Fabrication of thermoplastic ductile films of chitin butyrate/poly(ϵ -caprolactone) blends and their cytocompatibility

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ABSTRACT

We fabricate thermoplastic films of chitin butyrate (ChB)/poly(ϵ -caprolactone) (PCL) blends with different degree of miscibility (miscible (M), partially miscible (PM), and immiscible (IM)), and examined the feasibility as a cell scaffold system through evaluating mechanical properties and cytocompatibility. We found a remediation of the brittleness and an increase in ductility of ChB by blending PCL for the M and PM blends. The blend films were subjected to alkaline hydrolysis (2-M NaOH/37 °C/48 h) with expectation of the improvement of the surface hydrophilicity and cell accessibility. ATR-FTIR spectroscopy of the alkaline-treated PM and IM films revealed that PCL component and ester side-chains

of acyl chitin were selectively removed from the surface domain. L929 fibroblast cells well adhered and proliferated on these films. Therefore, the materials possess a great potential for the utilization as a thermoplastic cell scaffold in tissue engineering by adequate selection of the degree of miscibility and post treatment.

Keywords: chitin butyrate / poly(ϵ -caprolactone) / blend / tensile property / cytocompatibility

1. Introduction

Chitin has a large potential for developing specially functionalized materials in medical and sanitary fields, because of its excellent biological properties such as biodegradability, biocompatibility, and antibacterial and wound healing activity. Despite its promising potential, the major imperfections of chitin are poor thermal processability and less film ductility albeit its stiffness, which limit its application in tissue engineering (Jayakumar, Prabakaran, Nair, & Tamura, 2010; Khor & Lim, 2003; Muzzarelli, 2009). Meanwhile, poly(ϵ -caprolactone) (PCL), approved as biodegradable and biocompatible polyester, has high ductility, but its poor hydrophilicity, slow degradation kinetics, and no natural cell recognition sites greatly restrict its application in the biomedical field.(Prabhakaran et al., 2008) Various kinds of physicochemical and surface modification techniques have been used to overcome these shortcomings (Dubas, Kittitheeranun, Rangkupan, Sanchavanakit, & Potiyaraj, 2009; He et al., 2012; Jeong, Jun, Choi, Nho, Lee, & Shin, 2008; Zhu, Gao, Liu, & Shen, 2002). Even though PCL has attracted interest as a blending partner of chitinous (Chen, Sun, & Ren, 2005; Honma, Senda, & Inoue, 2003; Sarasam & Madihally, 2005; Senda, He, & Inoue, 2002; Wan, Wu, Cao, & Dalai, 2008) to obtain more advanced biocompatible materials by complementing each other's contradictory features, it seems to

53 have been difficult to obtain their physically tough intimate mixtures on the nanoscopic
54 scale.

55 Recently, we have successfully attained a series of thermodynamically miscible blends
56 of such a combination (Sugimoto, Kawahara, Teramoto, & Nishio, 2010). Namely, we
57 prepared solvent-soluble acyl chitin derivatives having a normal acyl group and
58 investigated effects of the side-chain length (side-chain carbon number, N ; 2–6) and the
59 degree of substitution (DS) on the miscibility of solution cast blends with PCL. NMR
60 analysis quantitatively demonstrated the acylations not only for C3/C6 hydroxyl protons but
61 also for C2 amino proton(s), where amide-DS for the N -acylation and ester-DS for the
62 O -acylation were determined as the average numbers of acyl substitution associated with
63 C2-NHCOR/C2-NH₂ and C3-OH/C6-OH, respectively, per glucosamine residue of chitin.
64 It was revealed that the acetate ($N = 2$) was immiscible with PCL even in the highly
65 acetylated state of total-DS (= amide-DS + ester-DS) = 3.8, while other acyl chitin products
66 ($N = 3$ –6) showed miscibility with PCL when totally high-substituted grades of them
67 (total-DS ≥ 3) were used. The degree of miscibility was enhanced definitely with an
68 increase in ester-DS, but it made less correlation with amide-DS. Fig. S1 summarizes the
69 result of the miscibility estimation, clearly indicating that the degree of miscibility was
70 adaptable by N as well as by DS.

71 In the present study, we examined the feasibility of using chitin butyrate (ChB, $N =$
72 4)/PCL (50/50 in weight) based films prepared by hot-pressing as a thermoplastic flexible
73 cell scaffold system. The chitin derivative ChB has recently been evaluated for biomedical
74 applications using its fibrous forms prepared via a dissolution-regeneration process
75 (Draczynski, Bogun, Mikolajczyk, Szparaga, & Krol, 2013; Muzzarelli et al., 2005; Pant et
76 al., 2013). Contrastively, in this study, the thermoplasticity was introduced to the chitinous
77 component by intimately combining PCL as an external plasticizer, potentially providing the
78 scaffold of varied three-dimensional forms. We investigated how the blend miscibility

