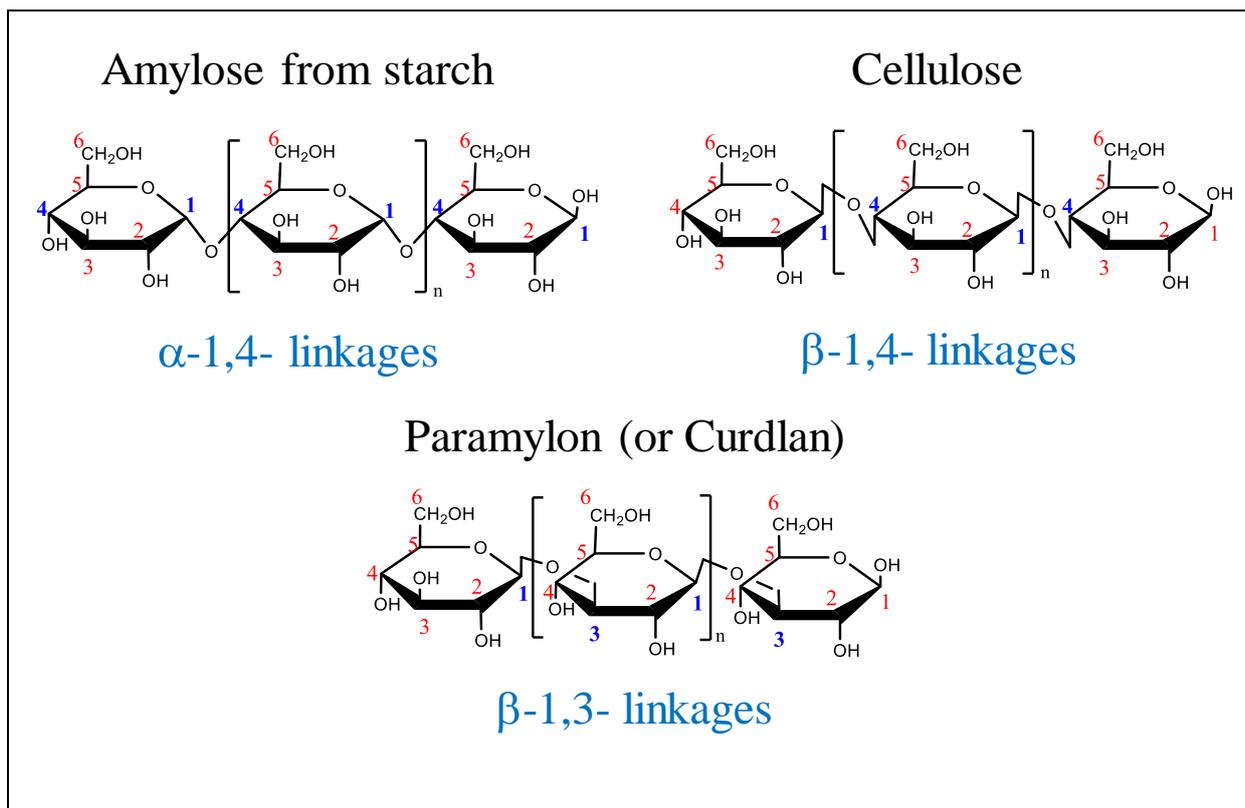
21 **Keywords:** β -1,3-glucan, crystallinity, solvents, processing, chemical modification.

22

23 **Introduction**

24 Among the natural glucans (defined in this review as polysaccharides that are homopolymers
25 of glucose) that possess a linear macromolecular structure, those involving β -1,3 linkages
26 between glycosidic repeating units are the scarcest. Contrary to cellulose, involving β -1,4
27 linkages, or amylose from starch, based on α -1,4 linkages, which are referred to by the same
28 names whatever was their botanical, bacterial, or fungal origin; linear β -1,3-glucans have been
29 given different names depending on their origin, the most common being curdlan and
30 paramylon (Stone, 2009): paramylon is a storage polysaccharide deposited as microscopic
31 granules in the cytoplasm of euglenoids, in particular the microalga *Euglena gracilis*
32 (abbreviated *E. gracilis* in the following). It was discovered in 1850 by German Professor J.
33 Gottlieb who named it. “paramylon” associates the two latin words « par » (standing by) and
34 « amylon » (starch) (Kiss et al., 1987; Barsanti et al., 2011), because besides being an isomer
35 of starch, with a similar granule form, it is not stained by iodine (Clarke & Stone, 1960). Despite
36 this early discovery, paramylon remained a laboratory curiosity for a long time.

37 In contrast, curdlan was discovered in 1966 during studies of exopolysaccharides production
38 by bacterium *Alcaligene faecalis* when a mutant was serendipitously found to produce it
39 (Harada et al., 1966). It was named after its ability to curdle, forming curd-like gels when heated
40 in water. It was rapidly used as food additive for this property, since the end of the 1980's in
41 Asia (McIntosh et al., 2005). This gel forming ability has been ascribed to a partial water
42 solubility of native curdlan (Chen & Wang, 2020). In contrast, and although being composed
43 of macromolecules with the same linear β -1,3 structure, paramylon granules extracted from
44 euglenoids are completely insoluble in water and do not have such gel forming properties.



45

Figure 1: Macromolecular structure of linear glucans

46 Curdlan is found in various bacteria as an exopolysaccharide. It can be produced by
 47 fermentation with a high yield, and is easy to recover (McIntosh et al., 2005). Therefore, it has
 48 been produced industrially since the early 1990's. Registered by the Food and Drug
 49 Administration (FDA) in 1996, it remains mainly used as stabilizer, thickener, and texturizer in
 50 food, but also in pharmaceutical products. However, its unique biological activities are drawing
 51 more and more attention, as evidenced by a recent review article (Chen & Wang, 2020).

52 As an opposition, until the last decade, the cultivation of euglenoids, and consequently the
 53 production of paramylon, remained mostly confined to research laboratories. However, the
 54 present decade may see paramylon's development at industrial scale. Facilities for edible mass
 55 outdoor cultivation of *E. gracilis* exist in Japan since 2005. With the emergence of this
 56 microalga cultivation's industry, the research on paramylon has intensified more and more: If
 57 we consider the selected papers focused on paramylon cited in the present review, only 22 were

58 published between 1960 and 2010, while 57 papers were published in the last decade (20 for
59 2010-2015, 30 in 2015-2020, and already 7 papers in 2021).

60 Regarding potential applications, there has been a primary focus on food and health sectors.
61 Paramylon was approved by the U.S. Food and Drug Administration in 2017 (*GRAS Notice for*
62 *Paramylon Isolate from Euglena Gracilis*, 2017). A battery of toxicological studies was
63 conducted on a dried preparation of *E. gracilis* biomass and pure paramylon to support their
64 safe food use (Simon et al., 2016): it was shown that they are non-immunogenic and well
65 tolerated (Del Cornò et al., 2020) when taken orally as food supplements. Generally speaking,
66 the different biological activities of paramylon, and its resulting potential applications for
67 health, are very similar to those of curdlan, which have been recently reviewed in detail (Chen
68 & Wang, 2020). Along with remarkable anti-tumor (Quesada et al., 1976) and antimicrobial
69 (Kottuparambil et al., 2019) properties, paramylon's alleged anti-inflammatory (Choi et al.,
70 2013; Russo et al., 2017) and immunostimulatory activities are the most promising (Barsanti et
71 al., 2011). Paramylon granules effectively interact with pattern recognition receptors in animal
72 and plant models (Barsanti & Gualtieri, 2019). The biopolymer was shown to promote the
73 healing of epithelial wounds *in vitro*, and to suppress the acute inflammatory reaction caused
74 by alkali burns in rats (Choi et al., 2013). Studies also showed a protecting role against acute
75 liver damage induced by carbon tetrachloride (Sugiyama et al., 2009), and an inhibiting effect
76 on the development of atopic dermatitis lesions in mice (Sugiyama et al., 2010).

77 The first available commercial products are dietary fibers based on unprocessed raw paramylon
78 granules, or even raw *E. gracilis* biomass, as immune-stimulating agent (Skov et al., 2012;
79 Kataoka et al., 2002). However, the specific role of paramylon remains unclear. A recent study
80 suggest that it is not a potent immunomodulator by itself (Phillips et al., 2019), and that other
81 molecules present in *E. gracilis* biomass and paramylon granules contribute to this bioactive
82 property (Phillips et al., 2019).

83 In the present review, our final aim is to focus on the various processing routes leading to new
84 potential paramylon based products, from nanofibers to biobased thermoplastics. However, we
85 will start by briefly considering the cultivation of *E. gracilis* for the production of paramylon.
86 Then we will take a closer look at the biopolymer's macromolecular structure, and to its
87 crystallinity which is key for processing strategies. We will discuss the different paramylon
88 solvents reported. Finally, the different processing routes, with and without chemical
89 modification of the biopolymer, will be detailed together with the corresponding potential
90 applications.

91 **1) Paramylon production by cultivation of *Euglena gracilis*.**

92 *E. gracilis* is a unicellular microalga belonging to the Euglenaceae family, or euglenoids, which
93 is pervasive in nature. It is found in swimming pools, ponds, and most freshwater, or brackish
94 biotopes. Contrary to bacteria, euglenoids do not present any pathological risk to humans. *E.*
95 *gracilis* can be safely grown in large quantities by different ways including photoautotrophic
96 (using sunlight and CO₂), heterotrophic (using an external organic carbon source) or a
97 combination of both (Rodríguez-Zavala et al., 2010; Grimm et al., 2015).

98 In general, microalgae are an important potential source of metabolites including
99 polysaccharides, proteins, lipids and pigments. In the case of *E. gracilis*, various metabolites of
100 interest such as dietary proteins, lipids, wax esters, tocopherol, carotenoids, and paramylon can
101 be produced, as highlighted in various recent review articles (Gissibl et al., 2019; Kottuparambil
102 et al., 2019). Paramylon is the unique reserve polysaccharide of the microalga. It is found in
103 proportion ranging from 50 to 80% of the cell's dry weight (Watanabe et al., 2013). Other
104 euglenoids such as *Euglena cantabrica* also contain paramylon, with similar content from 34
105 to 80% (Muñoz-Almagro et al., 2020).

