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Description	

1 Elucidating the degradation mechanism of a self- 2 degradable dextran-based medical adhesive

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17 Abstract

18 We developed a self-degradable medical adhesive, LYDEX, consisting of periodate-oxidized
19 aldehyde-functionalized dextran (AD) and succinic anhydride-treated ϵ -poly-L-lysine (SAPL). After
20 gelation and adhesion of LYDEX by Schiff base bond formation between the AD aldehyde groups
21 and SAPL amino groups, molecular degradation associated with the Maillard reaction is initiated,
22 but the detailed degradation mechanism remains unknown. Herein, we elucidated the degradation
23 mechanism of LYDEX by analyzing the main degradation products under typical solution conditions
24 *in vitro*. The degradation of the LYDEX gel with a sodium periodate/dextran content of 2.5/20 was
25 observed using gel permeation chromatography and infrared and ¹H NMR spectroscopy. The AD
26 ratio in the AD-SAPL mixture increased as the molecular weight decreased with the degradation
27 time. This discovery of LYDEX self-degradability is useful for clarifying other polysaccharide
28 hydrogel degradation mechanisms, and valuable for the use of LYDEX in medical applications, such
29 as hemostatic or sealant materials.

31 Keywords

32 hydrogel, LYDEX, degradation, dextran, poly-L-lysine, Maillard reaction.

33 1 . Introduction

34 Surgical tissue adhesives are commonly used as sealants to stop bleeding from organs and tissue
35 sutures and prevent air leakage from cut surfaces during thoracic surgery. Tissue adhesives
36 composed of natural or synthetic polymers, or a combination, have been developed (Li et al., 2014).
37 Fibrin-based sealants are highly biocompatible due to their blood coagulation properties, but weak
38 adhesive strengths (MacGillivray, 2003) limit their applications, with the risk of viral infection also
39 reported (Canonico, 2003; Hino et al., 2000; Ortel et al., 1994; Siedentop et al., 2001; Silver et al.,
40 1995). Conversely, cyanoacrylate, a synthetic adhesive, exhibits high stiffness and adhesive strength,
41 but inhibits *in vivo* healing of the diseased area due to systemic inflammatory reactions (Ramond et
42 al., 1986) and high cytotoxicity (Bhatia et al., 2007). This is because of the involvement of aldehyde
43 compounds in the gelation and degradation of the adhesive. Hence, their areas of application are
44 limited, and they cannot be used in direct contact with the central nervous system or blood vessels.
45 The combination of natural and synthetic polymers is represented by adhesives that contain gelatin
46 cross-linked with formaldehyde and glutaraldehyde. Although the adhesive strength is sufficiently
47 high, the preparations of these adhesives include toxic aldehyde compounds as cross-linking agents
48 (curing components), with their use limited by their biotoxicities (Erasmı et al., 2002; Fürst &
49 Banerjee, 2005; LeMaire et al., 2002).

50 As none of these are ideal tissue adhesives for repairing damaged elastic and soft tissues, extensive
51 research and development has recently been conducted to design biocompatible, biodegradable,
52 flexible sealants to form leak-free seals in soft tissues.

53 Elvin et al. reported that a photocrosslinked fibrinogen-based hydrogel based on Ru-catalyzed
54 photooxidation of tyrosine residues (Fancy & Kodadek, 1999) showed improved adhesion strength,
55 extensibility, and tensile strength compared to those of previously approved products (C. M. Elvin et
56 al., 2010; Christopher M. Elvin et al., 2009). Elvin et al. synthesized a photocrosslinkable gelatin
57 sealant via a similar reaction and observed the blood and air leak prevention effects in lung tissue
58 using a sheep model (Christopher M. Elvin et al., 2010). Taguchi et al. reported an improvement in
59 the pressure resistance by introducing dodecyl groups into pollock-derived gelatin to increase
60 hydrophobic interactions (Mizuno et al., 2017). Several types of sealants have been developed using
61 albumin, such as gelatin, from various animal sources. For example, the United States Food and
62 Drug Administration approved BioGlue (CryoLife, Inc.) (Chao & Torchiana, 2003) consisting of
63 bovine albumin and glutaraldehyde, and Progel (Daboll, Inc.) (Fuller, 2013; Kobayashi et al., 2001),
64 a composite sealant containing human albumin and a polyethylene glycol (PEG) cross-linker with
65 two NHS-activated ester groups. When the two components are mixed, the primary amine group of
66 the albumin lysine residue reacts rapidly with the succinimidyl succinate group to form a cross-
67 linked structure within 1 min.

68 Research regarding sealants containing polysaccharides, which are widely used in medicine and food
69 as natural polymers, is also active. As sealants, chitosan derivatives were prepared by reacting
70 chitosan with lactobionic acid in the presence of water-soluble carbodiimide (Ishihara et al., 2002;
71 Ono et al., 2000). Moratti et al. reported the hemostatic and adhesive properties of gels consisting of
72 succinylated chitosan and dextran aldehyde (Liu et al., 2009). Imine bonds are formed between
73 amino and aldehyde groups, yielding gels. Elisseeff et al. reported a gel-based sealant consisting of
74 chondroitin sulfate with aldehyde groups combined with poly(vinyl alcohol-*co*-vinylamine) (Reyes
75 et al., 2005).

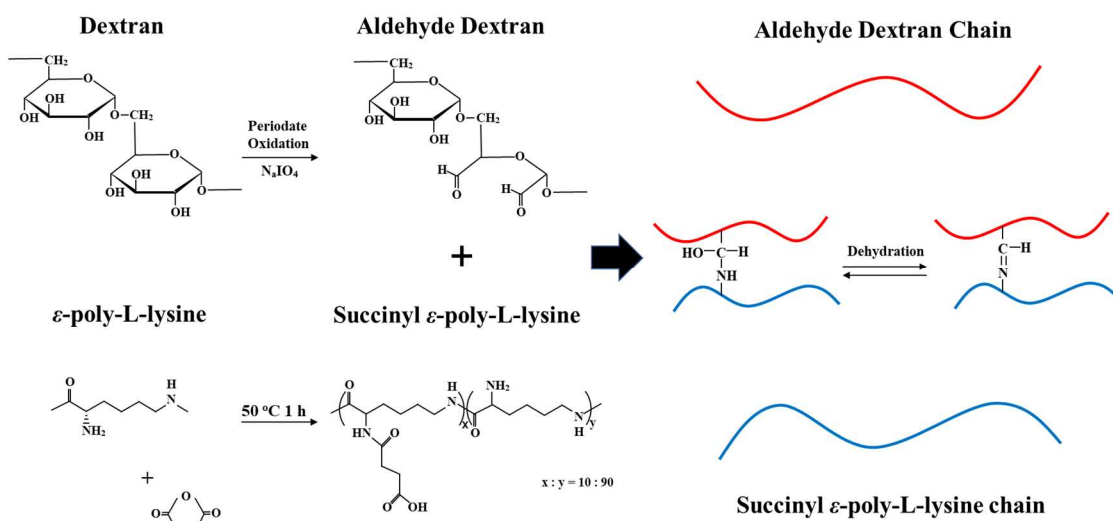
76 Polyurethane- (Gilbert et al., 2008), PEG- (Hill et al., 2001; Wallace et al., 2001), and polyester-
77 based (Sakai et al., 2013) synthetic sealants have been developed as wound closure technologies
78 suitable for clinical applications, owing to their excellent adhesion strengths and tunable mechanical
79 properties.

80 Additionally, we developed a novel dextran-based self-degradable medical adhesive, LYDEX, which
81 exhibits high adhesive performance and flexibility, low toxicity, and no risk of viral infection, to
82 meet clinical requirements (Araki, Tao, Nakajima, et al., 2007; Araki, Tao, Sato, et al., 2007; Hyon et
83 al., 2014; Matsumura et al., 2014). LYDEX is prepared using natural dextran and ϵ -poly-L-lysine (ϵ -
84 PLL) polymers. In addition, as various dosage forms (liquid, powder, sheet, and disc) may be
85 selected, numerous medical applications have been studied, including hemostatic (Naitoh et al.,
86 2013; You et al., 2014), sealant (Araki, Tao, Nakajima, et al., 2007; Araki, Tao, Sato, et al., 2007),
87 and anti-adhesion materials (Kamitani et al., 2013; Takagi, Araki, et al., 2013; Takagi, Tsuchiya, et
88 al., 2013), and endoscopic wound dressing (Bang et al., 2019).