influences the tensile mechanical property of the thermally molded films. Actually, however, mechanical properties of blend systems are sometimes deteriorated for the combinations of high degree of miscibility. Meanwhile, in general, the film toughness and ductility can be accomplished by high dispersion of microcrystallite and introduction of cross-linkages. Therefore, we will discuss the mechanical performance of the blend films in connection not only with miscibility but also with the crystallinity of the PCL component and scale of homogeneity of the blend components. Subsequently, we performed an alkaline treatment (2-M NaOH at 37 °C for 48 h) for the thermally molded blend films, expecting the improvement of the surface hydrophilicity and accessibility for cells because of a possible enrichment of the surface with bioactive chitinous compound. We characterized surface morphology and chemical compositions of the alkaline-treated films and explored the cytocompatibility of mouse fibroblast cells (L929) using the blend films.

2. Experimental

2.1. Materials

As our preceding paper (Sugimoto et al., 2010), an original material of chitin isolated from crab shells (Fluka 22720 Chitin Lot & Filling code: 405226/1 12900) was according to a Hackman's method (Hackman, 1954). The nominal molecular weight of the chitin material is 400,000 and the viscosity average degree of polymerization (DP) was determined to be 380 (Sugimoto et al., 2010). The degree of deacetylation (DD) of the purified chitin was 5.0 %, determined by infrared spectroscopy (Sannan, Kurita, Ogura, & Iwakura, 1978). PCL with a nominal weight-average molecular weight of 70,000–100,000 was purchased from Wako Pure Chemical Industries, Ltd., and it was used after purification by dissolution in tetrahydrofuran (THF, Wako Pure Chemical Industries, Ltd.) and reprecipitation into distilled water. Other solvents and chemicals used in this study were purchased from

Wako Pure Chemical Industries, Ltd.; these were all of guaranteed reagent grade and used without further purification.

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2.2. Preparation of chitin butyrate

The purified chitin was acylated with a butyryl chloride in *N,N*-dimethylacetamide (DMAc)-LiCl solution containing triethylamine in a manner similar to that adopted in the previous studies (Kusumi, Inoue, Shirakawa, Miyashita, & Nishio, 2008; Nishio, Matsuda, Miyashita, Kimura, & Suzuki, 1997; Sugimoto et al., 2010). ¹H NMR data were used for the determination of a total degree of substitution (total-DS), amide-DS, ester-DS, and DD, where amide-DS and ester-DS denote the average number of butyryl substitution associated with C2-NHCOR/C2-NH₂ and C3-OH/C6-OH, respectively, per glucosamine residue of chitin and total-DS is the sum of amide-DS and ester-DS. These quantifications were conducted in the same way as that adopted in an earlier study (Sugimoto et al., 2010). Table 1 tabulates the four parameters evaluated for the three ChB products prepared in this study. In this table, the highest total-DS (3.45) may seem strange. However, it is correct because there occurred not only amidation of amine groups but also replacements for *N*-acetate with another butyryl group, which was supported by a combination of spectroscopic analyses of FT-IR and NMR (Sugimoto et al., 2010). In fact, the amide-DS was estimated to be >1 for the all ChB samples used in the present study. Eventually, DD values were determined to be 39–72 %, raising noticeably from an initial value 5 % for the purified chitin. Hereafter, the obtained ChB of total-DS = *x* and ester-DS = *y* is encoded as ChB_{*x*(*y*)}. A weight-average DP of ChB_{3.45(1.86)} was determined to be 212 by gel permeation chromatography with THF as eluent (Sugimoto et al., 2010).

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Table 1 Characterization of the ChB samples used in the present study.

Sample code	total-DS	ester-DS	amide-DS	DD	<i>T_g</i> /°C	miscibility*
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				(%)		with PCL
ChB _{2.34(1.19)}	2.34	1.19	1.15	39	134	IM
ChB _{2.93(1.76)}	2.93	1.76	1.16	46	125	PM
ChB _{3.45(1.86)}	3.45	1.86	1.59	72	97	M

130 *Abbreviations M, PM, and IM indicate miscible, partially miscible, and immiscible,
131 respectively.

132

133 2.3. Blend preparation

134 ChB/PCL blends in 50/50 in a weight percent ratio were prepared in film form from mixed
135 polymer solutions by solvent evaporation with N,N-Dimethylformamide (DMF) as a
136 common solvent. 3-wt% solutions of ChB and PCL were prepared separately and mixed
137 with each other in 50/50 in a weight percent ratio. The as-cast samples were washed with
138 distilled water and dried at 40 °C under vacuum for more than 3 days. Subsequently, film
139 sheets were prepared by using a SA-302 hot-pressing apparatus (Tester Sangyo Co., Ltd.).
140 For the molding, (i) the as-cast samples were first sandwiched between two Teflon release
141 sheets with a stainless spacer, 0.1-mm thick; (ii) the set was inserted between two stainless
142 plate and pressure was applied at 180 °C to each molten blend gradually to reach 5 MPa in
143 3 min; after that, it was increased quickly to 15 MPa, and this application was maintained for
144 30 s; (iii) as soon as the pressure was released, the sample was another compressing
145 apparatus and quickly cold-pressed at 25 °C and 15 MPa for 2 min. After being released
146 again from the compressed state, the molded polymer sheet was finally conditioned at 23
147 °C and 50 % RH for 2 weeks.