106 The robustness and versatility of *E. gracilis* allow the production of paramylon within the
107 framework of environmental-friendly schemes (bioremediation, wastewater treatment, CO₂
108 trapping ...), taking advantage of the various trophic conditions depending on the available
109 resources (light, carbon ...): euglenoids can grow with a remarkably high tolerance to stressful
110 environments: They are acidophilic and can tolerate high concentrations in heavy metals.
111 Therefore, they may be used for bioremediation of contaminated waters (Krajčovič et al., 2015),
112 while producing valuable metabolites, including paramylon. The heterotrophic cultivation of *E.*
113 *gracilis* can involve nutrients recovered from wastewater (Aida et al., 2016; Rubiyatno et al.,
114 2021): For example, the hydrothermal conversion of solid sludge residues lead to ammonia and
115 phosphoric acid rich culture media allowing higher growth rates than a standard AF-6 culture
116 medium. The authors pointed out the high tolerance of *E. gracilis* to ammonia, which
117 concentration was ten times higher than in AF-6 medium (Aida et al., 2016).

118 One typical problem with heterotrophic microalgae cultivation is the noxious proliferation of
119 bacteria. However, in the case of *E. gracilis*, this can be beneficial to paramylon productivity:
120 Co-cultivation with bacteria *Pseudoalteromonas* in heterotrophic conditions (Jeon et al., 2020)
121 can increase paramylon yield by 34%. Last but not least, due to its fast growing acidophilic
122 nature, *E. gracilis* is particularly interesting for the direct conversion of CO₂ into biomass
123 (Piiparinen et al., 2018). The gas can be directly injected in the liquid cultivation media. The
124 resulting decrease of pH is beneficial to the specific growth rate (SPR) of the microalga.
125 Maximum SPR values of 0.9 per day are reported at pH 3 under continuous light, with a
126 considerable CO₂ consumption of about 2.4 g per g of biomass (Piiparinen et al., 2018).

127 This makes paramylon a potentially carbon neutral biopolymer. The storage polysaccharide is
128 produced by *E. gracilis* in autotrophic, heterotrophic or mixotrophic conditions (Krajčovič et
129 al., 2015). However, aerobic conditions are required. Anaerobic conditions lead to different
130 storage molecules such as wax esters, myristic acid being the main component (Inui et al.,

131 1983). It is indeed possible to induce the conversion of wax esters to paramylon by switching
132 form anaerobic to aerobic conditions (and vice versa). **Table 1** sums up the impact of the *E.*
133 *gracilis* cultivation conditions on the paramylon dry weight content of microalga biomass.
134 Photoautotrophic conditions lead to the lowest values: 20 % or lower depending on light
135 intensity and duration of cultivation (Grimm et al., 2015; Cook, 1963) because, as a storage
136 polysaccharide, its biosynthesis in photoautotrophic conditions only corresponds to excess
137 energy (Grimm et al., 2015). Therefore, paramylon content can be enhanced by increasing light
138 intensity (Cook, 1963): For low intensities, protein production is predominant, while for very
139 high light intensities, paramylon content becomes higher. However, such photoautotrophic
140 conditions should be used only if no organic carbon source is available, or if one wants to avoid
141 the cost of such nutrients.

142 When cheap nutrients are available, such as biomass waste (Aida et al., 2016), heterotrophic
143 conditions are generally more interesting. Indeed, as shown on **Table1**, higher paramylon
144 content can be obtained in purely heterotrophic conditions (50-80% paramylon with an organic
145 carbon source, in absence of light and CO₂). In presence of light, the paramylon content tends
146 to be lower, either for purely photo-heterotrophic conditions (with an organic carbon source, in
147 absence CO₂) (Matsuda et al., 2011), or for mixotrophic conditions (with an organic carbon
148 source and CO₂) (Grimm et al., 2015). However, this concentration greatly depends on the
149 duration of the cultivation because the accumulation of paramylon continues during the
150 stationary phase (Grimm et al., 2015). Paramylon contents of 50% after 7 days have been
151 recently reported for mixotrophic cultivation using sewage effluents as cultivation media
152 opening the perspective of transforming wastewater treatment plants into sustainable
153 paramylon production facilities (Rubiyatno et al., 2021). Note that the data in **Table 1** concerns
154 only wild type *E. gracilis* strains. Mutant strains were reported to accumulate up to 90% of
155 paramylon when grown in the dark, with an adequate carbon source (Barsanti et al., 2001). It

156 should also be noted that cultivation strategies can also be developed in order to optimize the
 157 coproduction of paramylon and other metabolites of interest: For example α -Tocopherol which
 158 requires light. In such a case, mixotrophic conditions offer a better compromise than purely
 159 heterotrophic ones (Grimm et al., 2015).

160 **Table 1:** Influence of *Euglena gracilis*'s cultivation conditions on the paramylon dry weight
 161 content of microalga biomass.

Cultivation conditions			Cultivation days	Paramylon content (%)	Ref.
Aerobic	Light (continuous)	Photo-autotrophic $1000 \mu\text{mol.m}^{-2}.\text{s}^{-1}$	4	10	(Grimm et al., 2015)
			7-10	23	
		Photo-heterotrophic $108 \mu\text{mol.m}^{-2}.\text{s}^{-1}$	3	33	(Matsuda et al., 2011)
			Mixotrophic $1000 \mu\text{mol.m}^{-2}.\text{s}^{-1}$	7	40
	10	72			
	Darkness	Heterotrophic	3	58	(Matsuda et al., 2011)
			7	75	(Grimm et al., 2015)
			10	83	(Grimm et al., 2015)
Anaerobic	Light or darkness	Photo-heterotrophic or heterotherotrophic	Wax esters (no paramylon)		(Krajčovič et al., 2015)

162

163 In early studies, authors remarked that paramylon is found in the cells as microscopic granules,
164 insoluble in water, and highly resistant to chemical or enzymatic attack, except for very specific
165 enzymes (Meeuse, 1964). Thus, rather than solubilizing the biopolymer, it can be recovered in
166 its native granular state. This requires disrupting the cells and eliminating the other metabolites
167 which are mostly lipids, pigments and proteins. Paramylon granules can then be recovered by
168 centrifugation, before being dried (Clarke & Stone, 1960) or freeze-dried (Kobayashi et al.,
169 2010). This extraction process can be applied to fresh *E. gracilis* biomass (Zhong et al., 2021)
170 or previously lyophilized biomass (Tanaka et al., 2017). However, there is no standard method.
171 The exact nature of the different steps, and the order in which they are performed, varies from
172 one author to another:

- 173 - The most common cell disruption method is mechanical. It typically involves sonication
174 in an aqueous medium where the biomass is dispersed (Tanaka et al., 2017). However,
175 enzymatic cell dissociation using trypsin has also been reported (Grimm et al., 2015)
176 though it seems rather used for quantification of paramylon content, rather than for bulk
177 extraction.
- 178 - Hydrophobic compounds (lipids and pigments) are usually removed using polar
179 solvents. Acetone or ethanol are the most widely used, but other solvents such as
180 chloroform or chloroform/ethanol mixtures have been reported (Clarke & Stone, 1960).
- 181 - Protein extraction can be done using detergents such as sodium dodecyl sulfate or
182 sodium lauryl sulfate, either at low temperature for a relatively long time (e.g. 24 hours
183 at 20-40°C) or at higher temperature with shorter times (e.g. 30 minutes at 70-100 °C)
184 (Tanaka et al., 2017; Kobayashi et al., 2010).

185 Such paramylon granules extraction methods are also used to monitor microalga cell's
186 paramylon content during cultivation (Šantek et al., 2012; Grimm et al., 2015). However, as
187 they require several steps and hours, more sophisticated techniques have been developed: Real-

188 time direct quantification of the intracellular components at the single cell resolution has been
189 reported (Wakisaka et al., 2016). However, a potentially cheaper and less-than-one-hour-long
190 alternative consists in disrupting the cells, and using specific fluorescent DNA aptamers having
191 a binding affinity with paramylon granules (Kim et al., 2020) for their quantification.

192 **2) Macromolecular and crystalline structure of paramylon**

193 In this section, we will see how paramylon is structured at different scales starting from the
194 macromolecular structure of the biopolymer up to the granules. The various differences with
195 curdlan will be discussed.

196 The macromolecular structure of paramylon is generally presented as perfectly linear, with only
197 β -1,3 linkages between glucosidic repeating units (Stone, 2009). However, according to a recent
198 report of the US Food and Drug Administration (*GRAS Notice for Paramylon Isolate from*
199 *Euglena Gracilis*, 2017), a detailed analysis shows that the β -1,3 linkages content is closer to
200 94%. The other linkages identified are mostly β -1,4 and β -2,3 linkages, each in the 2 to 3 %
201 range. Therefore, small “defects” are present in paramylon’s linear macromolecular
202 architecture. The same applies for curdlan, but with different “defects” since it is known to
203 contain few β -1,6 linkages (McIntosh et al., 2005).

204 The size of curdlan and paramylon macromolecules are also significantly different. The
205 reported average molecular weight for paramylon is typically between 100 and 500 kDa
206 (Koizumi et al., 1993; Barsanti et al., 2011), which corresponds to a number of glucose units
207 between 600 and 3000. This value is about 4 times lower than for curdlan, for which molecular
208 weights of 2000 kDa (\approx 12000 glucose units) have been measured by viscosimetric
209 measurements (Futatsuyama et al., 1999). More recent measurements by size-exclusion
210 chromatography with multi-angle laser-light scattering method confirmed the ratio of
211 approximately 4 between the average molecular weights of paramylon and curdlan (Tamura et

212 al., 2009). However, number and weight average number of glucosidic units obtained by this
213 technique were about half of those obtained by viscosimetric measurements (Tamura et al.,
214 2009).

215 Last but not least, paramylon strongly differs from curdlan in terms of native crystallinity. A
216 crystallinity index of approximately 88% was estimated for paramylon granules from density
217 measurements, compared to only 30% for native curdlan (Marchessault & Deslandes, 1979).
218 Moreover, the native crystalline structures of the two biopolymers are fundamentally different,
219 despite they both involve a parallel arrangement of the chains, that take different helical
220 conformations: Native paramylon only involves highly stable and water insoluble triple helices
221 conformers, while native curdlan microfibrils also involve less stable, and water-soluble, single
222 helices conformers (Chen & Wang, 2020). This explains why, unlike paramylon, curdlan is
223 partially water-soluble, the later rearrangements of the chains and microfibrils leading to
224 gelation (Chen & Wang, 2020).