89 Dextran within LYDEX predominantly consists of α -1,6-linked glucose polymers synthesized by the
90 heterolactic acid bacteria *Leuconostoc mesenteroides*. Dextran is widely applied in the biomedical
91 field because of its biocompatibility (Cadée et al., 2000; Ferreira et al., 2004), low toxicity (Hyon et
92 al., 2014), simple modification (Mehvar, 2000), and enzyme-mediated degradability *in vivo*
93 (Khalikova et al., 2005). In addition, dextran contains numerous hydroxyl groups, which provide
94 high hydrophilicity and may be used in chemical functionalization (Lévesque & Shoichet, 2007;
95 Maia et al., 2005; Massia & Stark, 2001; Matsumura & Rajan, 2021; Mehvar, 2000).

96 To develop LYDEX, we employed a modification strategy wherein sodium periodate was used to
97 introduce aldehyde groups into dextran, which reacted with amino groups to form imine bonds
98 (Schiff base), leading to hydrogel formation (Figure 1). Specifically, aldehyde-functionalized dextran
99 (AD) was mixed with ϵ -PLL (Araki, Tao, Nakajima, et al., 2007; Araki, Tao, Sato, et al., 2007). This
100 hydrogel showed *in vitro* degradation without degradable cross-linking points (Matsumura et al.,
101 2014). In addition, the development of hydrogels as biomedical adhesives and scaffolds using
102 aldehyde-modified polysaccharides by periodate oxidation (Malaprade reaction) (Malaprade, 1928)
103 has been previously reported (Drury & Mooney, 2003).

104 Studies on LYDEX degradation are underway. The oxidative cleavage of dextran using sodium
 105 periodate (Malaprade reaction) (Malaprade, 1928) is long-known, with the ring cleaved to form two
 106 aldehyde groups accompanied by partial degradation of the main chain (Maia et al., 2005). The
 107 structure of oxidized dextran is diverse, not unitary, and an intermolecular hemiacetal model
 108 between three types of molecules was proposed, using NMR structural analysis (Drobchenko et al.,
 109 1993; Maia et al., 2005, 2011). We investigated the possibility of an intramolecular hemiacetal
 110 model based on the assumption that the aldo–enol transition occurred, as the proton of the aldehyde
 111 group of oxidized dextran was undetected using NMR spectroscopy (Chimpibul et al., 2016). In
 112 addition, oxidized dextran undergoes irreversible reactions with amino groups, resulting in cross-
 113 linking and gelation due to imine bond formation; when the Amadori rearrangement occurs, the 1,6-
 114 glycosidic bond becomes unstable and rapidly cleaves the C–O bond of the main chain adjacent to
 115 the imino group, resulting in a decrease in molecular weight (Mw) within an hour and swelling of
 116 the gel (Hyon et al., 2014; Maia et al., 2005, 2011). Subsequently, the partial hemiacetal structure
 117 generated by periodate oxidation reacts with amino groups and undergoes Amadori rearrangement,
 118 leading to the cleavage of the glucose unit ring and molecular degradation (Chimpibul et al., 2016)
 119 (Figure S1). A hydrogel exhibiting controlled degradation of dextran derivatives via this degradation



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124 mechanism was designed and showed potential as a drug-releasing substrate (Nonsuwan et al., 2019;
 125 Nonsuwan & Matsumura, 2019). Furthermore, this degradation mechanism may be applied to induce
 126 molecular degradation of cellulose; oxidized cellulose is useful as a low-toxicity biodegradable
 127 scaffold (Chimpibul et al., 2020).

128 Although the gelation of the aldehyde of oxidized dextran and the amino groups is followed by the

129 Maillard reaction and molecular degradation, the degradation mechanism and products of LYDEX
130 have not been fully elucidated. For clinical application, clarification of the degradation products,
131 mechanisms, and *in vivo* kinetics is necessary to confirm their effects on biological safety.
132 Furthermore, clarification of the *in vitro* degradation behavior of LYDEX is useful for elucidating
133 the *in vivo* degradation and absorption mechanisms. Therefore, in this study, the main degradation
134 products of LYDEX in aqueous media are analyzed experimentally to elucidate the degradation
135 mechanism.

136

137 **2. Material and methods**

138 **2-1. Materials**

139 Dextran (70 kDa) was obtained from Meito Sangyo (Nagoya, Japan) and ϵ -PLL (4 kDa, 25 wt.%
140 aqueous solution, free base) from JNC (Tokyo, Japan). Sodium periodate, succinic anhydride (SA),
141 and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan). All chemicals were used
142 without further purification unless otherwise stated.

143

144 **2-2. Preparation of AD**

145 AD (oxidant/dextran ratio: 2.5/20 w/w) was prepared by oxidizing dextran with sodium periodate
146 according to the method reported (Mo et al., 2000).

147

148 **2-3. Preparation of SA-treated PLL (SAPL)**

149 AD reacts with the primary amino group of ϵ -PLL at a neutral pH to form a hydrogel. To control this
150 reactivity and due to its low toxicity (Chao & Torchiana, 2003), SA was used to synthesize SAPL. ϵ -
151 PLL is an oligomer of the amino acid L-lysine with 25–35 primary amino groups per molecule, but
152 SAPL consists of an α -carboxyl group bonded to an ϵ -amino group. Several amino groups of ϵ -PLL
153 were acylated by SA addition according to a previously reported method (Hyon et al., 2014).

154

155 **2-4. Characterization of AD and SAPL**

156 **2-4-1. Aldehyde content determination**

157 The aldehyde content of AD was evaluated using a simple iodometric titration method used in a
158 previous study (Hyon et al., 2014). Briefly, *ca.* 1% w/v aqueous AD solution (10 mL) was added to
159 an I₂ solution (20 mL, 0.05 M), followed by NaOH addition (20 mL, 1 M). The oxidation reaction
160 proceeded for 15 min. After H₂SO₄ addition (15 mL, 6.25% v/v), the I₂ consumption was determined
161 by titration with 0.1 M Na₂S₂O₃ using one drop of 20% w/w starch solution as an indicator. The
162 aldehyde group reacts with 1 mol of I₂ under alkaline conditions to form a carboxylic acid, and 1
163 mol of I₂ reacts with 2 mol of S₂O₃²⁻ ions. Three readings were obtained for each titration (n = 3).

164 The aldehyde content (mol/ Aldehyde Glucose Unit (AGU)) was calculated using Equation 1.

$$165 \quad \text{Aldehyde content} = \frac{C_{\text{Na}_2\text{S}_2\text{O}_3} \times ([V_B] - [V_S]) \times M_{\text{WAGU}} \times [D]}{[W] \times 2 \times 1000} \quad (\text{mol/AGU}) \quad (1)$$

166 where [VB] is the titration volume of the blank, [VS] is the titration volume, [D] is the dilution rate
167 of the sample, and [W] is the anhydrous-equivalent sample volume.