148

149 2.4. Measurements

150 DSC was performed by using a Hitachi High-Tech Science Corporation
151 DSC6200/EXSTAR6000 apparatus. The measurements were carried out on 7-mg film

152 samples under a nitrogen atmosphere after calibrating the temperature readings with an
153 indium standard. The samples were first heated from $-150\text{ }^{\circ}\text{C}$ to $200\text{ }^{\circ}\text{C}$ at a scanning rate
154 of $20\text{ }^{\circ}\text{C}/\text{min}$ (first heating scan) and then immediately quenched to $-150\text{ }^{\circ}\text{C}$ at a rate of ~ 80
155 $^{\circ}\text{C}/\text{min}$. The second heating scans were run from $-150\text{ }^{\circ}\text{C}$ to $200\text{ }^{\circ}\text{C}$ at a scanning rate of
156 $20\text{ }^{\circ}\text{C}/\text{min}$. For estimation of the glass transition temperature (T_g) of each sample, the
157 midpoint of the discontinuity in heat flow was recorded using the second heating scan.
158 The melting temperature (T_m) and the enthalpy of fusion (ΔH_f) were determined from the
159 maximum and the area of the melting endotherm, respectively, in the first heating scan.

160 Wide-angle X-ray diffraction (WAXD) measurements were made for the film specimens
161 with a Rigaku Ultima-IV diffractometer at $20\text{ }^{\circ}\text{C}$ in a reflection mode. Nickel-filtered $\text{CuK}\alpha$
162 radiation was used at 40 kV and 40 mA. The diffraction intensity profiles were collected in
163 the range of $2\theta = 10\text{--}30\text{ }^{\circ}$.

164 Solid-state NMR experiments were carried out at $20\text{ }^{\circ}\text{C}$ with a Varian Unity INOVA 400
165 WB NMR apparatus operated at a ^{13}C frequency of 100.6 MHz, using the aforementioned
166 film specimens of PCL and blend samples. The magic angle spinning rate was 15 kHz.
167 ^{13}C CP/MAS spectra were measured with a contact time of 7.0 ms. A 90 ° pulse width of
168 $2.8\text{ }\mu\text{s}$ was used with 2048 FID signal accumulations. In quantification of proton
169 spin-lattice relaxation times (T_1^{H}), a contact time of 1 ms was used and a proton
170 spin-locking time τ ranged from 0.1 to 1 s. 1024 scans were accumulated for the T_1^{H}
171 measurement.

172 For tensile tests, specimens were cut from the sheet of blends to dumbbell-type test
173 pieces (JIS K6251: length and width of the narrow portion, 12 mm and 2 mm, respectively)
174 with a thickness of 0.1 mm. Tensile behavior was examined for these specimens at $23\text{ }^{\circ}\text{C}$
175 by using a Shimadzu Autograph AGS-5kNG. The strain rate and span length were 0.75
176 mm/min and 15 mm, respectively. Three specimens of each blend sample were employed
177 for the measurement and the averaged data were adopted.

178 Contact angle was measured with an optical contact angle meter (Kyowa Interface
179 Science Co., Ltd., Drop Master 500) by $\theta/2$ method at 25 °C. Distilled water (1.5 μ L) was
180 dropped carefully onto the surface of the blend sheets. An average value was obtained by
181 measuring the same sample at five different positions.

182 The surface morphology of the specimens was observed using a field emission
183 scanning electron microscopy (FE-SEM) (S-4800, Hitachi High-Technologies Corporation)
184 at 1.5 kV. The samples were coated with Pt by sputtering.

185

186 *2.5. Alkaline treatment*

187 Three 1-square-cm specimens cut from the above thermally molded sheet was placed in a
188 test tube containing 2-M NaOH aqueous solution. The alkaline hydrolysis was carried out
189 at 37 °C for 48 h in a rotary shaker (100 rpm). After that, the specimens were taken out
190 and washed thoroughly with ethanol, then dried in vacuo at 23 °C until their weights
191 reached constancy. Those specimens of the respective ChB/PCL blends were evaluated
192 for the weight loss in the alkaline hydrolysis. The surface composition of the specimens
193 was estimated by using a Shimadzu IR Prestige-21 equipped with a Shimadzu AIM-8800
194 infrared microscope combined with an attenuated total reflection (ATR) prism made of Ge.