225 The native crystalline arrangement of the helices is also different: In native curdlan, the simple
226 helices are arranged in orthorhombic crystalline cells (Okuyama et al., 1991), while in
227 paramylon the triple helices are arranged in hexagonal crystalline cells (Marchessault &
228 Deslandes, 1979; Chuah et al., 1983). It has to be noted that this difference was not clear until
229 the early 1990's (Okuyama et al., 1991) because the X-Ray Diffraction early studies did not
230 involve native curdlan, but recrystallized samples. Indeed, such recrystallized curdlan adopts
231 the same triple helices/hexagonal structure as paramylon (Marchessault & Deslandes, 1979;
232 Marchessault et al., 1977).

233 **Figure 2**, drawn after (Marchessault & Deslandes, 1979) and (Chuah et al., 1983), gives a closer
234 look at this multi scale crystalline structure of paramylon:

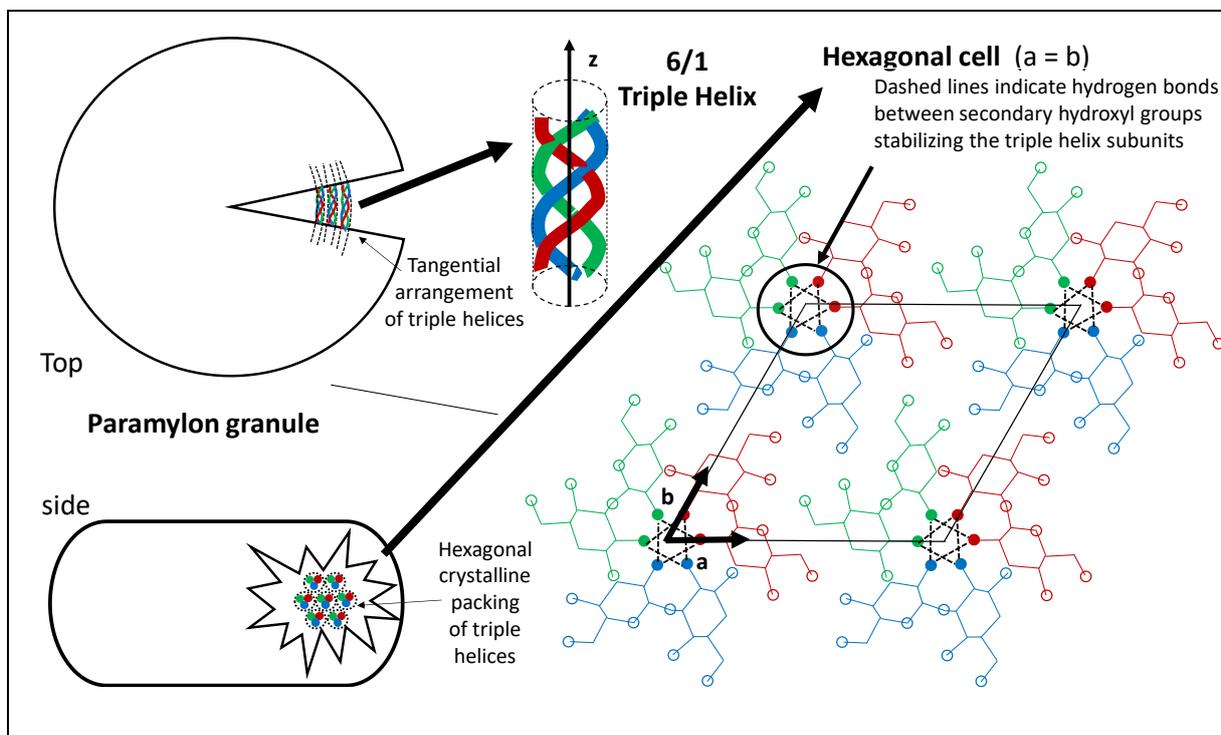
235 - At the lower scale, groups of 3 chains are assembled forming a triple helix (6/1 right
236 handed (Chuah et al., 1983) with an advance of approximately 2.9 Angstrom per

237 monomer unit (Marchessault et al., 1977).) These triple helix structural units are very
238 stable, mostly due to internal hydrogen bonds that involve only the C2 secondary
239 hydroxyl groups of each glucosidic unit (Chuah et al., 1983). These bonds are assumed
240 to form a hexagonal shape perpendicular to the helix axis (as shown with dotted line on
241 **figure 2**). However, according to an alternative model, other hydrogen bonds not
242 perpendicular to the axis may involve secondary C2 OH groups in different x,y planes
243 (Miyoshi et al., 2004). This alternative model is theoretically more stable for β -1,3-
244 glucans containing more than ten glucosidic units (Miyoshi et al., 2004).

- 245 - Parallel triple helices then form the hydrogen bonded hexagonal crystalline structure
246 (Deslandes et al., 1980). The dimensions of the hexagonal unit cell depend on moisture
247 content. Only paramylon occurs in the (almost fully) dry high density form, while water
248 molecules are always present in the unit cell for recrystallized curdlan (Marchessault &
249 Deslandes, 1979; Chuah et al., 1983).

250 The transition between dry and hydrated polymorphs of paramylon is reversible with a large
251 hysteresis: upon drying, the transition from hydrated to dry polymorph takes place around 30%
252 relative humidity, and around 70% relative humidity the other way round (Kobayashi et al.,
253 2010). One molecule of water per glucopyranoside residue is present in the hydrated form for
254 which the in-plane dimensions are $a = b = 15.56 \text{ \AA}$, while $c = 18.78 \text{ \AA}$ is equal to the repeating
255 length of the triple helices (Deslandes et al., 1980). Upon drying, the dimensions of the cell are
256 shrunk to $a = b = 14.41 \text{ \AA}$ and $c = 5.87 \text{ \AA}$ (Chuah et al., 1983), so that this later corresponds to
257 1/3 of the repeating length of the triple helices. Such a transition to the dry polymorph can also
258 occur upon heating due to water evaporation, therefore impeding any possibility for the melting
259 of the crystalline structure, without thermal degradation of the biopolymer, as shown by recent
260 hot compression molding experiments on paramylon and curdlan (Kawahara et al., 2020).

261 The paramylon granules can have different shapes depending on the euglenoid strain. At least
 262 six different morphological variations have been reported, from disks to rods (Monfils et al.,
 263 2011). For *E. gracilis*, the shape is typically flat cylindrical with reported diameters around
 264 $5\mu\text{m}$ and thicknesses about $3\mu\text{m}$ (Anraku et al., 2020). The granules are actually surrounded
 265 by a biomembrane. Inside these paramylon native granules, schematically represented on
 266 **Figure 2**, the triple helices are arranged tangentially, while curdlan have a more spherulite-like
 267 microstructure (Marchessault & Deslandes, 1979). It is assumed that paramylon is first
 268 biosynthesized as elementary microfibrils with low crystallinity (Kiss et al., 1987, 1988): as the
 269 granule matures, the microfibrils assemble to form the final highly ordered crystalline structure
 270 with concentric deposition of aggregates of elemental nanofibers of 4-10 nm. Recently,
 271 morphometric characterizations by focused ion beam/scanning electron microscopy (FIB/SEM)
 272 tomography (Anraku et al., 2020) confirmed that the paramylon particles have a very dense
 273 structure without large void spaces. A specific surface area (SSA) of $2.438\text{ m}^2/\text{g}$ was measured
 274 by BET which is close to starch granules from potatoes and corn.



275 **Figure 2:** Multiscale structure of native paramylon.

276 Summing up, paramylon is similar to curdlan but presents structural differences at different
277 scales, from the “defects” present at the macromolecular structure level of the chains, to the
278 native crystalline arrangement. However, the key difference is probably the presence of water-
279 soluble single-helices in curdlan, explaining its gel forming properties, while paramylon is
280 completely water insoluble. Therefore, the processing of paramylon into materials requires the
281 use of specific solvents and/or chemical modifications.

282 **3) Solvents of paramylon**

283 Like cellulose or starch, paramylon is water insoluble and has very few known solvents. This
284 limited solubility can be explained not only by the stability of the crystalline structure which is
285 extensively hydrogen bonded (Deslandes et al., 1980) but also, and mostly, by the stability of
286 the individual triple helices, due to the intermolecular hydrogen bonds between C2 secondary
287 OH groups (Chuah et al., 1983) (**Figure 2**). Indeed, some of the oldest known and most
288 frequently used solvents of paramylon are alkaline aqueous solutions (NaOH (Clarke & Stone,
289 1960; Manners et al., 1966) or KOH (Vogel & Barber, 1968)). They can dissolve the
290 biopolymer either “completely” in the form of random coils, or “partially” as individual triple
291 helices, depending on the alkaline concentration: In dilute alkaline solutions, paramylon
292 granules are disrupted into elemental 4 nm microfibrils (Kiss et al., 1988). Triple helices disrupt
293 for NaOH concentrations higher than 0.22 M. This transition can be detected by adding Congo
294 Red to the solution since the dye forms a complex with the triple helices (Shibakami et al.,
295 2013a). Note that such a triple helix to random coil transition at extreme pH is reversible upon
296 lowering alkaline concentration. For paramylon the reverse random coil to triple helices
297 transition takes place for NaOH concentrations around 0.17–0.20 M allowing the formation of
298 nanofibers by self-assembly (Shibakami et al., 2013a).

299 Concentrated NaOH solutions are also used as a medium for homogeneous chemical
300 modification of paramylon (Daglio et al., 2019; Shibakami et al., 2016) and for purification of

301 paramylon by solubilization and precipitation by addition of ethanol (Russo et al., 2017).
302 Recently, aqueous solutions of curdlan in various states from triple helices to random coils and
303 intermediate state were obtained by first solubilizing the biopolymer in NaOH solutions
304 followed by neutralization with HCl solutions of the same concentration (Yan et al., 2020). This
305 approach can also be applied to paramylon. Indeed, a Japanese patent application from 2011
306 (Arashida et al., 2011) describes the preparation of “amorphous“ paramylon (actually partially
307 recrystallized as single helices) by a similar solubilization/neutralization process. This process
308 leads to a low apparent density paramylon powder ($0.1\text{g}\cdot\text{cm}^{-3}$) with significantly increased
309 water and oil absorption performances compared to raw paramylon granules, improving
310 potential benefits as dietary fibers (Arashida et al., 2011).