168

169 **2-4-2. Carboxyl group content determination**

170 The carboxyl group content in ϵ -PLL was evaluated using the ninhydrin method (McGrath, 1972).

171 The acylated ϵ -PLL solution (10% w/v) was diluted 400-fold using distilled water, and subsequently,
172 0.1 mL of the dilution, 1 mL of ninhydrin solution (0.8 g ninhydrin and 0.12 g anhydrous
173 hydrindantin in 30 mL 2-methoxyethanol), and 2 mL of acetic acid buffer solution (0.1 M acetic acid
174 and 0.2 M sodium acetate, pH 5.5) were added to glass tubes, which were heat-sealed. After coloring
175 at 100 °C for 3 min, the absorbance at 570 nm was recorded at 25 °C using an ultraviolet (UV)-
176 visible spectrophotometer (UVmini-1240; Shimadzu, Kyoto, Japan). Three readings were obtained
177 for each sample (n = 3). Separately, polylysine solution was colored using a ninhydrin solution and
178 measured in the same manner. A calibration curve ($a\lambda$, $b\lambda$) was prepared using the obtained
179 absorbances, and the residual amino group content is calculated using Equation 2.

$$180 \quad R_{\text{amino}} = \left(\frac{A_{\lambda} - b_{\lambda}}{a_{\lambda}} \right) / c_{\text{SAPL}} \times 11 \div 10 \times 100 \quad (\%) \quad (2)$$

181

182 **2-4-3. Preparation and degradation of LYDEX**

183 LYDEX was prepared using AD powder, with an oxidant/dextran ratio of 2.5/20 (w/w), and SAPL
184 powder in a 4:1 mass ratio. To prepare a LYDEX gel, 40 mg of LYDEX powder was added to the
185 well of a silicone mold (ϕ : 10 mm, LADD Research Industries, Williston, VT, USA) with 60 μL of
186 saline solution, and 120 μL of saline solution was added to form a hydrogel, which was allowed to
187 stand for 3 min. To degrade the gel, the gel was removed from the silicone mold and placed in a
188 brown glass bottle (30 mL, Nichiden-Rika Glass, Kobe, Japan) containing 12 mL of saline solution,
189 and shaken for 1 d, or 1, 2, or 4 wk in a water bath shaker (37 °C, 100 rpm). This solution was then
190 separated into filtrate and residue using a membrane filter (0.45 μm A045A047A, Advantech, Tokyo,
191 Japan). The residue was washed with approximately 1 mL of water and dried under reduced
192 pressure. The residue was weighed, and the filtrate was subjected to absorption spectroscopy using a
193 Shimadzu UV-1800 spectrophotometer.

194

195 **2-4-4. Degradation product analysis using gel permeation 196 chromatography (GPC) fractionation and spectroscopy**

197 LYDEX gels were subjected to degradation for 2 wk and filtered. The fraction of each GPC peak

198 was collected and analyzed. The sample volume used for preparative GPC was 5 mL, the injection
199 volume was 100 μ L per injection, and the number of preparative GPC runs was 50. TSKgel
200 G4000PW_{XL} and TSKgel G2500PW_{XL} columns were used. Two milliliters of the obtained fractions
201 were placed in dialysis tubes (Biotech CE Trial Kit, Repligen, Waltham, MA, USA; MWCO: 100–
202 500D), immersed in approximately 1 L of ion exchange water at 4 °C, and gently agitated for
203 approximately 20 h (during the ion exchange, the water was changed once). The dialyzed samples
204 were placed in eggplant flasks, frozen in a freezer, placed in a desiccator (5 L capacity), and
205 sublimated under vacuum (approximately 6 h). The remaining solids were collected and subjected to
206 infrared (IR) and ¹H NMR spectroscopy. IR was performed using the KBr tablet method (4 mm ϕ)
207 using an IRPrestige-21 (Shimadzu, resolution: 4 cm⁻¹, measurement range: 400–4000 cm⁻¹,
208 cumulative number: 64 times), and ¹H NMR spectroscopy was conducted using a JNM-ECA 400
209 (400 MHz, JEOL, Tokyo, Japan). The lyophilized sample was dissolved in approximately 0.75 mL
210 of D₂O (internal standard: none). Mw fractionation was performed using Shimadzu Prominence LC-
211 20AD HPLC LC-Solution TSKgel G4000PW_{XL} and TSKgel G2500PW_{XL} columns, and absorption
212 at 323 and 210 nm was measured using a UV detector, whereas refraction was measured using a
213 differential refractometer. The Mw fractionation ranges of the columns ranged from 2000 to
214 300 000 Da for G4000PW_{XL} and <3000 Da for G2500PW_{XL} for PEG. Therefore, G4000PW_{XL} was
215 used to examine the degradation products of the high-molecular-weight LYDEX, and G2500PW_{XL}
216 was used to examine oligomers, smaller monosaccharides, and amino acids, which were the higher-
217 degradation products. GPC measurement conditions were as follows: mobile phase, 0.9% NaCl;
218 flow rate, 0.6 mL/min; column temperature, 35 °C. Pullulan (STANDARD P-82, Showa Denko,
219 Tokyo, Japan) was used as the reference standard.

220

221 **3. Results and discussion**

222 **3-1. AD and SAPL characterization**

223 Dextran was oxidized using sodium periodate. The reagents were added in predetermined amounts to
224 establish an oxidant:dextran ratio of 2.5/20 (w/w). The aldehyde content of the produced AD was
225 determined using iodine titration. The desired product was obtained, with an aldehyde content of
226 0.28 mol/AGU.

227 Several of the amino groups of ϵ -PLL were acylated with SA. The reagents were added in
228 predetermined amounts to establish a reaction rate of 10 ± 5 mol%. According to ninhydrin
229 measurement, the desired product was obtained, with an SA reaction rate of 12 mol%. These results
230 are consistent with those of previous studies (Hyon et al., 2014; Matsumura et al., 2014), and these
231 parameters were utilized in this study because polymers with these parameters showed good
232 degradabilities and low cytotoxicities.

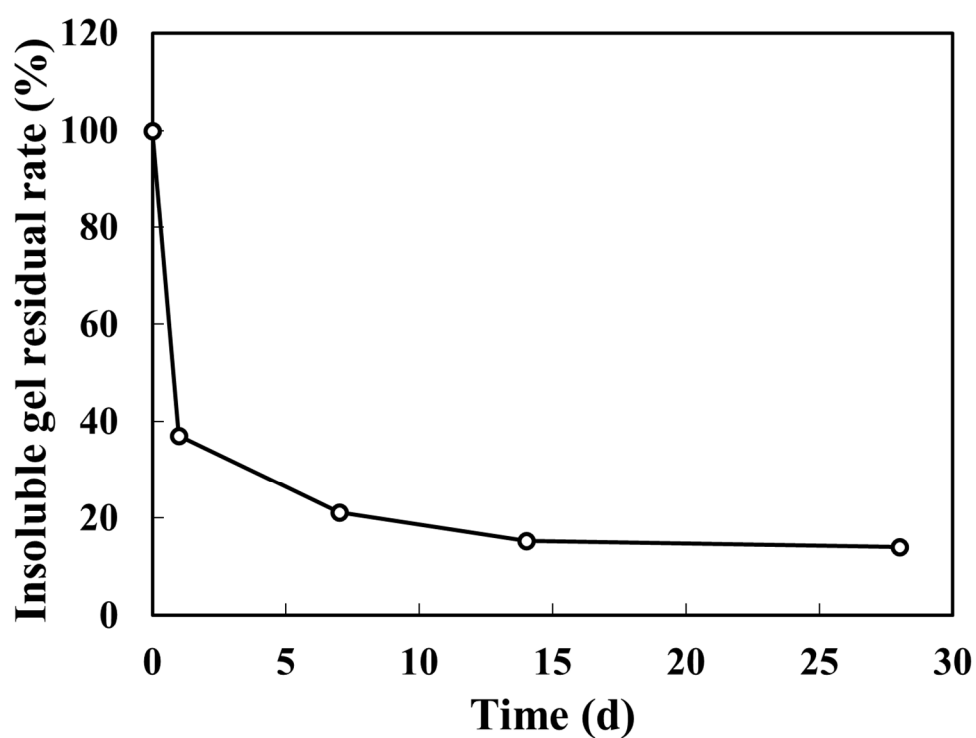
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234 **3-2. Degradation product analysis using GPC and gel degradation over**
235 **time**

236 The degradation of the LYDEX gel in physiological saline was analyzed using GPC. Figure 2 shows
237 the gel degradation with time. The gel degrades rapidly on the first day of degradation to 35%
238 residual, and degradation slows thereafter. The residual is 15% after 4 wk. The gel is not visually
239 confirmed after 5 d.