195

196 *2.6. Cytocompatibility test*

197 L929 fibroblast cells (European Collection of Cell Cultures) derived from mouse were
198 cultured on the specimens of the original ChB and PCL and their blends before and after
199 alkaline hydrolysis, in Eagle's minimal essential medium (Eagle's MEM, Nissui
200 Pharmaceutical Co.) at 37 °C in a humidified atmosphere of 95-% air and 5-% CO₂. 1000
201 mL of the Eagle's MEM included 15 mL of supplemental mixture solution containing
202 10.0-wt% fetal bovine serum (FBS, Sigma Co.), 3-wt% L-glutamine, and 8-wt% sodium
203 hydrogen carbonate. Prior to the cell adhesion studies, all films were sterilized by

immersing in Eagle's MEM containing Kanamycin for 5 h. The L929 cells were seeded onto the sterilized specimens at a population density of 1.0×10^5 cells/mL for the cell-adhesion investigations. After cultivation, the films were gently rinsed twice with phosphate buffered saline to remove cells weakly adhered on the surface. Subsequently, the specimens were soaked in the 2.5 wt% glutaraldehyde solution at 4 °C for 12 h, and then dehydrated with 10-min exposures to successive 30, 50, 70, 90, and 100 % concentrations of ethanol.

211

212 **3. Results and discussion**

213

214 *3.1. Sample overview*

215 Table 1 lists the characterization data for the ChB samples used in the present study as well
216 as their miscibility with PCL. Blend pairs of these ChB samples were classified into
217 immiscible (IM), partially miscible (PM), and miscible (M) ones in their amorphous states by
218 differential scanning calorimetry (DSC). The thermograms of the second heating scan
219 were shown in Fig. S2. The immiscibility was judged from an observation of no significant
220 shift in glass transition temperature (T_g) for both components. For M blends, a single T_g
221 was detected and varied between the T_g values of the two constituent polymers, depending
222 on the blend compositions. PM blends gave rise to just a slight shift in the T_g of the ChB
223 component but an elevation in the PCL T_g . The T_g shift for PM and M was prominent only
224 at compositions rich in ChB, as has been experienced before,^[14] while 50/50 blend samples
225 were used in the present study.

226 Fig. 1 demonstrates the visual appearances for the film specimens of ChB_{3.45(1.86)} and
227 50/50 (in weight) blends in different miscibility states. For preparing the specimens,
228 solution cast blend films were hot-pressed at 180 °C and cut into dumbbell-type test pieces
229 for tensile measurements. The ~0.1-mm-thick film specimens were visually homogeneous

230 and exhibited transparency. The highest transmittance was observed for the ChB_{3.45(1.86)}
 231 film (78 % at 550 nm). The transparency of ChB was diminished by blending PCL, which
 232 usually gives white opaque thermally-molded sheets due to coexistence of macroscopic
 233 crystalline texture and amorphous domain. The transmittance at 550 nm decreased in the
 234 order of miscibility and was estimated to be 52, 39, and 24 % for the M, PM, and IM blends,
 235 respectively. This result may reflect a heterogeneity in the scale of more than ~100 nm
 236 depending on the degree of miscibility, but it is difficult to simply explain the tendency
 237 because the visual appearance would be varied not only by a mixing scale of the blend
 238 components but also with the difference of the PCL crystalline organization. The
 239 yellow-brownish color observed for the IM blend might be derived from relatively less
 240 thermoplasticity of the ChB component (ChB_{2.34(1.19)}).

242
 243 **Fig. 1.** Visual appearance of the hot-press molded (180 °C) films of ChB_{3.45(1.86)} and IM,
 244 PM, and M blends (ChB/PCL = 50/50 in weight).

246 3.2. Crystallinity

247 For the miscibility judgment by estimating T_g of the blends, we conducted DSC analysis
 248 (Fig. S2) in the second heating scan after rapid cooling (~80 °C/min) from a molten state.
 249 On the other hand, the film specimens provided for the tensile testing preserved another

250 thermal history (stored at 23 °C for 2 weeks after thermal molding). All DSC thermograms
 251 (Fig. 2a) in the first heating scan for the specimens show a melting endotherm and no
 252 cold-crystallization exotherm, indicating sufficient development of PCL crystal in the storage
 253 period.
 254

255
 256 **Fig. 2.** (a) DSC thermograms (first heating scan) and (b) WAXD profiles of ChB_{3.45(1.86)}, IM,
 257 PM, and M blends (ChB/PCL = 50/50 in weight), and PCL, measured for their film
 258 specimens stored at 23 °C for 2 weeks after thermal molding.
 259

260 Using the DSC thermograms illustrated in Fig. 2a, the crystallinity index X_c can be
 261 calculated per net weight of PCL component, from the following equation:

$$262 \quad X_c = \Delta H_m / (0.5\Delta H_m^0) \quad (1)$$

263 where $\Delta H_m^0 = 136$ J/g (Khambatta, Warner, Russell, & Stein, 1976) is the heat of fusion of
 264 100 % crystalline PCL and 0.5 is the weight fraction of the PCL component. The result is

265 listed in Table 2. As shown there, both the T_m and X_c of the blends decreased with
 266 increasing degree of miscibility. In general, the decreasing action can be primarily
 267 ascribed to a morphological effect such as lowering of the size of crystallites for all the
 268 blend pairs. In addition, for the M and PM blend systems, there may be a contribution of
 269 the so-called thermodynamic diluent effect (Scott, 1949) by the acyl chitin, even though
 270 quantitative analysis of isothermal crystallization behavior is necessary to discuss in detail.
 271 However, the possible highest diluent effect for the M blend probably gave rise to the
 272 largest range of reduction for the T_m and X_c .

