Solid-State NMR Studies of Polysaccharide Systems

Jiří Spěváček, Jiří Brus*

Summary: Two polysaccharide systems were studied by solid-state NMR methods: (*i*) *Chitin/glucan complexes*. The ^{13}C NMR spectra have shown that in samples isolated from the mushroom *Pleurotus* sp., the glucan content was always higher in stems than in pilei. While carbonyl lineshape in complex isolated from *Aspergillus niger* mycelium shows similar hydrogen bonding as in neat chitin, a significantly higher amounts of hydrogen bonding between carbonyl groups of chitin and hydroxy groups of glucan was found in complexes isolated from *Pleurotus* sp. (*ii*) *Biodegradable starch/polycaprolactone (PCL) blends*. From the relaxation times $T_1(H)$ and $T_{1\rho}(H)$ it follows that blends starch/PCL, starch/ester oligomers and starch formate/ester oligomers are phase-separated even on the scale 20–110 nm. On the contrary, starch formate/PCL blend is phase-separated on the scale 1–9 nm but homogeneously mixed on the scale 20–90 nm. Therefore formylation of starch significantly improves its miscibility with PCL.

Keywords: biodegradable polymers; chitin/glucan complex; polysaccharides; solid-state NMR; starch/polycaprolactone blends

Introduction

Polysaccharides represent a significant group of biopolymers and solid-state NMR can provide important information in their structural characterization, similarly as with synthetic polymers. Two different systems containing polysaccharides which fall in the category of glucans (polymers of glucose) were investigated by solid-state NMR methods in the present study. The first one are chitin/glucan complexes isolated from the fruit bodies of edible mushroom *Pleurotus* sp. It is well known that fungal cell walls contain, besides chitin, hemicelluloses and mannans, also β-glucans. In last 15 years increased attention has been devoted to β-glucans isolated from the cell walls of fungi, which can act as nonspecific modulators of the immune system and have found applications as immunoadjuvants, antitumor and radioprotective agents.[1] Such structural features as β-$(1\rightarrow3)$linkages in the backbone of the glucan, and additional β-$(1\rightarrow6)$ branching points are needed for antitumor activity. Higher-molecular-weight glucans have been reported to be more effective against tumors than those of low molecular weight.[2,3] Significant reduction of cholesterol and glucose in blood due to β-glucans has been also described.[4] In fungi glucans form water-insoluble complexes with chitin. Until now very little information exists on the structure of these complexes.[5] Therefore, in the present study we tried to contribute to the structural characterization of insoluble chitin/glucan complexes isolated from the mushroom *Pleurotus* sp. and *Aspergillus niger* mycelium by solid-state ^{13}C NMR spectroscopy.

The second system discussed in the present article are biodegradable starch/polycaprolactone (PCL) blends. Research on biodegradable polymers has received increasing attention in recent years and PCL is one of the most often investigated systems. However, since this polymer is still expensive and does not match all the

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technical requirements for possible applications, its blends with starch, which is a cheap abundant resource, are also investigated (cf. references in ref. [6]). The miscibility in these blends is one of the most relevant parameters related to blend properties. In the present study, the results of the measurements of ^{1}H spin-lattice relaxation times T_1(H) and ^{1}H spin-lattice relaxation times in the rotating frame $T_{1\rho}$(H), as obtained using ^{13}C CP/MAS NMR spectroscopy on starch/PCL and starch formate (starch modified by formylation)/PCL blends,[6] are discussed from the point of view of the blend morphology (domain size). Similar approach was recently used to characterize the morphology in starch/crosslinked poly(acrylic acid) blends[7] or in investigations of the blends of PCL with other synthetic polymers.[8–10]

Experimental Part

Samples

Chitin/glucan complexes were isolated from the mushroom *Pleurotus* sp. as insoluble fraction after extraction according to the modified procedure of Freimund et al.,[11] which involves extraction with 80% ethanol, boiling water and subsequent extraction with a 1M NaOH.[12] Dried mycelium of *Aspergillus niger* was deproteinised and then deacetylated under alkaline conditions. Samples of neat α-chitin (Fluka), chitosan (Fluka) and β-glucans isolated from the yeast were also studied for comparison.