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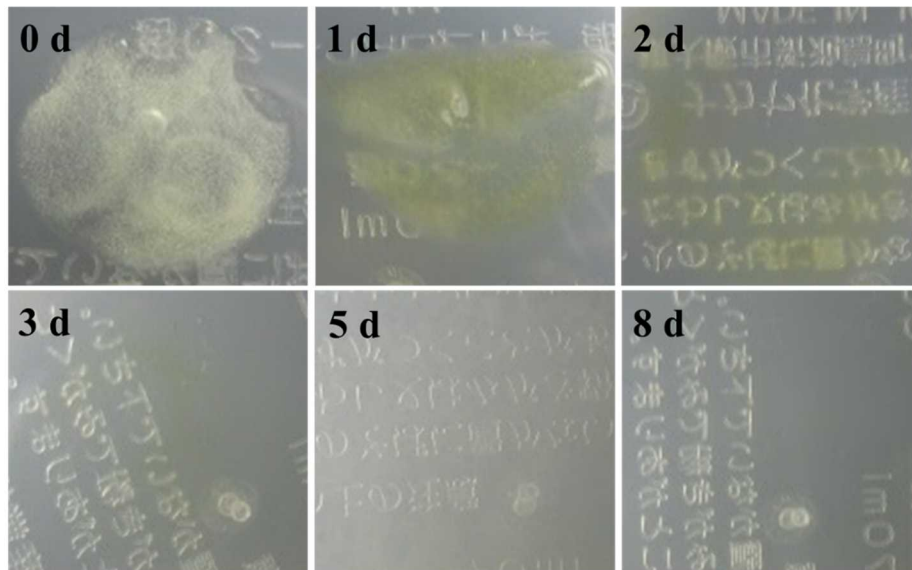
(A)



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(B)



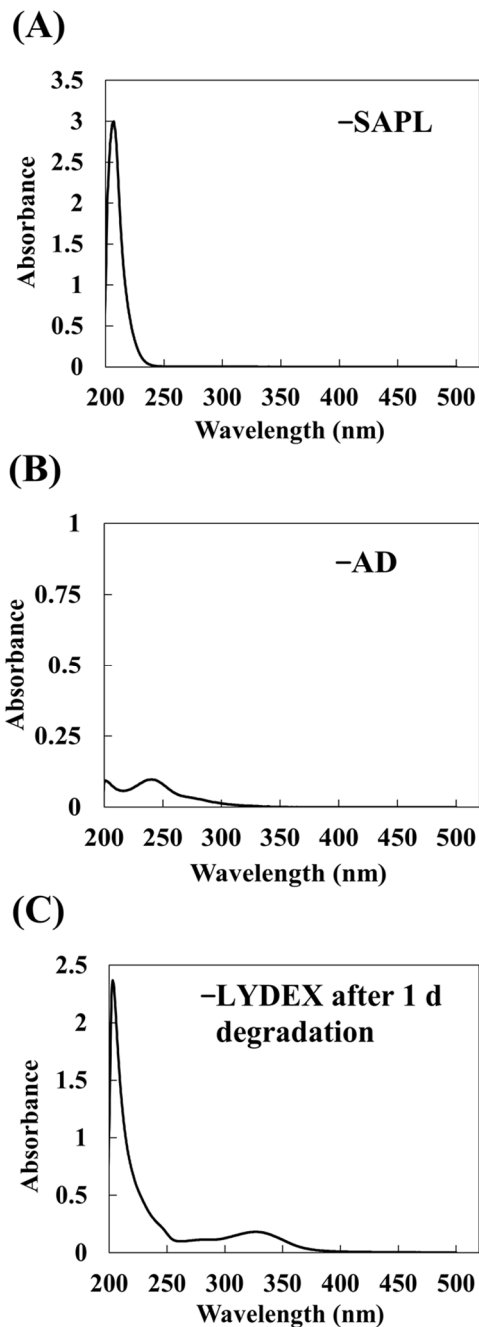
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245 Figure 2. (A) Residual rate of LYDEX gel over time, (B) Degradation of LYDEX gel at 0, 1, 2, 3, 5,
246 and 8 d at 37 °C in saline solution.

247 The absorption spectra of the filtrates of SAPL, AD, and LYDEX after 1 d of degradation are shown
248 in Figure 3. A 210 nm absorption peak is observed for SAPL, whereas AD shows almost no
249 absorption, with only a slight absorption maximum at approximately 240 nm. The 240 nm peak is
250 due to a pH-dependent enol (Drobchenko et al., 1993). In addition, LYDEX immediately after
251 gelation shows absorption only at 210 nm. Conversely, the LYDEX gel after 1 d of degradation
252 shows absorption at approximately 210 and 323 nm. It gradually turns yellow due to the Maillard
253 reaction (Chimpibul et al., 2016; Matsumura et al., 2014), and 323 nm is the absorption of this
254 yellowing. Therefore, we conducted UV detection following GPC at 210 and 323 nm.

255



256

257 Figure 3. Absorption spectra of SAPL, AD, and the LYDEX gel degradation products.

258 The GPC chromatograms detected using refractive index (RI) detection are shown in Figure 4A.

259 There is a small peak at a retention time (RT) of 10.4 min (M_w = more than several million grams

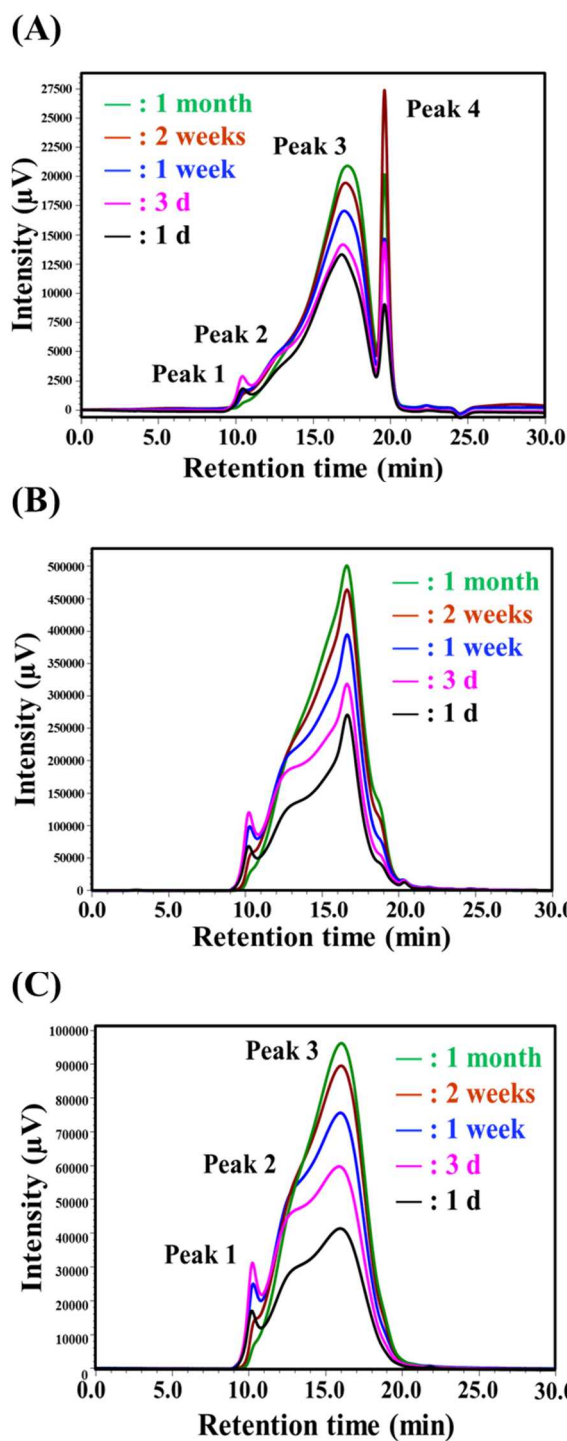
260 per mole), a peak at 12.5 min RT (M_w = 145 900 g/mol), a large peak at 17.0 min RT (M_w = 11 200

261 g/mol), and a sharp peak at 19.6 min RT ($M_w \leq 2300$ g/mol). All peak areas increase with time. For

262 UV detection following GPC at 323 (Figure 4B) and 210 nm (Figure 4C), the peaks at 10.4, 12.5,

263 and 17.0 min RT are observed at both wavelengths, but not the peak at 19.6 min RT. The peak at

264 10.4 min RT represents degradation products of $M_w =$ more than several million grams per mole that
265 are not separated by the TSKgel G4000PW_{XL} column and appear in the exclusion limit. However,
266 the peak at 17.0 min RT is much broader than that at 10.4 min RT, and as shown in Figure 2,
267 LYDEX gel degrades rapidly and then slowly, suggesting that the degradation of LYDEX gel occurs
268 as soon as the gel collapses and solubilizes, up to the M_w peak at 17.0 min RT ($M_w = 11\ 200$
269 g/mol). Meanwhile, there is an increase in the peak at 19.6 min RT ($M_w \leq 2300$ g/mol) with time,
270 and the fraction represented by this peak exhibits no UV absorption, suggesting that the
271 compositions of the fractions with RTs of 19.6 and 17.0 min are different. The M_w s of the
272 degradation products are not uniformly distributed, and after 17.0 min RT, no degradation products
273 with $M_w = 3000$ g/mol are observed up to RT 19.6 min ($M_w \leq 2300$ g/mol).