273

274 **Table 2** Thermal and crystallographic property of ChB/PCL blends (50/50 in weight) and
 275 PCL, measured for their film specimens stored at 23 °C for 2 weeks after thermal molding

Sample code	$T_m/^\circ\text{C}$	$\Delta H_m/\text{J/g}$	$X_{c(\text{PCL})}$ (%)	D/nm
M blend	58	20.6	30.3	25.4
PM blend	59	23.4	34.5	11.6
IM blend	61	32.2	47.4	33.7
PCL	65	92.5	68.0	29.4

276

277 Fig. 2b displays WAXD profiles obtained at 23 °C for the film specimens with the
 278 identical thermal history (stored at 23 °C for 2 weeks after thermal molding). For the
 279 unblended PCL sample, three distinctive diffraction peaks observed at $2\theta = 21.4^\circ$, 22.0° ,
 280 and 23.7° , which are indexed as (110), (111), and (200), respectively (Chatani, Okita,
 281 Tadokoro, & Yamashita, 1970). The average crystallite size of the PCL component was
 282 estimated from the full-width half maximum (FWHM) of a WAXD peak at $2\theta = 21.4^\circ$ derived
 283 from the (110) plane using the Scherrer's formula:

$$284 \quad D = 0.94 \lambda / (\beta \cos\theta) \quad (2)$$

285 where λ ($= 0.154$ nm) is the wavelength of the X-ray used, β is the FWHM of the peak and
 286 $\cos\theta$ is the Bragg diffraction angle. The results of the calculation were tabulated in Table 2.
 287 As seen in this table, the D decreased by blending the ChB having more or less miscibility
 288 with PCL, and the value was specifically small for the PM blend. This decrease indicates
 289 an overall reduction in size and/or regularity of the formed PCL crystal for these blends.
 290 The difference in dimension and dispersion of the PCL crystallites was also suggested by a
 291 contrast between spherulitic morphologies of M and PM blends (see Supporting
 292 Information, Fig. S3). Eventually, for the PM blend, the PCL crystallite with small
 293 correlation length, in other words, the microcrystallite of PCL was well dispersed in the
 294 molded blend films.

295

296 *3.3. Quantification of domain size by T_1^H measurement*

297 In order to further elucidate the microscopic morphological situation in several tens of
 298 nanometers, we tried to evaluate a mixing scale of the blend components using solid-state
 299 NMR. As is well established, ^1H spin-lattice relaxation time (T_1^H) measurements in
 300 solid-state ^{13}C NMR spectroscopy provide information about some microphase structure of
 301 polymers in a scale of a few tens of nanometers. ^{13}C CP/MAS spectra and the peak
 302 assignments are shown in Fig. 3. The relaxation process was monitored for the peak
 303 intensities of C3/C4/C5 pyranose carbons and C4' methyl carbon of the ChB component
 304 and for those of C3"/C4" and C5" methylene carbons of the PCL component. T_1^H values
 305 can be obtained practically by fitting the carbon resonance intensity to the following
 306 exponential equation

$$307 \quad M(\tau) = M_0 (1 - 2 \exp(-\tau / T_1^H)) \quad (3)$$

308 where $M(\tau)$ is the magnetization intensity observed as a function of the spin-locking time τ
 309 and the initial intensity is given by $M(0) = -M_0$. As a general rule, if two polymer
 310 components are in a homogeneous mixing state on the scale over which ^1H spin diffusion

311 can take place in a time T_1^H , the T_1^H values for different protons belonging to the respective
 312 components may be equalized to each other. The result of the T_1^H measurements is
 313 summarized in Table 3. In the M and PM blends, the T_1^H of ChB diminishes appreciably
 314 while that of PCL rather rises, so that these values almost coincide with each other. Such
 315 an attunement was not observed for the IM blend. It is therefore reasonable to assume
 316 that the two constituent polymers in the M and PM blends are coexistent in a range where
 317 the mutual ^1H -spin diffusion is permitted over a period of the respective homogenized T_1^H ,
 318 e.g., 1.00–1.13 s.
 319

320
 321 **Fig. 3.** Solid-state ^{13}C CP/MAS NMR spectra of ChB_{3.45(1.86)}, M blend, and PCL.
 322