311 Other reported solvents of paramylon working at room temperature are listed in **Table 2**.
312 Formaldehyde and concentrated acids, such as H_2SO_4 (55%) and anhydrous formic acid, were
313 reported as solvents in early studies (Clarke & Stone, 1960). Formic acid was later used for
314 production of films by evaporation casting despite it has the drawback of depolymerising
315 paramylon by acid hydrolysis (Clarke & Stone, 1960; Kawahara & Koganemaru, 2006).
316 Moreover, for acidic solvents there seem to be no evidence in literature that the triple helices
317 are disrupted.

318 Some solvents able to destabilize hydrogen bonds allow disrupting the triple helices structure,
319 in particular dimethylsulfoxide (DMSO): It has been used for producing oriented curdlan fibers
320 for XRD studies by dissolution and coagulation in methanol (Chuah et al., 1983) and to obtain
321 paramylon single helices by solubilisation followed by lyophilisation of the DMSO solution
322 (Aketagawa et al., 1993). However, DMSO is essentially used for viscosimetric measurements,
323 for which it is preferred to NaOH solutions. For this latter, a study with curdlan samples of
324 various molecular weights (including small molecular weights comparable to that of
325 paramylon) showed that the viscosity strongly depends on the alkaline concentration, and the

326 resulting state of the macromolecules (random coils or triple helices) (Futatsuyama et al., 1999).
327 As an opposition, macromolecules are always in the form of random coils in DMSO
328 (Futatsuyama et al., 1999), resulting in viscosities that depend only on curdlan concentration,
329 molecular weight, and temperature. Therefore, the Mark-Houwink parameters have been
330 determined for curdlan in DMSO at 25°C (Futatsuyama et al., 1999) allowing the determination
331 of the average molecular weight by viscosimetry. This method has also been applied to
332 paramylon molecular weight evaluation (Kawahara & Koganemaru, 2006). Note that mixtures
333 of water with cadoxen (cadmium and ethylenediamine based solvent) have been also reported
334 as efficient solvents of curdlan (Futatsuyama et al., 1999) for viscosimetric measurements and
335 so may be used also for paramylon. However the authors recommend to use DMSO
336 (Futatsuyama et al., 1999).”

337 More precise measurements can be made using SEC/MALLS technique using a mixture of 1%
338 LiCl in 1,3-Dimethyl-2-imidazolidinone (DMI) as eluent (Tamura et al., 2009). DMI/LiCl is
339 indeed an efficient solvent of both curdlan and paramylon, but requires heating at 100°C.
340 Dimethylformamide (DMF)/ LiCl has also been recently reported as a solvent for paramylon
341 homogeneous modification at 70°C (Seok et al., 2021)

342 The other reported hydrogen bonding solvents, listed in **Table 2**, also require heating in order
343 to completely dissolve paramylon. N,N-Dimethylacetamide with a small amount of lithium
344 chloride (typically DMAc/ LiCl 8g.l⁻¹) has been used as an efficient media for homogeneous
345 chemical modification (Shibakami, 2017; Shibakami et al., 2014). Recently, a mixture of
346 DMAc with and ionic liquid (1-butyl-3-methylimidazolium chloride) with a
347 [Bmim][Cl]/DMAc volume ratio of 5:2 was used as a solvent of paramylon (Yasuda et al.,
348 2018). No details are given about the state of the paramylon molecules in the solution. However,
349 recent works on the solubility of curdlan in another imidazolium ionic liquid (1-ethyl-3-
350 methylimidazolium acetate ([Emim][OAc])) suggest complete solubility as random coils (Bai

351 et al., 2019). Therefore, ionic liquids may be a new family of solvent for paramylon.
352 Nevertheless, depolymerization may be an issue: significant molecular mass decrease was
353 observed for curdlan after 2 hours at 80°C in [Emim][OAc] (Suzuki et al., 2021).

354 Summing up, due to its crystalline structure and its triple helix conformation (stabilized by
355 interchain hydrogen bonds involving the C4/C6 –OH groups, and the C2-OH groups,
356 respectively), paramylon is only soluble at extreme pH (acidic or basic) or in solvents able to
357 form strong hydrogen bonds. Moreover, its solubility can be either complete, with individual
358 paramylon macromolecules taking a random coil conformation, or partial with remaining triple
359 helices, depending on the ability of the solvent to completely or partially disrupt the different
360 interchain hydrogen bonds.

361

Table 2: Solvents of paramylon reported in literature; with their respective known drawbacks and applications.

Solvent	State of macromolecules	Known drawback	Main applications	References
Formaldehyde	unknown	toxicity	none	(Clarke & Stone, 1960)
DMSO	random coil	none	viscosimetry	(Futatsuyama et al., 1999) (Kawahara & Koganemaru, 2006)
			recrystallization of paramylon monohelices	(Aketagawa et al., 1993).
KOH	unknown	unknown	none	(Vogel & Barber, 1968)
NaOH < 0.22 M	triple helix	incomplete solubilization	extraction	(Clarke & Stone, 1960; Manners et al., 1966)
			fractionation into microfibrills	(Kiss et al., 1988)
			Self-assembly	(Shibakami et al., 2013a)
NaOH > 0.22 M	random coil	None	purification,	(Russo et al., 2017)
			“amorphisation”	(Arashida et al., 2011)
			chemical modification	(Daglio et al., 2019; Shibakami et al., 2016)

H₂SO₄ (55%)	unknown	depolymerization	none	(Clarke & Stone, 1960)
Formic acid (90%)	unknown	depolymerization	solvent casting	(Kawahara & Koganemaru, 2006)
DMAc / LiCl	random coil	temperature ($\approx 100^{\circ}\text{C}$)	chemical modification	(Shibakami et al., 2014)
BMIMCl/ DMAc	unknown	temperature (60-70°C)	film regeneration	(Yasuda et al., 2018)
BMI/LiCl	random coil	temperature ($\approx 100^{\circ}\text{C}$)	chromatography	(Tamura et al., 2009)
DMF/LiCl	random coil	temperature ($\approx 70^{\circ}\text{C}$)	chemical modification	(Seok et al., 2021)

363

4) Processing of paramylon without chemical modification

The preservation of paramylon's unique β -(1,3) linear macromolecular structure allows targeting applications as materials that take advantage of two intrinsic properties: the biological activity, and the mechanical properties induced by its triple helix based crystallinity. According to some authors, the synergistic optimization of these properties may however be difficult to achieve since biological activity tends to be enhanced by an amorphous structure, while mechanical reinforcement requires a high crystallinity (Kawahara, 2014).

Almost all the processes described in the scientific and patent literature involve at least two steps: First, native paramylon granules (or previously purified paramylon powder) is solubilized. Then in a second step, paramylon is regenerated in the targeted shape (powder, fibers, films) by elimination of the solvent, either by evaporation when it is volatile or by washing with a non-solvent of paramylon. However, in a recent study solvent free processing of paramylon and curdlan was intended: Both polymers were hot pressed into films at temperatures around 200°C, with optimal conditions around 220 and 230°C for paramylon and curdlan, respectively (Kawahara et al., 2020). However, the XRD data suggest that the crystalline structure never completely melts. The persistence of some peaks and the appearance of new ones at high temperatures rather suggest rearrangements, which differ between curdlan and paramylon samples. In addition, significant degradation and subsequent uncontrolled crosslinking reaction occur. However, this hot press process leads to materials with mechanical properties comparable to those of Polyamide 12 in terms of flexural modulus and strength, and to those of epoxy resins in terms of elongation at break (Kawahara et al., 2020).

In 2006, Kawahara & Koganemaru (Kawahara & Koganemaru, 2006) were the first to show the feasibility of film casting from paramylon solutions in 90% formic acid. Transparent/yellow films were obtained by slow evaporation of the solvent. X-ray diffraction and tensile tests indicated that the films were mostly amorphous with glassy behaviour (Young's modulus \approx 1

389 GPa and elongation at break < 15%). Partial recrystallization was observed by annealing under
390 water steam, leading to higher stiffness and lower elongation. A decrease of films mechanical
391 properties was observed for very long residence time in formic acid solution (> 72 hours) before
392 film casting. It was ascribed to depolymerisation hydrolysis, leading to paramylon oligomers,
393 as confirmed by viscosimetric measurements (Kawahara & Koganemaru, 2006). The same
394 authors tried, without success, to produce paramylon fibers by wet spinning using a solution of
395 Na₂SO₄ as coagulation bath. Blending with polyvinyl acetate (PVA) (initially co solubilized in
396 formic acid) improved the spinnability, but resulted in low mechanical properties due to
397 incompatibility of the PVA/paramylon mixture (Kawahara & Koganemaru, 2006).

398 Formic acid was also used as a co-solvent of paramylon and silk fibroin for the preparation of
399 blend films (Arthe et al., 2020). Despite, the high hydrophobicity of the protein, the blends
400 obtained seemed to be compatible, according to AFM and SEM observations showing no
401 heterogeneities at the micrometre scale. Infrared spectroscopy did not show specific interactions
402 between the biopolymers. However, a synergy was observed in terms of mechanical properties:
403 for a 75:25 protein:paramylon ratio, the stiffness of the blend films was twice higher than that
404 of the pure biopolymers films (Arthe et al., 2020). The authors showed that the blends present
405 a combination of properties (low water absorption/swelling capacity, blood compatibility and
406 non-toxicity) suitable for wound healing applications. This kind of potential application was
407 also reported for pure paramylon 20- μ m thick films produced using a [Bmim][Cl]/DMAc
408 mixture as a solvent (Yasuda et al., 2018). Due to the non-volatility of the ionic liquid, the
409 regeneration of paramylon films required washing with a non-solvent (methanol) and drying.
410 Unfortunately, no characterization of the films' structure and mechanical properties are
411 provided. Only their biological activity is investigated, showing wound healing properties by
412 inhibiting inflammatory aggression, suggesting potential wound dressing applications (Yasuda
413 et al., 2018).

414 Regarding the shaping of fibers, it can be first recalled that curdlan was shaped as oriented
415 fibers for XRD studies, by solubilisation in DMSO and coagulation in methanol (Chuah et al.,
416 1983). More recently, curdlan was shaped into fibers by wet spinning from solutions in 1-ethyl-
417 3-methylimidazolium ionic liquid [Emim][OAc] (Suzuki et al., 2021). The authors reported a
418 solubility of curdlan up to 9 wt.% in the ionic liquid at 80°C. The dope was extruded in air and
419 spin drawn in two different coagulating baths (water or ethanol). For optimized spinning
420 conditions, the semi-crystalline transparent fibers obtained combined relatively high Young's
421 modulus (≈ 2 GPa) and high elongation at break (up to 40%). A similar processing route may
422 be applied for the production of paramylon fibers. However, the authors reported a significant
423 decrease of the molecular mass of curdlan, which was almost divided by two. This suggest a
424 depolymerisation of the biopolymer while in solution in the ionic liquid. In the case of
425 paramylon which already has much lower molecular masses than curdlan, this may be an issue.