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Figure 4. Gel permeation chromatograms obtained using G4000PW_{XL} columns, of the products within the LYDEX degradation treatment solution; (A) refractive index detection, (B) ultraviolet (UV) detection at 323 nm, (C) UV detection at 210 nm.

3-3. GPC detection value ratio of each component

To observe the change in the formation behaviors of the LYDEX degradation products over time, the ratios of the three GPC detection values of LYDEX gel are compared with those of the raw materials AD and SAPL (Table 1). Differential refractometry of AD shows a single peak at 15.9 min RT (Mw = 73 000 g/mol, Figure S2), but almost no UV absorption is observed at 323 and 210 nm. AD is a polysaccharide, despite aldehyde group introduction, and the Mw of the starting material, dextran, is 70 000 g/mol, and hence these results are reasonable. The RI of SAPL (Figure S3) displays bimodal peaks at 17.3 (Mw = 11 000 g/mol) and 18.5 min RT (Mw = 3800 g/mol), in addition to UV absorption at 210 nm (Table 1). At 323 nm, very small bimodal peaks are observed. GPC was performed in the same manner for ϵ -PLL without the introduction of carboxyl groups (COOH 0% PLL) and polylysine with an increased carboxylation rate of 65% (COOH 65% PLL). The chromatograms reveal single peaks at 18.3 min RT (Mw = 4300 g/mol) for COOH 0% PLL and 16.9 min RT (Mw = 12 000 g/mol) for COOH 65% PLL. COOH 65% PLL is used as a cryoprotectant for cells (Matsumura & Hyon, 2009). ϵ -PLL is a polyamino acid consisting of 25–35 lysine amino acids that are ϵ -linked. SAPL is prepared by carboxylating 10% of these amino groups using SA. In COOH 65% PLL, 65% of the amino groups are carboxylated by increasing the amount of SA in the reaction. In COOH 65% PLL, the amino and carboxyl groups form aggregates via ionic bonding, and the Mw suggests that it is a trimer. In SAPL, 10% of the amino groups are carboxylated to form approximately 1/3 of the aggregate; thus, it exhibits bimodal peaks. For the

Table 1. Gel permeation chromatography detection value ratios of aldehyde-functionalized dextran (AD), succinic anhydride-treated poly-L-lysine (SAPL), and LYDEX against refractive index (RI) intensity.

	<i>RI detection</i>	<i>Ultraviolet (UV, 323 nm)</i>	<i>UV (210 nm)</i>
AD	1	0.0	0.2
SAPL (front peak)	1	0.1	70
Gel degradation peak	RT 10.4 min	1	13
	RT 12.5 min	1	13
	RT 17.0 min	1	4
	RT 19.6 min	1	0.0

RT, retention time.

The peak area of each component is expressed as a relative value when the RI value is set to 1, calculated using UV intensity / RI intensity (unit: mv).

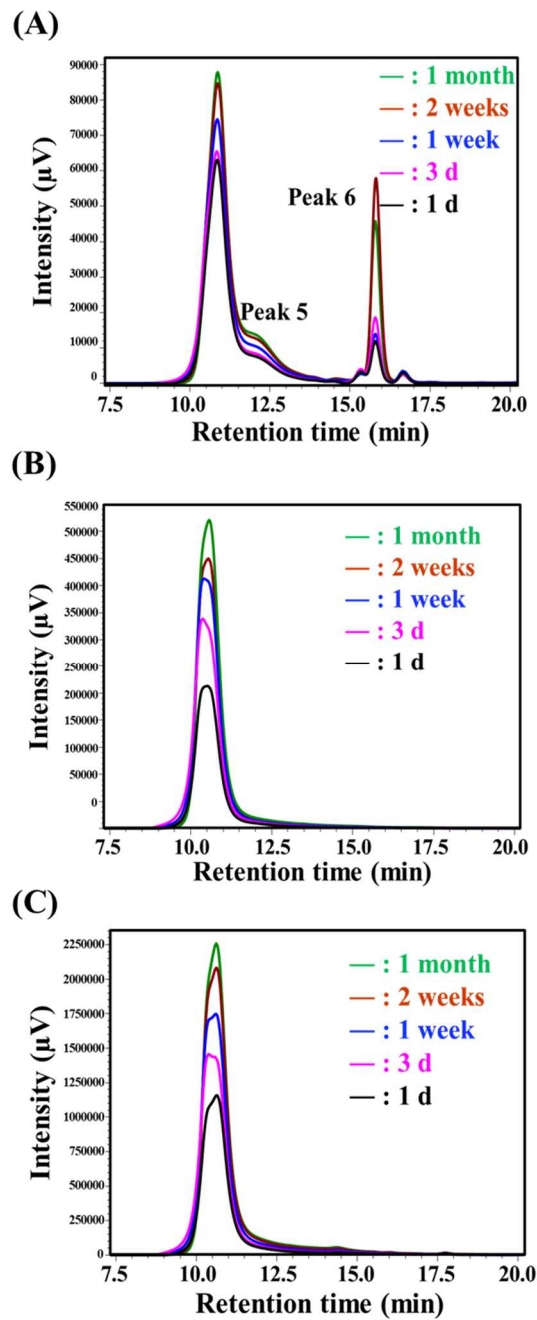
307 degradation products of LYDEX gel, detection of the small peaks at 10.4 min RT using UV at 323
308 and 210 nm is more sensitive than RI detection. Even at 17.0 min RT, UV is more sensitive than RI,
309 but less sensitive than UV at 10.4 min RT. At 19.6 min RT, no UV absorption is observed at 323 nm,
310 and even at 210 nm, the sensitivity is lower than that at 10.4- and 17.0-min RT.
311 Comparing AD and SAPL, only the degradation product of LYDEX gel exhibits UV absorption at
312 323 nm, suggesting that UV absorption occurs following the Maillard reaction of the Schiff base. As
313 there is no UV absorption at 323 nm at 19.6 min RT, there is therefore no Maillard reaction
314 component from the imine bond (Schiff base) in the fraction represented by this peak. In addition,
315 because the Mw of SAPL is 4000 g/mol, the degradation product is an oligodextran structure, similar
316 to a degradation product of AD undergoing the Maillard reaction. Although the initial reaction
317 pathway of the Maillard reaction is long known (Hayashi & Namiki, 1986; Hodge, 1953), the Schiff
318 bases are isomerized via Amadori rearrangement to ketoamines, which are likely connected to the
319 side chains of SAPL.

320

321 **3-4. Temporal behaviors of main degradation products**

322 To evaluate the temporal changes in the small-molecule products within the LYDEX gel degradation
323 treatment solution, GPC was performed using a TSKgel G2500PW_{XL} column (Figure 5).

324



325

326 Figure 5. Gel permeation chromatography, using a G2500PW_{XL} column, of the products within

327 LYDEX degradation treatment solution; (A) refractive index detection, (B) ultraviolet (UV)

328 detection at 323 nm, (C) UV detection at 210 nm.