323 **Table 3** Values of T_1^H /s for ChB_{2..93(1.76)}, ChB/PCL (50/50 in weight) blends, and PCL

Sample code	ChB		PCL		L/nm
	pyranose	C4'	C3''/C4''	C5''	
	C4/C5/C3	(13.2 ppm)	(24.7	(28.2 ppm)	
		(74.7 ppm)	ppm)		

ChB _{2..93(1.76)}	1.41	1.37	-	-	-
M blend	1.07	1.12	1.13	1.11	43.9–52.1
PM blend	1.06	1.00	1.03	1.04	42.4–50.4
IM blend	1.35	1.40	0.96	0.96	-
PCL	-	-	0.90	0.98	-

An effective path length L of the spin diffusion in a time T_1^H is given by the following equation (McBrierty & Douglass, 1981):

$$L \approx (6D T_1^H)^{0.5} \quad (4)$$

where D is the diffusion coefficient, taken to be $4\text{--}6 \times 10^{-16} \text{ m}^2/\text{s}$ for PCL (Huang & Yang, 2005). Substitution of the T_1^H data obtained above into this equation leads to the assessment of L as an upper limit of the heterogeneous domain size in the relevant blends. By simple application of this relation with the T_1^H data, the diffusion path lengths are calculated as $L = 43.9\text{--}52.1$ and $42.4\text{--}50.4$ nm for the M and PM blends, respectively. Thus, these blends were found to be actually homogeneous with an upper limit of ca. 50 nm in the T_1^H measurements. The homogeneity of the amorphous domain for the M blend has already been ensured being on a couple of tens nanometers by the T_g detection with DSC. In the PM blend, it follows that the homogeneity lies on a certain scale between 20 and 50 nm.

3.4. Tensile mechanical properties

Fig. 4 shows the stress-strain curves obtained for the specimens of the ChB_{3.45(1.86)}, PCL, and 50/50 blends of ChB/PCL at 23 °C. The numerical values determined by the tensile test were listed in Table 4. Basically, original mechanical properties of the blend components are in contrast with each other: stiff and brittle for ChB of relatively high strength and modulus; flexible and ductile for PCL with low modulus. The results revealed

345 that there was essentially no improvement for the IM blend pair due to poor interfacial
 346 adhesion between the blend components. On the other hand, for the M and PM blends,
 347 we found a remediation of the brittleness and an increase in ductility of ChB by blending
 348 PCL. However, the tensile properties of these blend films were not simple arithmetic
 349 average of the original materials, but were strikingly influenced by the degree of miscibility.
 350 Namely, the elongation at rupture for the PM blend films was estimated to be > 200 %,
 351 which was extraordinarily larger than that for the M blend. The PM blend also exhibited a
 352 lower Young's modulus in comparison with the other blends.
 353

354
 355 **Fig. 4.** Stress-strain curves for ChB_{3.45}(1.86), IM, PM, and M blends (ChB/PCL = 50/50 in
 356 weight), and PCL.

357
 358 **Table 4** Tensile mechanical properties of ChB_{3.45}(1.86), ChB/PCL (50/50 in weight) blends
 359 in various miscibility states, and PCL, measured for their film specimens stored at 23 °C for
 360 2 weeks after thermal molding. **Numbers shown in parentheses are standard deviations.**

Sample code	Young's	Yield stress	Elongation	Stress at
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	modulus /MPa	/MPa	at rupture (%)	rupture /MPa
ChB _{3.45(1.86)}	631 (20)	-	3.5 (0.3)	24.3 (1.6)
M blend	445 (16)	16.9 (0.2)	28.1 (3.0)	15.0 (1.8)
PM blend	170 (3)	7.9 (0.1)	215 (26)	12.7 (1.5)
IM blend	425 (8)	-	2.7 (0.3)	15.2 (0.4)
PCL	285 (16)	13.9 (0.7)	>333	>13.9

361

362 Such contrasting results for the M and PM blends can be explained as follows. For the
363 M blend system, an interface region can be taken to be absent on a scale of larger than 20
364 nm. However, because of a possible interaction between the chitinous and PCL
365 molecules, a restriction of the deformation of the PCL molecules might give rise to a
366 moderate improvement of the ductility, even if the T_g of the blend is lower than the test
367 temperature. On the other hand, for the PM blend, there might be a balance in crystallite
368 dimension and domain size, favorable for the improvement of ductility: (1) the ductility might
369 be improved by the miniaturization of the PCL crystallite as an effective physical
370 cross-linker and/or nanofiller and (2) the scale of mixing between 20 and 50 nm might bring
371 about appropriate interaction between the ChB and PCL molecules so as not to interfere
372 with the flexibility of PCL but to provide a domain as if acting as cross-linking.

373 Accordingly, we found that a striking difference in the mechanical property for the
374 ChB/PCL blends was produced by the variation of the degree of miscibility based on the
375 alteration of DS, where the PM blend exhibited the highest ductility.