426 Paramylon nanofibers can be obtained by two methods : electrospinning (Kawahara, 2014) and
427 self-assembly (Shibakami et al., 2013a): Fully amorphous paramylon nanofibers were obtained
428 by electrospinning of paramylon solutions in formic acid. In this work the author takes
429 advantage of the unavoidable hydrolysis kinetics of paramylon by formic acid to optimize the
430 viscosity of the solution so that the appropriate range of Berry number (i.e. intrinsic viscosity \times
431 concentration) for electrospinning is matched. The nanofibers obtained had diameters ranging
432 from 50 to 500 nm (average value 192 ± 135 nm). Being fully amorphous, they were too brittle
433 for mechanical reinforcement. However, the author proposed to use them for the production of
434 biologically active amorphous micropowder by grinding (Kawahara, 2014).

435 A different approach for the production of crystalline nanofibers is a bottom up approach taking
436 advantage of the intrinsic self-assembly behaviour of β -1,3-glucan chains (Shibakami et al.,
437 2013a) . This ability had been previously described for other β -1,3-glucans with pendant chains
438 such as schizophyllan (Sletmoen & Stokke, 2008), but its application to nanofibers production

439 is only possible with paramylon chains, which do not present branching. Indeed, the control of
440 alkaline solvent NaOH concentration allows triggering the transition of paramylon solubilized
441 macromolecules from individual random coil, at high concentration, to triple helix rods, when
442 the NaOH concentration is decreased. Thus for linear chains, large nanofibers can be obtained
443 apparently consisting of more than 100 triple helices (Shibakami et al., 2013a). It is striking
444 that these large nanofibers seem to be constituted of smaller 4 nm nanofibers which is the size
445 of elemental nanofibrils reported when paramylon granules are disrupted in dilute alkaline
446 solutions (Kiss et al., 1988). Moreover, it has to be noted that, as shown on **Figure 2**, the
447 formation of triple helix and its stabilisation by internal hydrogen bonds involves only the C2
448 secondary hydroxyl groups of each glucosidic unit (Chuah et al., 1983). Consequently, the
449 remaining hydroxyl groups, which are external to the helices, can be chemically modified
450 without losing the self-assembly properties (Shibakami et al., 2013b). Unmodified paramylon
451 nanofibers prepared by such a self-assembly approach showed an anti-fibrotic effect on liver
452 damage induced by carbon tetrachloride (Kusmic et al., 2018). These 10 nm thick nanofibers
453 were efficient at a dosage about 20 times lower than in previous studies involving paramylon
454 granules (Sugiyama et al., 2009). Nanofibers also effectively interact with pattern recognition
455 receptors in animal and plant models (Barsanti & Gualtieri, 2019).

456 The above literature results demonstrate that it is possible to use solvent based processes in
457 order to transform native paramylon granules into different shapes (powder, films, fibers and
458 nanofibers) and blends with other polymers, while preserving its native chemical structure (only
459 the molecular weight is decreased in some cases). This solvent based processing also allows
460 modifying the physical structure of paramylon chains, which can become partially or
461 completely amorphous. However, when mechanical properties were characterized, amorphous
462 structures tend to result in brittleness, both for nanofibers and films. In addition, the available
463 data does not allow evidencing a relationship between an amorphous structure and enhanced

464 bioactive properties. Indeed, for nanofibers (the only shape for which both fully amorphous and
465 highly crystalline structures are reported), the bioactive properties of electrospun amorphous
466 nanofibers were not characterized, while crystalline self-assembled nanofibers showed
467 improved bioactivity compared to native paramylon granules.

468 Concurrently, solvent free processing trials by hot press up to 230°C showed that paramylon is
469 not thermoplastic. Indeed, despite semi-crystalline films with mechanical properties
470 comparable to engineering polymers could be obtained by this process, this was not possible
471 without simultaneous thermal degradation and uncontrolled crosslinking. The absence of
472 melting of paramylon's crystalline structure below its degradation temperature may be related
473 to the shrinkage of the hexagonal cell upon drying, which is likely to make it more stable upon
474 heating, thus preventing melting.

475 **5) Chemical modifications of paramylon**

476 Each sugar residue of β -1,3-glucan chains presents three hydroxyl groups that can be used as
477 modification sites located the C2, C4 and C6 carbons (**Figure 1**). The primary C6 -OH group
478 is more reactive (Chen & Wang, 2020). The various chemical modifications reported in
479 literature are either non-selective or regioselective. They are conducted either in homogeneous
480 conditions, using some of the solvents listed in **Table 2**, or in heterogeneous conditions after
481 dispersion of paramylon in non-solvents. The two main kinds of modified paramylons described
482 in literature are ionic derivatives (paramylon sulfates, salts of paramylon ethers and one salt of
483 paramylon acid) (**Table 3** and **Table 4**), and different types of paramylon esters and mixed
484 esters (including some bearing an ionic group) (**Table 4** and **Table 5**).

485 However, before focusing on these two main categories, we have to mention a recent alternative
486 approach, in which crosslinked hydrogels were obtained by reacting paramylon and curdlan
487 with ethylene glycol diglycidyl ether (EGDGE) (Matsumoto et al., 2021). The reaction between

488 the epoxy functions of the crosslinker and the hydroxyl groups of the biopolymers was
489 performed in a 1M NaOH aqueous solution at room temperature during 48 hours. After
490 neutralization with acetic acid and washing with water, self-standing gels were obtained. The
491 authors tested different initial paramylon concentrations and varied the polysaccharide:EGDGE
492 ratio. Though the crosslinking efficiency was rather low, with a maximum of 43% of the initial
493 EGDGE content present in the hydrogels, the hydrogels showed high deformability in
494 compression, up to 94% strain at break, and remarkable shape recovery when deformed at 80%
495 strain. However, the shape recovery for hydrogels obtained by crosslinking of paramylon was
496 lower than that of curdlan based hydrogels. In addition, a higher initial concentration of
497 polysaccharide is required in the case of paramylon. These results were ascribed to the higher
498 molecular weight of curdlan, leading to entanglements even at low concentrations (Matsumoto
499 et al., 2021). It should be pointed out that the same research group produced curdlan based
500 organogels by subsequent acetylation of crosslinked hydrogels (Enomoto-Rogers et al., 2016).
501 These organogels were hydrophobic and swell in chloroform. The same approach could
502 potentially be applied to crosslinked paramylon.

503 **5.1) Paramylon sulfate, ether, and acid ionic derivatives**

504 The various reported anionic and cationic paramylon derivatives (except esters) obtained by
505 homogeneous and heterogeneous reactions are listed in **Table 3**. To our knowledge the first
506 reported modified paramylons are anionic and cationic derivatives (Koizumi et al., 1993). The
507 authors report a significant increase of water solubility of all the ionic derivatives, which results
508 in an inhibitory activity against human immunodeficiency virus (HIV) for sulfated paramylon
509 with a sulphur content above 4%. The highest anti-HIV activities were obtained for sulphur
510 contents in the 12.6% to 14.0% range (Koizumi et al., 1993), which corresponds to values of
511 degree of substitution (DS) between 1.2 and 1.7. However, the authors do not give information
512 about the chemical modification conditions. Similar properties were previously reported for

513 sulfated curdlan (Yoshida et al., 1990). The homogeneous modification of curdlan was
514 performed in solution in DMSO, by reaction with piperidine-N-sulfonic acid. The DS values
515 ranged from 0.8 to a maximum of 1.6 (100% for C6, $\approx 5\%$ for C4 and 40% for C2) (Yoshida et
516 al., 1990). Given that paramylon is also soluble in DMSO and presents the same
517 macromolecular structure as curdlan (with shorter chains), we assume that similar non-
518 regioselective sulfatation reaction conditions can be applied to paramylon. However, we do not
519 know if this was the case in the work on sulfated paramylon (Koizumi et al., 1993).

520 More recently, anionic paramylon ether derivatives were obtained by carboxymethylation,
521 leading to sodium salts of carboxymethyl paramylons that are not only water-soluble, but also
522 showed nanofiber forming ability (Shibakami et al., 2016). The modification was performed
523 both in homogeneous and heterogeneous conditions. Homogenous reaction with chloroacetic
524 acid in concentrated NaOH leads to low DS ≈ 0.13 . Note that slightly higher DS ≈ 0.25 were
525 obtained in the same homogeneous conditions by other authors who suggest applications as
526 pharmaceutical tablets disintegrant (Daglio et al., 2019).

527 Heterogeneous carboxymethylation in a mixture of 2-propanol and NaOH aqueous solution
528 allowed a better control of the DS with values ranging from as low as 0.015 up to 0.78
529 (Shibakami et al., 2016). However, this heterogeneous reaction required the previous
530 preparation of “amorphous” paramylon by solubilisation in NaOH aqueous solution followed
531 by neutralization with HCl as described in a patent application from 2011 (Arashida et al.,
532 2011). The nanofiber forming ability depends on the DS range: For $0.07 < DS < 0.78$, the
533 nanofiber forming was observed in solution in water, whereas for lower $0.015 < DS < 0.07$, a
534 nanofiber gel is formed in water, leading to aerogels maintaining their fibrous structure when
535 freeze-dried. (Shibakami et al., 2016). The formation mechanism of these nanofibers was not
536 investigated but involves previously water solubilized chains. Therefore, it is probably similar

537 to the self-assembly mechanism described by the same group for non-derivatized paramylon
538 (Shibakami et al., 2013a).