329

330 Using the TSKgel G2500PW_{XL} column, peaks 5 and 6 at 12.2- and 15.8-min RT, respectively, are

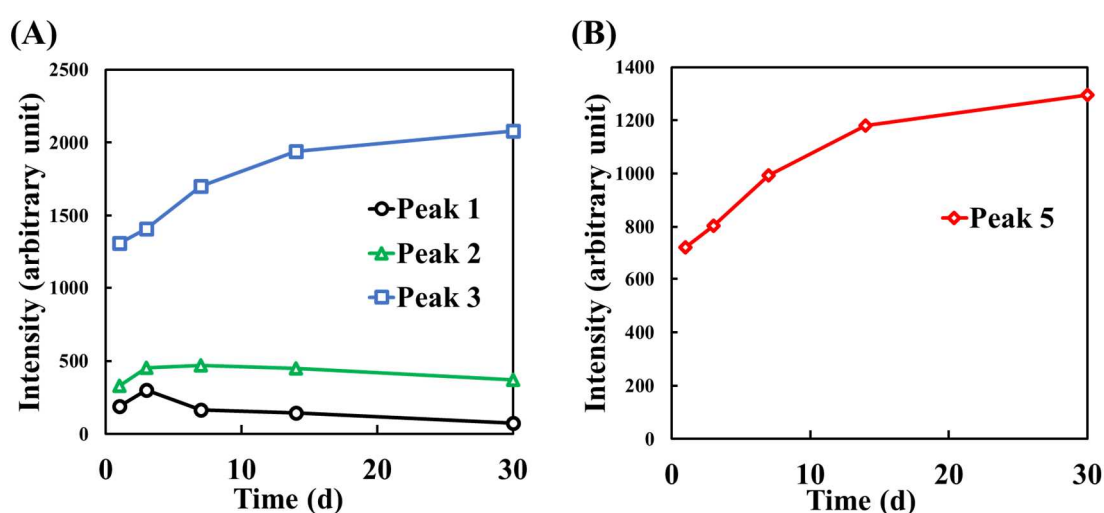
331 the characteristic peaks, in addition to the peak at 10.7 min RT that elutes near the exclusion limit of

332 the column. Of these peaks, 4 and 6 are consistent with the RT of NaCl, and their irregularities,

333 rather than changes over time, suggest that NaCl and low-molecular-weight compounds may elute
334 together.

335 Figure 6 shows the changes in the RI signals of peaks 1, 2, 3, and 5 with increasing degradation
336 time, as shown in Figures 4 and 5. Peak 1 increases initially, but then slowly decreases. The trends of
337 peaks 3 and 5 are similar, with slight increases even after 1 mo. The trend of peak 2 is similar to that
338 of peak 1, increasing initially and remaining constant for a while, but then gradually decreasing. The
339 Mw distribution values for each peak show no significant changes, suggesting the formation of a
340 metastable substance over time (Table S1). First, peak 1 represents macromolecules with Mws of >1
341 million grams per mole, which initially form and then gradually degrade when dissolved. Peak 2
342 represents compounds with Mws of several hundred thousand grams per mole, and their formation,
343 due to the degradation of the gel and compounds represented by peak 1, and their degradation in the
344 dissolved state occur in parallel, gradually decreasing over time. In addition, peak 3 represents
345 compounds with Mws of 10 000–20 000 g/mol, and peak 5 represents compounds with Mws of
346 several thousand, but they are constantly increasing with the convergence to the formation of these
347 compounds during the final stage of degradation.

348 Figure S4 shows the GP chromatogram of the degradation treatment solution (1 wk), obtained using
349 a TSKgel G2500PW_{XL} column. A large peak (10.8 min RT) is observed in the exclusion limit of the
350 column, followed by a gentle peak to a shoulder centered at 11.8 min RT (Mw = 2500 g/mol) and
351 peaks at 14.4, 15.3, 15.8, 16.6, and 17.2 min RT. The fractions below Mw = 2500 g/mol show almost
352 no UV absorption at 323 and 210 nm and represent degradation products of the oligodextran moiety
353



354

355 Figure 6. Changes in the degradation products (main peaks) over time in the degradation treatment
356 solution; (A) Refractive index (RI) detection following elution from the G4000PW_{XL} column and
357 (B) RI detection following elution from the G2500PW_{XL} column.

358

359 generated by degradation, because no Schiff base (C=N bond) is present, as described above.
360 Glucose reacts with proteins to produce glyoxal (Wells-Knecht et al., 1995) (Mw = 58.04 g/mol),
361 which is formed by autoxidation of glucose undergoing the Maillard reaction, glycolaldehyde
362 (Glomb & Monnier, 1995) (Mw = 254.39 g/mol), which is derived from Schiff bases, and
363 methylglyoxal (Thornalley, 1993) (Mw = 72.06 g/mol), 3-deoxyglucosone (Kato et al., 1989)
364 (Mw = 162.14 g/mol), and glucosone (Kawakishi et al., 1990) (Mw = 178.140 g/mol), which are
365 formed by the Schiff base and degradation of Amadori rearrangement products. Therefore, AD-
366 derived Amadori rearrangement products and their degradation products, which are smaller than
367 glucose, suggested to be emerging.

368

369 **3-5. IR spectroscopy**

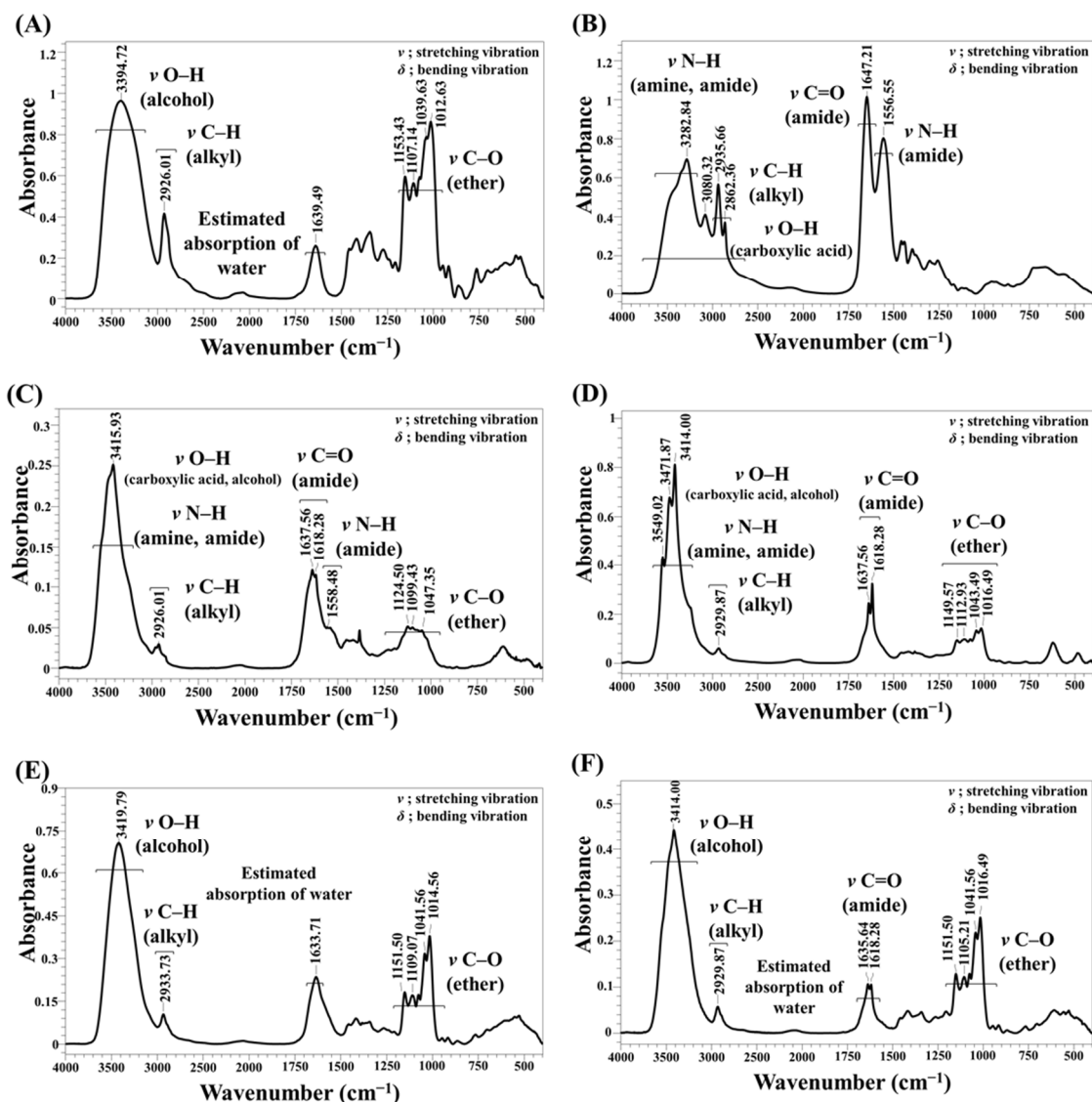
370 The IR spectra of the samples that showed the major peaks after preparative isolation using GPC,
371 dialysis, lyophilization, and concentration are shown in Figure 7. For comparison, AD and SAPL,
372 which are components of LYDEX, were also measured in the same manner. AD exhibits no
373 aldehyde group absorption ($\sim 1700\text{ cm}^{-1}$), instead showing strong ether bond absorption, suggesting
374 that the aldehyde group is converted to a hemiacetal (ether bond) via reaction with water, which is
375 the characteristic absorption. The lack of aldehyde group absorption is consistent with the study by
376 Maia et al. (Maia et al., 2005) and its absence for iodate-oxidized polysaccharides is generally due to
377 the formation of gem-diol with hydrated hemiacetal groups (Sloan et al., 1954).