376

377 3.5. Alkaline treatment and cytocompatibility

378 We examined an alkaline treatment (2 M NaOH at 37 °C) for the thermally molded films of
379 the ChB/PCL blends, expecting the improvement of the surface hydrophilicity and

accessibility by cells. When 48 h passed, even though the IM blend film became too brittle to remain the initial shape after the alkaline treatment, the other two blends maintained their original film forms. The weight loss values were 0.83, 14.2, and 76.0 wt% for the M, PM, and IM blends respectively. The M, PM, and IM blends before the alkaline treatment showed contact angle values of $88.0 \pm 6^\circ$, $85.0 \pm 2.7^\circ$, and $88.5 \pm 1.5^\circ$ for distilled water, respectively. After the alkaline treatment, a decrease in the contact angle values was observed for the M and PM blends ($74.7 \pm 0.9^\circ$ and $67.2 \pm 1.4^\circ$, respectively), which indicates an improvement of surface hydrophilicity for these samples by the alkaline treatment. However, it was difficult to obtain the data for the IM after the alkaline hydrolysis due to its brittleness.

To disclose what caused the weight loss and contact angle variation, the surface composition was examined by microscopic ATR-FTIR. Fig. 5 exemplifies the spectra. In ATR spectroscopy, the penetration depth (d_p) is defined as the distance from the germanium ATR prism-sample interface where the intensity of the evanescent wave decays to $1/e$ (approximately 37 %) of its original value. The d_p is expressed by

$$d_p = \lambda / 2\pi n_1 / (\sin^2 \theta - (n_2 / n_1)^2)^{0.5} \quad (5)$$

where θ is the angle of incidence of the light beam, λ the wavelength of infrared-light absorption, n_1 the refractive index of the ATR prism used, and n_2 the refractive index of the sample. In the present study, the d_p value can be estimated as ca. 0.17–0.41 μm for the spectra (4000–1600 cm^{-1}), if we substitute $\theta = 45^\circ$ and $n_1 = 4$ (for Ge), with a tentative value of $n_2 = 1.5$. The actual sampling depth d_s is defined by the full absorption of the evanescent wave and known to be about $3d_p$. Therefore, the spectra illustrated in Fig. 5 reveals the depth-dependent distribution of the chemical compositions ranging from 0.5 to 1.2 μm in depth for the film samples.

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Fig. 5. Microscopic ATR-FTIR spectra of film specimens (I) before and (II) after the alkaline hydrolysis (2-M NaOH at 37 °C for 48 h): (a) chitin, (b) ChB_{3.45(1.86)}, blends of (c) IM, (d) PM, and (e) M, and (f) PCL before the alkaline treatment; (g) IM, (h) PM, and (i) M blends after the alkaline treatment. ChB/PCL ratio of the original blend materials was 50/50 in weight.

411

412 In the spectra of the original materials (Fig. 5 (I)), specific absorption bands were
413 present in the spectral region associated with OH ($3100\text{--}3700\text{ cm}^{-1}$) and amide I (1655
414 cm^{-1}) for chitin (a) and with ester C=O (1720 cm^{-1}) for PCL (f), respectively. For
415 ChB_{3.45(1.86)} (b), remarkable developments in absorption were observed in 1740 cm^{-1} and
416 1710 cm^{-1} , being assigned to the introduced C=O of ester and N(COR)₂ moieties,
417 respectively (Sugimoto et al., 2010), although the former band shifted to the higher
418 wavenumber in comparison with the ester C=O band of PCL. The smaller wavenumber of
419 the signal of C=O for PCL is likely due to the crystallinity. In common with the observation
420 for the highly substituted ChB of total-DS > ~3 in our previous study (Sugimoto et al., 2010),
421 we found a suppression in the amide I band, as manifested for the spectrum (b) of
422 ChB_{3.45(1.86)}. Meanwhile, the spectra (c) and (e) of the IM and M blends, respectively,
423 before the alkaline hydrolysis appeared similar to that of PCL (f), suggesting the surface
424 segregation of the PCL component for the hot-press molded films.

425 In Fig. 5 (II), we also demonstrate the spectra (g)–(i) for the blend films after the alkaline
426 hydrolysis. Those three spectra can be classified into two groups according to the shape.
427 Namely, in the spectra of the IM (g) and PM (h) blends after the hydrolysis, we can see the
428 strong bands ascribable to chitinous OH ($3100\text{--}3700\text{ cm}^{-1}$) and amide I (1655 cm^{-1}) and a
429 moderate one at 1720 cm^{-1} of ester C=O. The ester C=O band was probably originated
430 with the PCL component because the band location is similar to the one of the plain
431 polyester (f). Therefore, for the surface region of these two alkaline-treated blend films up
432 to $\sim 1.2\text{ }\mu\text{m}$ in depth, we can assume a formation of chitin by an almost complete removal of
433 the butyryl moiety of ChB and an appreciable elution of the PCL component. These
434 observations are consistent with the noticeable weight loss by the alkaline hydrolysis, as
435 has been mentioned above. On the other hand, the spectrum for the alkaline-treated film
436 of the M blend (i) seems like that of the original blend (e). Taking into consideration the

437 much less weight loss (0.83 wt%) by the alkaline treatment for the M blend, there was just a
438 slight effect on the chemical composition for the film specimen by the alkaline treatment.