539 Anionic paramylon acid derivatives were also obtained by regioselective heterogeneous
540 oxidation of C6 -OH groups, mediated with 2,2,6,6-tetramethylpiperidine-1-oxyl radical
541 (TEMPO) in water at pH 10 with NaBr and NaClO (Tamura et al., 2009). Up to 100%
542 substitution of C6-OH groups was observed. The resulting (1→3)-β-D-polyglucuronic acid
543 sodium salt was water-soluble. However, this was not the case for the other lower DS values
544 studied (1% to 43% substitution of C6-OH groups) which remained insoluble in water, pointing
545 out the need for a minimum degree of such regioselective substitution for water solubility. This
546 contrast with the anionic ether paramylon derivatives may be due to the acid linkage. The C2
547 secondary OH groups being unmodified, these paramylon derivatives were able to form
548 nanofibers (Kawahara, 2014) by the self-assembly mechanism discussed earlier (Shibakami et
549 al., 2013a). However, a strong decrease of molecular weight was also observed with a final DP
550 = 68 (Tamura et al., 2009). Note that such a small size probably also favours the water solubility.
551 Indeed, underivatized curdlan oligomers with DP = 32, obtained by other authors by hydrolysis
552 with H₂O₂, were also soluble in water (Zhu & Wu, 2019).

553 Cationic paramylon ether derivatives have been obtained by both homogeneous (Shibakami et
554 al., 2018) and heterogeneous reactions (Daglio et al., 2019). Homogeneous modification was
555 performed in concentrated NaOH aqueous solution with glycidyltrimethylammonium chloride,
556 leading to different DS, from 0.01 to 0.64 depending on the paramylon:ammonium salt ratio
557 (Shibakami et al., 2018). Freeze-dried cationic paramylon samples were put into water showing
558 poor dispersibility for DS < 0.06, and very good solubility for DS > 0.31. For intermediate DS
559 values (0.07 < DS < 0.16) the cationic paramylon chains spontaneously formed nanofibers after
560 dissolution, leading to gelation. Freeze-drying of the gel allowed XRD characterization of the
561 nanofiber whose crystallinity is close to native paramylon, suggesting that the secondary C2

562 hydroxyl groups, allowing triple helices self-assembly and stabilization, were hardly modified,
563 and that the more reactive C6 OH group was mostly substituted for these intermediate DS
564 (Shibakami et al., 2018). Concurrently, cationic paramylons with $DS > 0.31$ and $DS < 0.06$
565 showed outstanding transparent film forming ability by casting from homogeneous solutions
566 and heterogeneous solutions, respectively (Shibakami et al., 2018). For $DS > 0.3$ the cationic
567 paramylons may also be used as antidiabetic agents due to their ability to selectively absorb
568 hydrophobic bile salts. Upon administration to diet induced obese mice, a reduction of
569 mesenteric and body fat was observed with a significantly higher secretion of glucagon-like
570 peptide-1 compared to with cellulose reference. (Shibakami, Shibata, et al., 2019).

571 Recently, higher DS (0.85-1.22) cationic paramylon nanofibers were successfully dispersed in
572 organic solvents such as methanol and binary volatile solvent systems allowing the casting of
573 films (Shibakami, 2021b). Introduction of phenolphthalein showed that, when the films were
574 immersed in alkaline solution, this dye molecule was released faster with increasing DS
575 (Shibakami, 2021b), suggesting potential applications in controlled dye release.

576 More recently, the heterogeneous modification of paramylon was performed with (3-chloro-2-
577 hydroxypropyl) trimethylammonium chloride (CHPTAC) in water at 90°C , in presence of
578 NaOH and NaBH_4 to prevent depolymerisation (Daglio et al., 2019). Only one $DS \approx 0.30$ was
579 obtained. In agreement with previous report (Shibakami et al., 2018), this cationic derivative
580 was apparently not fully soluble in water. A solubility in 0.03 mol.l^{-1} Tris/HCl buffer (pH 8.0)
581 is reported (Daglio et al., 2019). However, the modified polysaccharide showed efficient
582 flocculation properties on kaolin suspension, comparable to commercial cationic
583 polyacrylamides (Daglio et al., 2019).

Type	media	reactant type	Reference	Derivative properties
Homogeneous modification				
Anionic.	DMSO(*)	piperidine-N-sulfonic acid (*)	(Koizumi et al., 1993).	Anti HIV activity (1.2 < DS < 1.7)
Anionic	Aqueous NaOH	Chloroacetic acid	(Shibakami et al., 2016; Daglio et al., 2019).	Tablets disintegrant (DS ≈ 0.18-0.25)
cationic	Aqueous NaOH	Glycidilammonium chloride	(Shibakami et al., 2018)	Nanofibers (0.07<DS<0.16) Transparent Solvent cast films (DS > 0.31 or DS < 0.06)
			(Shibakami, 2021b)	Nanofibers dispersible in organic solvents (DS ≈ 1.22) Controlled dye release films
Heterogeneous modifications				
Anionic	2-propanol/Aqueous NaOH mixture	Chloroacetic acid	(Shibakami et al., 2016).	Controlled DS from 0.015 to 0.80 Nanofibers forming ability.
Anionic	Water (pH 10)	TEMPO (regioselective)	(Tamura et al., 2009).	Water-soluble Nanofibers (DS≈1 for C6)
cationic	Water	Chlorinated ammonium chloride	(Daglio et al., 2019)	Flocculant (DS ≈ 0.30)

Table 3: Paramylon ionic derivatives obtained by homogeneous or heterogeneous reactions in various media (*: not specified by authors, assumed similar to previous anionic modification of curdlan (see text)).

584
585

586 **5.2) Paramylon esters derivatives**

587 This section is dedicated to the various modifications involving the substitution of paramylon -
588 OH groups by an ester linkage to a pendant group $R = -O - (C=O) - X$, which can be of different
589 size and nature (including hydrophobic alkyl chains but also hydrophilic ionic groups).

590 To our knowledge the first reported modification of this kind was heterogeneous acetylation (X
591 = - CH_3) by reaction with acetic anhydride in a mixture with acetic acid ($\approx 2:1$ acid/anhydride
592 ratio) with sulfuric acid catalysis (Shibakami et al., 2012). A $DS = 0.18$ was obtained resulting
593 in the formation of a gel for hydrated paramylon granules, whereas for anhydrous granules, the
594 authors observed the transformation of the shape of the suspended paramylon granules from
595 native spheroidal shape to doughnut-like shape. This unexpected result was ascribed to the
596 solubility of acetylated paramylon in acetic acid, and to the preferential solubilization of the
597 acetylated chains located in the center of the granule, progressively leading to a hole in it
598 (Shibakami et al., 2012). Note that in this case, the “product” is not a paramylon derivative and
599 had no foreseen applications.

600 In what follows, we will focus first on homogeneous modification reactions, which are the most
601 widely studied, and then on heterogeneous reactions in other media.

602 **5.2.1) Homogenous esterification**

603 The various reported esters and mixed esters of paramylon obtained by homogeneous
604 modification in DMAc/LiCl and DMF/LiCl are listed in **Table 4**. In these solvents of
605 paramylon, the reaction with anhydrides can be conducted in presence of pyridine.

606 Acetylation leads to different properties depending on the DS. The first report concerns $DS >$
607 2.01 , leading to semi-crystalline films (Shibakami et al., 2015b): depending on the subsequent
608 purification, acetylated paramylon with different DS were obtained. They were fully soluble in
609 either chloroform ($DS = 2.01$) or dichloromethane ($DS = 2.37$), allowing the production of self-

610 standing films by solution casting. The optically transparent films obtained were semi-
611 crystalline, with a higher crystallinity for higher DS, resulting in Young's modulus and
612 elongation at break comparable to those of acetylated cellulose (Shibakami et al., 2015b).
613 However, it should be noted that their crystalline structure was different from that of native
614 paramylon, with the suspected formation of mono helices by these highly acetylated
615 paramylons (Shibakami et al., 2015b). More recently, lower DS values were investigated
616 (Shibakami, 2021a): the same chemical modification conditions were used, but varying reaction
617 time in order to control the DS (Shibakami, 2021a). For the intermediate $1.59 < DS < 2.01$
618 range, acetylated paramylons displayed a glass transition with a low crystallinity, whereas for
619 lower range ($1.16 < DS < 1.42$) highly crystalline materials, without any observed glass
620 transition, were obtained. Summing up, this suggests the following overall trend for acetylated
621 paramylons: i) high crystallinity similar to pristine paramylon in the lower DS range, ii) lower
622 crystallinity with an amorphous phase for intermediate DS, and iii) increasing crystallinity with
623 presence of mono helices in the higher DS range.

624 Besides, acetylated paramylon with DS lower than 2 were able to form nanofibers by triple
625 helices self-assembly. For $DS = 1.42$, nanofibers dispersible and stable in chloroform were
626 obtained, opening perspectives for industrial applications (Shibakami, 2021a). Note that more
627 "classically" water-soluble anionic paramylon esters salts ($-X = -CH_2-CH_2-(C=O)-O^- Na^+$),
628 able to self-assemble as nanofibers, had previously been obtained by reaction with succinic
629 anhydride in the same homogeneous conditions (in presence of triethylamine), followed by
630 dialyze (Shibakami et al., 2013b). For these succinylated paramylons, the partial DS of the C2,
631 C4 and C6 were 0.23, 0.05 and 0.18, respectively, thus leaving enough intact C2 -OH groups
632 for the formation of triple helices in NaOH solutions (Shibakami et al., 2013b).

633

634 Paramylon propionate was also recently obtained by reaction with propionic anhydride (Seok
635 et al., 2021) resulting in DS of 1.6 and 2.0, with a preferential substitution of the C6 primary
636 and C4 secondary hydroxyl groups. These paramylon monoesters were subsequently modified
637 by reacting the C2 hydroxyl group with L-lactide or ϵ -caprolactone, resulting in poly(lactic
638 acid) (PLA) or polycaprolactone (PCL) grafted side chains. These grafted paramylon
639 derivatives were thermoplastic, with glass transition and melting temperatures close to those of
640 pure PLA and pure PCL, respectively. However, the mechanical properties were significantly
641 lower (Seok et al., 2021). Nevertheless, this approach opens the perspective of making polymer
642 blends between these grafted paramylon esters and pure PLA or PCL.