378 The characteristic IR signals of amides are amide I ($1600\text{--}1800\text{ cm}^{-1}$), amide II (1470--
379 1570 cm^{-1}), amide III ($1250\text{--}1350\text{ cm}^{-1}$), and amide A ($3300\text{--}3500\text{ cm}^{-1}$). SAPL is characterized by
380 strong absorption, particularly at approximately 3400 and 1620 cm^{-1} due to the amide group, which
381 are consistent with the reported signals of PLL (De Campos Vidal & Mello, 2011). The spectrum of
382 the compounds represented by preparative peak 1 shows the characteristic absorptions reflecting the
383 structures of AD and SAPL. For the compounds represented by preparative peak 2, similar to peak 1,
384 the peaks reflecting the structures of AD and SAPL are observed, but the amide II band of SAPL at
385 approximately 1500 cm^{-1} is completely absent, and the ether absorption increased slightly,
386 suggesting that the AD component increased relative to that of the fraction represented by peak 1.

387 For the compounds represented by preparative peak 3, the absorption of amide reduced and the
388 absorption of ether, which reflects the structure of AD, is observed, thereby rendering the spectrum
389 even closer to that of AD than that of peak 2.

390 For the compounds represented by preparative peak 5, as for preparative peak 3, absorption
391 reflecting the structure of AD is strong, and the spectrum is close to that of AD, but the amide
392 absorption due to SAPL ($\sim 1620\text{ cm}^{-1}$) is also observed. In summary, the spectrum of the compounds
393 represented by preparative peak 1 is close to that of SAPL, the spectra of the compounds represented

394 by preparative peaks 3 and 5 are close to that of AD, and the compounds represented by preparative
 395



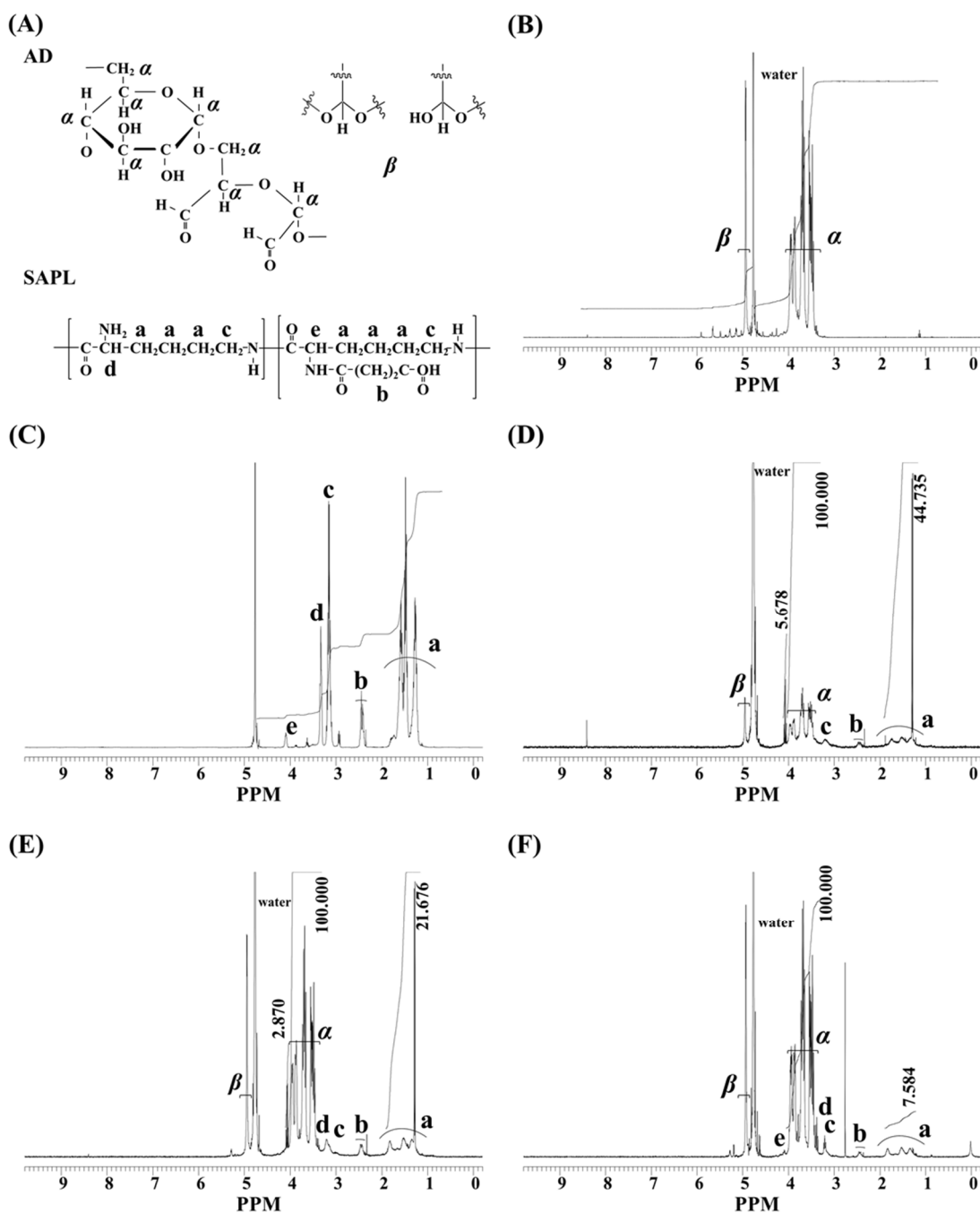
396
 397 Figure 7. (A) Infrared (IR) spectrum of AD. (B) IR spectrum of SAPL. (C) IR spectrum of the
 398 fraction represented by preparative peak 1, (D) peak 2, (E) peak 3, and (F) peak 5.
 399

400 peak 2 include AD and SAPL. Therefore, as the preparative peak shifts from 1 to 2 to 3 and 5 to the
 401 low-molecular-weight region, the structure shifts from relatively rich in the SAPL skeleton to rich in
 402 the AD skeleton. This refers not only to the whole structure but also to the substructure.
 403

404 3-6. NMR spectroscopy

405 The ¹H NMR spectra of the preparative samples represented by peaks 2, 3, and 5 are shown in
 406 Figure 8. For comparison, AD and SAPL, the raw materials of LYDEX, were also measured in the

407 same manner. Preparative peak 1 was not analyzed due to insufficient sensitivity for the small



408

409 Figure 8. (A) Chemical structures of AD and SAPL. (B) ^1H NMR spectrum of AD. (C) ^1H
 410 NMR spectrum of SAPL. (D) ^1H NMR spectrum of the compounds represented by
 411 preparative peak 2, (E) peak 3, and (F) peak 5.