439 Fig. 6 displays FE-SEM images of the blend films, which were acquired before and after
440 the alkaline hydrolysis over a period of 48 h. The as-prepared films showed an essentially
441 smooth surface with lines originating from the surface shape of Teflon substrates for
442 thermal molding. On the other hand, the surface morphology of the alkaline-treated films
443 was basically rough and exhibited three different geometries depending on the miscibility.
444 Namely, we can see several radial textures on the surface of the M blend film (a), which
445 might be the spherulitic textures exposed by an etching effect after the removal of the
446 chitinous and amorphous PCL components by the alkaline treatment. This observation is
447 consistent with the microscopic ATR-FTIR data, where the PCL component is mainly
448 present on the surface. For the PM blend (b), the surface after alkaline treatment
449 exhibited relatively random asperity on larger scale. By comparing enlarged images of the
450 M and PM blends, a remarkable eluviation was observed for the latter one. According to
451 the above ATR-FTIR data, it may be reasonable to assume that what we see is mainly
452 composed of chitin. On the other hand, the IM blend (c) presented a further random and
453 inhomogeneous surface with aggregation of granular particles, as shown in the enlarged
454 one.

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Fig. 6. FE-SEM images of (a) M, (b) PM, and (c) IM blends (the original ChB/PCL weight ratio, 50/50) before and after the alkaline treatment.

To evaluate cell adhesion and proliferation, L929 fibroblast cells were seeded on the blend films. In Fig. 7, FE-SEM micrographs demonstrate the morphologies of cells that were cultured for 48 h on the as-pressed films of ChB_{3.45(1.86)}, PCL, and the blends as well as the alkaline-treated ones of the blends. On the films without the alkaline treatment, some of the cells were spread and flattened, but many were still round, and this indicated an absence of strong cell-films interaction. On the other hand, for the alkaline-treated blend films except the M blend, the adherence of cells to the surfaces was densely distributed in comparison with those without the alkaline treatment. This observation indicates the evidence of the anchoring ligands reaching out to help support the cells on the

469 film surface of the alkaline treated PM and IM blend films.
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471
 472 **Fig. 7.** FE-SEM images of cell growth on the films of (a) ChB_{3.45}(1.86), (b) PCL, and (c) M,
 473 (d) PM, and (e) IM blends (the original ChB/PCL weight ratio for the blends, 50/50). The
 474 images of the blend films (c)–(e) include the ones after the alkaline treatment. Scale bars
 475 denote 20 μm.

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 477 Resultingly, we consider that the high porosity of these two alkaline-treated films
 478 provides more structural space for cell accommodation and makes the exchange of nutrient

479 and metabolic waste between the scaffold and environment more efficient. In addition, the
480 surface exposure of the chitinous component for the PM and IM blend films alkaline-treated,
481 confirmed by the ATR-FTIR analysis, not only improve the hydrophilicity of the films but also
482 provide the necessary active sites through which other biocompatible components such as
483 proteins, polysaccharides, cell growth factors, or peptides can be further immobilized.

484 From this evidence, the cell adhesion on the thermoplastic ChB/PCL blend films can be
485 improved via alkaline-hydrolysis and the important factor was the degree of miscibility for
486 the original blend films in order to promote cell attachment, cell proliferation, and proper cell
487 spreading. In the present study, it can be concluded that the PM blend is the most
488 appropriate scaffold from the balance of the specific ductile nature and the cytocompatibility
489 after the alkaline treatment.

490

491 **4. Conclusions**

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493 In the present study, thermoplastic transparent films of ChB/PCL blends were successfully
494 fabricated by hot-press molding. Even though no modification effect in mechanical
495 property was observed for IM blend, we found a remediation of the brittleness and an
496 increase in ductility of ChB for the M and PM blend with PCL. In particular, the PM blend
497 exhibited the highest ductility, which may be ascribable to a balance in the dimension and
498 dispersion of the PCL microcrystallites and the domain size of the blend components.
499 Subsequent alkaline treatment gave rise to the high porosity and the surface exposure of
500 chitinous component for the PM and IM blend films. Cell culture imaging confirmed that
501 the PM and IM blend after the alkaline treatment were the best in promoting the cell
502 attachment and proliferation. Taking into consideration the ductility and cytocompatibility,
503 the PM blend was a good candidate for use as thermoplastic scaffolding materials capable
504 of wide application.

505

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