643 The above homogeneous acetylation and succinylation reactions with anhydrides were also
644 applied as secondary modifications in order to obtain thermoplastic mixed esters (Shibakami et
645 al., 2014) and amphiphilic mixed esters (Shibakami, 2017). In both cases, paramylon was
646 previously esterified with long hydrophobic pendent chain esters ($-X_1 = -(\text{CH}_2)_n\text{-CH}_3$; $n=12$
647 or 14 or 16) by reaction with acyl chlorides. This long chain substituent is preferentially
648 substituted to the more reactive C6-OH group, so that the subsequent acetylation or
649 succinylation targets mainly the remaining secondary -OH groups:

- 650 - Paramylon acylates succinylated in salt form ($-X_2 = -\text{CH}_2\text{-CH}_2\text{-(C=O)-O}^- \text{Li}^+$) were
651 amphiphilic (Shibakami, 2017). Besides still showing ability to self-assemble as
652 nanofibers, these mixed esters induced high viscosity in aqueous solutions, in particular
653 when combining succinylation to higher DS_1 and acyl chain length (Shibakami, 2017).
654 Films prepared by soaking/casting in ethanol combined mechanical strength with
655 optical transparency and remarkable water absorption performances that may be used
656 for medical applications (Shibakami, 2017).
- 657 - Concurrently, acetylated paramylon acylates with a total DS in the 2.5 to 2.7 range
658 (acylation DS values were in the 0.28-0.61 range, while acetylation DS was > 2) showed

659 thermoplastic behavior (Shibakami et al., 2014), with melt flow rates and mechanical
660 properties comparable to commercial bioplastics such PLA and Polyamide11. It is
661 noteworthy that the reversed sequential modification (acetylation followed by acylation)
662 hinders the later due to the higher reactivity of the C6 primary hydroxyl group that, once
663 substituted by acetates, cannot be acylated. This results in a low acylation DS and no
664 thermoplasticity (Shibakami et al., 2014). It suggests that a minimum DS is required in
665 order to obtain thermoplastic paramylon esters. Regarding the type of long chain
666 substituent, the authors insisted on the potential of paramylon acetate myristate for
667 industrial production because of the possibility to obtain myristic acid from *E. gracilis*
668 cultivation in anaerobic conditions (Inui et al., 1983).

669 Another homogeneous modification strategy for the synthesis of high DS thermoplastic
670 paramylon mixed esters involves the simultaneous “one pot” reaction of paramylon with
671 multiple anhydrides, in the presence of 4-dimethylaminopyridine (Shibakami & Sohma, 2017).
672 It successfully lead to mixed esters with acetate ($X_1 = -CH_3$) and medium chain length acylate
673 ($X_2 = - (CH_2)_n - CH_3$) (with $n = 1, 2, 3$ or 4). Note that the multiple anhydride reactants are
674 obtained by previously mixing one anhydride with one acid: for example, propionic anhydride
675 with acetic acid, or inversely acetic anhydride with propionic acid. However, this affects the
676 final partial DS values, resulting in medium chain length rich or acetyl rich mixed esters with
677 different melt volume rate, melting and glass transition temperatures and mechanical properties.
678 For almost all mixed esters, films could be obtained by hot-pressing at temperatures above
679 200°C (Shibakami & Sohma, 2017). It was later shown that paramylon acetate propionate can
680 display melt spinnability at 240°C when the partial DS are in the ranges 0.5-0.7 and 2.2-2.5,
681 respectively (Shibakami, Sohma, et al., 2019). The tough monofilaments obtained may be used
682 in medical applications such as sutures.

683

Reactants	R= -O- (C=O) – X	Reference	Derivative properties
Acetic anhydride	X= -CH ₃ DS = 2.01-2.37	(Shibakami et al., 2015b)	Transparent self standing films
Acetic anhydride	X=-CH ₃ DS = 1.16-2.01	(Shibakami, 2021a)	Nanofibers stable in chloroform (DS = 1.42)
Succinic anhydride	X= - CH ₂ -CH ₂ -(C=O)-O ⁻ Na ⁺ DS = 0.46	(Shibakami et al., 2013b)	Water-soluble nanofibers
Propionic anhydride*	X= - (CH ₂)-CH ₃ DS = 1.60-2.00	(Seok et al., 2021)	Thermoplastic (after grafting with PLA or PCL, see text)
1: Acyl chlorides 2: Acetic anhydride (sequential reactions)	X ₁ = - (CH ₂) _n -CH ₃ ; n=12,14,16 DS ₁ = 0.28-0.61 X ₂ = -CH ₃ ; DS ₂ = 2.07-2.49	(Shibakami et al., 2014)	Thermoplastic
1: Acyl chlorides 2: Succinic anhydride (sequential reactions)	X ₁ = - (CH ₂) _n -CH ₃ ; n=12,14,16 DS ₁ up to 0.13 X ₂ =- CH ₂ -CH ₂ -(C=O)-O ⁻ Li ⁺ or -CH ₃ DS ₂ up to 0.46 (salt) or 2.77 (acetate)	(Shibakami, 2017)	Amphiphilic thickeners Water absorbing films (Acylate Succinate salts)
Multiple anhydrides (simultaneous reactions)	X ₁ = -CH ₃ DS ₁ = 0.36-1.24 X ₂ = - (CH ₂) _n -CH ₃ ; n= 1, 2, 3, 4 DS ₂ = 1.71-2.24	(Shibakami & Sohma, 2017)	Thermoplastic (hot-press)
Multiple anhydrides (simultaneous reactions)	X ₁ = -CH ₃ DS ₁ = 0.36-1.24 X ₂ = - (CH ₂) _n -CH ₃ ; n= 0,1,2 DS ₂ = 1.71-2.24	(Shibakami, Sohma, et al., 2019)	Melt spinnable (paramylon acetate proprionate, DS ₁ = 0.5-0.7 ; DS ₂ = 2.2-2.5)

684 **Table 4:** Paramylon esters and mixed esters obtained by homogeneous esterification_reactions in DMAc/LiCl, except * in DMF/LiCl.

685 **5.2.2) Heterogeneous esterification in other media**

686 Heterogeneous esterification of paramylon has also been reported in various media for the
687 production of monoesters and mixed esters (**Table 5**). It allows obtaining high total DS values
688 necessary for thermoplasticity, but with a better control of partial DS values. Thermal
689 transitions, mechanical properties and viscosity can thus be finely tuned. A “one pot” synthesis
690 approach of mixed esters consists in a heterogeneous reaction with two acids in trifluoroacetic
691 anhydride (TFAA) (Shibakami et al., 2015a). Using acetic acid and various long chain fatty
692 acids, the authors observed that the resulting paramylon derivatives (with total DS in the 2.59-
693 2.89 range) are actually soluble in TFAA so that the final solution was homogeneous. The
694 advantage of this approach is that it allows controlling the partial DS by tuning the feed ratio
695 of the two acids. In the case of myristic acid, this allows tuning the thermoplasticity properties
696 of the paramylon acetate myristate (Shibakami et al., 2015a). Indeed a more recent work shows
697 that paramylon acetate myristate with partial DS ≈ 0.35 lead to a good compromise between
698 moldability and mechanical properties, the myristate pendent group acting both as an “internal
699 plasticiser” and a “viscosity increaser” (Shibakami & Sohma, 2020).

700 This approach was also applied to the synthesis of fully substituted paramylon monoesters with
701 varying pendant chain length ($X = -(\text{CH}_2)_n-\text{CH}_3$; $n = 0,1,2,3,4,5,6,7,9,11$) (Gan et al., 2017).
702 Total substitution of the paramylon hydroxyl groups was achieved (DS = 3). Depending on the
703 substituted chain length, the thermoplastic polymers were semi-crystalline ($n = 0-5$ with melting
704 temperatures (T_m) values from 281 to 114 °C) or amorphous ($n = 7-11$; the glass transition
705 temperature (T_g) was not measured). Except for the acetate, all these paramylon derivatives
706 were thermoplastic above their glass transition or their melting temperature, allowing hot
707 pressing of films. Film casting from solutions in chloroform was also possible, with similar
708 mechanical properties (Gan et al., 2017). Thermal stretching of the films allowed improving
709 the mechanical performances of the semi-crystalline paramylon monoesters thanks to

710 orientation. Further investigation of the crystallinity of stretched fibers showed that, contrary to
711 native paramylon, the esterified chains take a 5/1 helical conformation, and that they are
712 arranged in pseudo-hexagonal unit cells (Gan et al., 2019). This five-fold conformation favors
713 the axial deformation of the fibers, which have low Young's moduli (2.5 to 1 GPa with
714 increasing pendent chain length), especially compared to cellulose triacetates fibers which have
715 a 3/2 helical conformation and Young's moduli ten times higher (Gan et al., 2019).

716 Partially substituted paramylon monoesters, with DS lower than 3, can be obtained by
717 heterogeneous reaction of acyl chlorides in pyridine (Shibakami & Sohma, 2018). The DS can
718 be controlled in the 1.50 to 3 range by acyl chloride/paramylon ratio. Contrary to fully
719 substituted monoesters, some of these lower DS derivatives were not only thermoplastic,
720 allowing hot pressing of films, but also presented a remarkable tackiness at room temperature.
721 Thus they may be used as pressure sensitive adhesives, with best performances observed for
722 paramylon myristates with DS \approx 2.6-2.9 (Shibakami & Sohma, 2018).

723 More recently, the same heterogeneous reaction of acyl chlorides with paramylon was
724 performed in N-methylpyrrolidone/pyridine mixtures (Zhong et al., 2021). DS values from 1.7
725 to 2.7 were obtained by varying the acyl chloride/paramylon ratio. The authors demonstrated
726 that the DS value can be easily characterized on particles of the paramylon derivatives by
727 terahertz spectroscopy, using specific signature peaks of paramylon which are shifted upon
728 modification (Zhong et al., 2021).

729

Media	Reactants	R= -O- (C=O) – X	Reference	Derivative properties
Acetic acid	Acetic anhydride	X= -CH ₃ ; DS=0.18	(Shibakami et al., 2012)	Doughnut-like particles
Trifluoroacetic anhydride	Acetic acid Long chain fatty acids	X ₁ = -CH ₃ DS ₁ = 1.84-2.24 X ₂ = - (CH ₂) _n -CH ₃ ; n=6,8,10,12,14,15,16 DS ₂ =0.35-1.05	(Shibakami et al., 2015a)	Thermoplastic (Acetate Myristate)
Trifluoroacetic anhydride	Various carboxylic acids	X = - (CH ₂) _n -CH ₃ ; n= 0,1,2,3,4,5,6,7,9,11 DS =3	(Gan et al., 2017)	Semi-crystalline thermoplastics (n =1-5) Amorphous thermoplastics (n =7-11) Hot pressed and solvent casted films. Thermally stretched crystalline fibers.
pyridine	Various acyl chlorides	X= - (CH ₂) _n -CH ₃ ; n=12,14,16 DS = 1.5-3	(Shibakami & Sohma, 2018)	Thermoplastic (hot pressed) Pressure sensitive adhesives (paramylon myristates)
N-methylpyrrolidone /pyridine mixture	Various acyl chlorides	X= - (CH ₂) _n -CH ₃ ; n=12,14,16 DS = 1.7-2.7	(Zhong et al., 2021).	Not studied.