412

413 amount within the preparative volume. AD is characterized by methine and methylene signals
 414 representing protons adjacent to O atoms on the dextran backbone (α in the figure) and signals

415 representing protons between hemiacetals (or acetals) (β in the figure). There are no signals
416 attributed to the aldehyde protons. The C2–C3 and C3–C4 bonds of the glucose moiety are removed
417 by oxidation, suggesting that C2 and C4, which are replaced by aldehydes, react with hydroxyl
418 groups and are converted to the hemiacetal structure (ether bond) (Aalmo et al., 1981; Ishak &
419 Painter, 1978; Yu & Bishop, 1967).

420 SAPL is characterized by signals such as those representing the adjacent methylene on the PLL
421 backbone (a), the methylene adjacent to the carbonyl group on the succinate backbone (b), the
422 methylene adjacent to the imine (c), and the proton on the C atom to which the amino group is
423 attached (d). There are signals attributed to the α proton (e). Matsumura et al. reported the similar
424 structural properties of succinylated PLLs at different carboxylation rates (Matsumura et al., 2013),
425 although the carboxylation rate of SAPL is different from those reported previously.

426 The compounds represented by the preparative peaks 2, 3, and 5 exhibit the characteristic peaks (α ,
427 β , a, b, c, and d) of AD and SAPL in all samples, suggesting that the structure is a hybrid of these
428 skeletons. However, the structures of the degradation products represented by each preparative peak
429 appear to shift from SAPL to AD skeleton-rich from preparative fraction 2 to 3 to 5 to the low
430 molecular weight region, which is consistent with the IR spectra. The methine/methylene group
431 increases from preparative fractions 2 to 3. Figures 7(D) and (E) show that the AD-derived peaks
432 increase as the degradation of LYDEX progresses. The progress of the Maillard reaction deduced
433 using previous studies (Chimpibul et al., 2016) (Figure S1(A)) and the methylene group as one of the
434 components of 3-deoxyosone that undergoes Strecker degradation via Amadori rearrangement
435 (Tressl et al., 1995) suggest that these peaks are due to an AD-derived degradation product.

436 Conversely, as SAPL is stable in water and AD undergoes the Maillard reaction, alteration of the
437 SAPL structure is unlikely, and the SAPL-derived methine/methylene group is observed. Thus, this
438 increase suggests that the degradations of AD and SAPL are related to the Maillard reaction.

439 For preparative fractions 2, 3, and 5, the peak integrals of the glucose-derived signals of AD
440 observed at 3.3–4.0 ppm are set to 100, and the peak integrals of the methylene-derived signals of
441 SAPL observed at 1.2–1.9 ppm are compared. The SAPL/AD ratio is 4:2:1 for the preparative
442 fractions 2:3:5.

443 Therefore, after AD and SAPL form a Schiff base, the main chain of AD is broken via Amadori
444 rearrangement and molecular degradation commences, with AD degrading into Amadori
445 rearrangement products and their degradation products; in addition, the Schiff bases of AD and
446 SAPL are loosely bound via C–N, but SAPL retains most of the PLL structure, and as PLL is stable
447 in aqueous solution (Hiraki, 1995), SAPL remains bound to a section of AD. This AD-bound SAPL
448 becomes a polymer with an Mw of approximately 4000–5000 g/mol, which forms dimeric or
449 trimeric aggregates, remaining undegraded.

450

451

452 **4. Conclusions**

453 To elucidate the degradation mechanism of LYDEX in aqueous media, the main degradation
454 products were analyzed, and the following degradation mechanisms were clarified. The analysis of
455 degradation products using GPC showed that LYDEX became >60% water-soluble in 1 d, with mild
456 subsequent solubilization. Degradation product analysis using GPC, followed by IR and ¹H NMR
457 spectroscopy, revealed products with mixed structures containing SAPL and AD moieties.

458 According to the IR and ¹H NMR spectra, the later the GPC elution, the higher the percentage of the
459 AD moieties, and in the fractions 2, 3, and 5 collected in this study, the SAPL/AD ratio changed
460 from approximately 4 to 2 to 1, respectively, based on the ¹H NMR peak integrals. The methylene
461 group increased from fraction 2 to 3 and was one of the components of 3-deoxyosone that underwent
462 Strecker degradation via Amadori rearrangement, suggesting that it was related to the Maillard
463 reaction.

464 The mixture of AD and SAPL became richer in AD as the Mw decreased. After the main chains of
465 the solubilized degradation products of LYDEX were cleaved via Amadori rearrangement
466 (Figure S1(B)), the structure of the AD-SAPL network, with Mws from several million to
467 145 000 g/mol, was potentially maintained for a while, wherein the SAPL skeleton remained
468 undegraded and the AD portion degraded to oligodextran, with an Mw of > 2300 g/mol. However,
469 the low-molecular-weight fractions of peaks 4 and 6 were not included in this analytical study
470 because of irregular fluctuations that suggested potential contamination by NaCl or other substances.
471 Specifically, peaks 4 and 6 increased with processing time, in addition to other degradation peaks in
472 the GP chromatograms, as shown in Figure 6. In addition, the RTs of these peaks are consistent with
473 that of NaCl and may therefore simply represent NaCl or organic compounds, such as sugars (alone),
474 generated by LYDEX degradation.

475 Although LYDEX gel should not degrade further in saline solution, the *in vivo* degradation and
476 absorption mechanism should be clarified in combination with data from *in vivo* kinetics and
477 implantation studies for clinical application.

478 Macromolecules, such as polyvinylpyrrolidone and albumin, enter cells via endocytosis
479 (Silverstein et al., 1977), and these macromolecules undergo digestion by endosomes formed there,
480 which fuse with lysosomes. A Schiff base-bound Adriamycin-oxidized dextran (using a 70 kDa
481 dextran) complex was used to study the intracellular form of Adriamycin, speculating that it is
482 absorbed into tissue cells via endocytosis and then undergoes low-molecular-weight conversion by
483 lysosomes (Munehika et al., 1994).

484 This suggests that, similar to LYDEX, degradation products formed via gel microfragmentation due
485 to self-degradation and high-molecular-weight degradation products are likely digested *in vivo*
486 through phagocytosis by phagocytes, such as macrophages, absorbed into the spleen and liver, and

487 degraded to low molecular weight via metabolism *in vivo*. When LYDEX gels are applied in the
488 gastrointestinal tract, such as endoscopic wound dressings, AD and AD-derived degradation products
489 may be reduced by enzymes to sugars, such as dextranase and isomaltose, several of which are
490 utilized in the metabolic cycle *in vivo*, and several are eliminated. Regarding the pharmacokinetics of
491 SAPL and SAPL-derived degradation products, low absorption of ¹⁴C-radiolabeled ε-PLL in the
492 gastrointestinal tract was observed in an absorption, distribution, metabolism, and excretion
493 (ADME) study using oral administration (Hiraki et al., 2003), and SAPL and SAPL-derived
494 degradation products may therefore pass through the fecal gastrointestinal tract without absorption.
495 Finally, hydrogels obtained by the reaction of AD with polymers bearing amino groups, such as
496 collagen, have been previously reported. However, the *in vitro* degradation behavior of LYDEX by
497 the reaction of AD with SAPL, as shown in this study, revealed LYDEX self-degradability for the
498 first time. This is extremely valuable for the study of molecular degradation mechanisms of
499 polysaccharide hydrogels and the development of medical applications, such as hemostatic agents,
500 sealants, and anti-adhesion materials.

501

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505

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509

510 **Conflict of interest**

511 Woogi Hyon and Suong-Hyu Hyon are founders of BMG, Inc.

512 Shuji Shibata, Etsuo Ozaki, and Motoki Fujimura are employees of BMG, Inc.

513

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