730

731

Table 5: Paramylon ester derivatives obtained by heterogeneous reactions in various media.

732

733 Summarizing, compared to pristine paramylon, the various derivatives described throughout
734 this section allow a much wider variety of processing routes: their solvent based processing into
735 films and nanofibers can be done either in water or in organic solvents, thanks to modified
736 solubility, while solvent free thermomechanical processing can become possible thanks to their
737 thermoplasticity without thermal degradation:

- 738 • Solubility in water was achieved by substituting paramylon's –OH groups with anionic
739 or cationic groups. The reported minimum DS values are typically lower than 1, but
740 depend strongly on the type of linkage and ionic group. As recalled by one author
741 (Tamura et al., 2009), historically, the first motivation for investigating ionic derivatives
742 was to enhance paramylon's biological activity, whose limited performances were
743 ascribed to its absence of solubility in water. For paramylon sulfates, a correlation is
744 indeed reported between water solubility and bioactivity. However, the optimum DS
745 (1.2-1.7) range is very high compared to that of most of the other anionic and cationic
746 derivatives studied since then. Actually, it is striking that the authors often did not focus
747 on the impact of solubility on their bioactive properties, but rather on their nanofiber
748 forming ability in water (which actually seems to be an efficient way to improve
749 paramylon's bioactive properties, as discussed in section 4 (Sugiyama et al., 2009)
750 (Barsanti & Gualtieri, 2019)) and on the production of films from these nanofibers.

751 The reported results suggests that the ability to form nanofibers in water requires the
752 ionic derivatives to combine: i) an absence of, or a low substitution of C2 –OH groups
753 in order to have stable triple helices (this implies a maximum total DS, 1 and 1.22 being
754 the highest reported for regioselective substitution of C6-OH groups, and non
755 regioselective substitution, respectively), and ii) A minimum total DS allowing the
756 destabilization of the native crystalline structure for a partial solubilization before
757 subsequent reassembly as nanofibers. This minimum total DS depends on the ionic

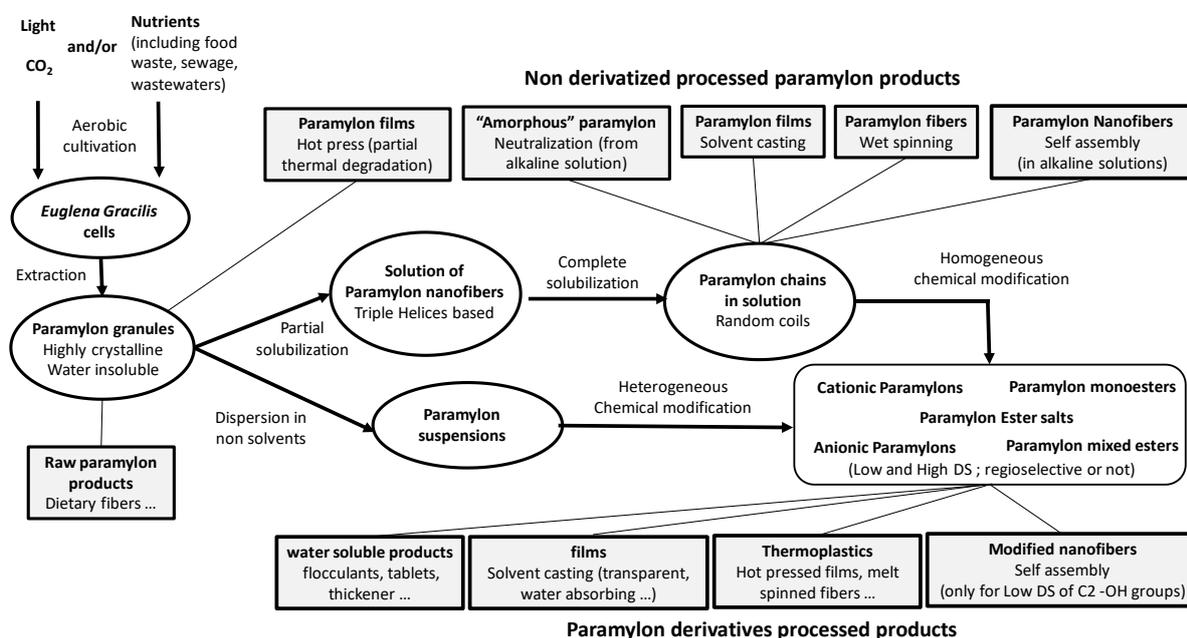
758 substituent and needs to be finely tuned in order avoid, or favor, gelation of the
759 nanofibers.

760 • Paramylon acetates can be similarly processed as nanofibers and films in organic
761 solvents. However, this requires higher minimum DS values than for ionic derivatives
762 in water (e.g. in chloroform, solubility is achieved at DS=2.01, while nanofibers are
763 stable of DS=1.42). This may be ascribed to the hydrophilic nature of paramylon's -OH
764 groups, which thus need to be highly substituted. Contrary to pristine paramylon,
765 depending on their DS, paramylon acetates can present a glass transition or a modified
766 crystallinity with presence of mono helices. However, thermoplasticity cannot be
767 achieved, even for DS=3.

768 • Thermoplasticity requires both a high DS (generally close to 3) and the introduction of
769 at least a fraction of longer aliphatic ester pendant groups allowing to reduce the
770 interactions between paramylon chains by playing an internal plasticiser role. It is
771 striking that the thermoplastic paramylon esters obtained can have melting temperature
772 as high as 280°C, without reported thermal degradation during hot-press processing.
773 This indicates that the substitutions also increase the thermal stability of paramylon.
774 Both mono and mixed esters can be used, with the following apparent rules in terms of
775 DS: i) If only one type of aliphatic ester pendant groups is used, a DS equal to 3 is
776 necessary for short chains (e.g. proprionates), while for long pendant chains (e.g.
777 myristates), the minimum DS can be lowered (down to ≈ 2.5); ii) long pendant chains
778 can also be combined with short chains, including acetates. However, the maximum
779 partial acetylation DS depends on the size of co-substituent: If long pendant chains are
780 used, a rather high partial acetylation can be used (DS ≈ 2), whereas with lower chain
781 length co-substituents, the maximum acetylation degree that can be tolerated becomes
782 lower, but the total DS should remain close to 3.

783 **Conclusions and perspectives**

784 With the emergence of microalga *E. gracilis* large scale cultivation, the linear β -1,3-glucan
 785 paramylon produced by this microorganism is likely to become more and more widely available
 786 in the coming years. Due to the high versatility and robustness of euglenoids, paramylon can
 787 be produced either in photoautotrophic or heterotrophic (or both), as well as within the
 788 framework of environmental-friendly schemes, such as bioremediation, wastewater treatment,
 789 and CO₂ trapping. This makes paramylon a potentially carbon neutral and sustainable feedstock
 790 for biobased products. However, as a storage polysaccharide, it is biosynthesized as highly
 791 crystalline, water insoluble, intracellular granules, in which β -1,3-glucan form stable triple
 792 helices. Beside its applications in this raw granular native form, such as dietary fibers, various
 793 processing routes have been developed, leading to both non-derivatized and chemically
 794 modified paramylon products, with various shapes and properties as summarized in **Figure 3**.



795
 796 **Figure 3:** Overview of paramylon processing routes and potential applications.

797 The use of specific solvents, allowing to disrupt the triple helices, gives the possibility to shape
 798 native paramylon into films and fibers without chemical modification. The reversibility of triple

799 helices disruption in aqueous alkaline solutions allows the production of nanofibers by a self-
800 assembly mechanism. This outstanding property of paramylon can persist when paramylon is
801 chemically modified by substitution of hydroxyl groups, except the C2 hydroxyl groups that
802 are involved in triple helices stabilization mechanism. Thus paramylon derivatives based
803 nanofibers can be produced including nanofibers dispersible in organic solvents.

804 Chemical modifications, in homogeneous or heterogeneous conditions, also allows obtaining
805 two classes of paramylon derivatives: i) a variety of water-soluble ionic derivatives particularly
806 interesting for health related applications; and ii) paramylon monoesters and mixed esters,
807 including thermoplastic and melt spinnable derivatives for the production of bioplastic films
808 and fibers.

809 **Suggestions and prospects**

810 Produced as intracellular granules, paramylon first needs to be extracted from *E. gracilis*
811 biomass. However, there is currently no standard method neither for the extraction of the
812 granules, nor for the purification of paramylon. In the perspective of large scale production, it
813 would be necessary to investigate the influence of the extraction and/or of the purification
814 conditions on paramylon's purity and molecular weight. Controlling the purity seems
815 particularly important for the use of paramylon as an immunomodulator, for which the role of
816 other molecules present in *E. gracilis* biomass and paramylon granules needs to be further
817 investigated (Phillips et al., 2019).

818 Regarding the reported solvents of paramylon, it appears that for some of them the exact state
819 of solubilized paramylon, as triple helices or random coils, is not clear. Therefore, it would be
820 interesting to investigate this point, particularly for ionic liquids. Indeed, beside DMSO and
821 concentrated alkaline solutions (which both allow the disruption of triple helices), ionic liquids
822 seem promising new candidates that remain poorly studied, despite their known versatility as

823 solvents of polysaccharides. Despite their potential influence on paramylon depolymerisation
824 (as observed for curdlan when solubilized in 1-ethyl-3-methylimidazolium acetate) which
825 should be further investigated, ionic liquids present the advantage of being tunable by changing
826 the chemical structure of the anion and cation moieties. These moieties can even be biobased
827 and/or biocompatible. Thus, ionic liquids can potentially be used, not only as solvents for non-
828 derivatising shaping of paramylon or as media for chemical modification, but even as final
829 components of paramylon materials (as plasticisers for example). Last but not least, ionic liquid
830 being efficient solvents of other polysaccharides and biopolymers, they may be used as co-
831 solvent for the processing of blends with paramylon.

832 Concurrently, the recently reported paramylon monoesters grafted with PCL or PLA chains
833 open perspectives for the melt processing of blends of paramylon derivatives with such
834 polyester bioplastics. The use of derivatised paramylon nanofibers as reinforcements in other
835 polymers would also be interesting to investigate.

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