Native wheat starch was provided by Roquette (France). Starch formate with degree of substitution ~1.2 was prepared from the dry native wheat starch and 99% of aqueous formic acid (Sigma Aldrich) by the procedure described elsewhere.[13,14] Polycaprolactone (PCL) Capa 6800 (molecular weight $M_w = 80\,000$) was provided by Solvay. Ester oligomers (1,6-hexane-diol adipate and phthalate; molecular weight $M_w = 2\,700$) were provided by Durez. The starch (or starch formate)/PCL (or ester oligomers) blends were extruded with a twin screw extruder (Brabender, DSK 42/6) controlled by a Lab-Station with a screw rotation speed of 30 rpm and temperatures of respectively 85, 90 and 95 °C from hopper to slot die (4'50 mm^2). The neat starch, starch formate as well as starch (starch formate)/PCL (ester oligomers) blends were dried under vaccuum for 8 h at 50 °C before measurements. After this treatment, a water content close to 1% was measured by modified Karl Fischer method (ISO 5381:1983) both in the neat starch (starch formate) and blend samples studied.

NMR Measurements

Solid-state ^{13}C CP/MAS (cross polarization/magic angle spinning) NMR spectra were measured at ambient temperature on a Bruker Avance 500 spectrometer at 125.8 MHz with spinning frequency 8–11 kHz and contact time 2 ms, i.e., under conditions that allow quantitative analysis.[15,16] All investigated systems are rather rigid and the only component where some interference between the higher mobility and CP might hypothetically exist is amorphous phase of PCL or ester oligomers. However, the fact that for the neat PCL the ^{13}C CP/MAS NMR spectrum is very similar to the fully relaxed ^{13}C MAS spectrum measured without CP with 45° pulses and 1200 s relaxation delay[17] shows that this is not the case. Chemical shifts in the ^{13}C NMR spectra were referred to the carbonyl line of glycine (with a signal at 176.0 ppm from TMS) by sample replacement. The ^{1}H rotating-frame spin-lattice relaxation times $T_{1\rho}$(H) and spin-lattice relaxation times T_1(H) were measured at ambient temperature via ^{13}C detection from ^{13}C CP/MAS NMR spectra. The experimental scheme with a variable spin-lock time in the range 0.1–10 ms after the proton signal excitation followed by constant contact time was used in $T_{1\rho}$(H) measurements; the proton spin-locking field in frequency units was 80 kHz. T_1(H) values were measured using the combination of cross-polarization and saturation recovery pulse sequence.

Results and Discussion

Chitin/Glucan Complexes

Figure 1 shows ^{13}C CP/MAS NMR spectra of the neat α-chitin (Fluka) and neat β-glucan isolated from the yeast. The assignment of signals to various carbon types follows that in the literature.[18,19] The signal at 33 ppm is due to impurities, residual proteins and/or lipids.[19,20] In consequence of the fact that chitin is also polymer of glucose derivative, *N*-acetylglucosamine, the range of the resonances of C1-C6 carbons (55–104 ppm) is similar in both systems, though not identical. However, for chitin it is characteristic the presence of acetyl groups on C2 carbons which are manifested by the separate signals of methyl and carbonyl carbons. These signals then can be used to determine the composition of chitin/glucan complexes.

^{13}C CP/MAS NMR spectra of chitin/glucan complexes, isolated from the stem and pileus of the mushroom *Pleurotus* sp., are shown in Figure 2. The comparison of these spectra with those obtained for neat α-chitin, chitosan and β-glucans, as well as with the spectra of neat chitin and β-glucans recently reported in the literature,[18–20] confirmed that investigated samples are really chitin/glucan complexes and enabled us to determine their composition. Higher relative intensities of methyl and carbonyl signals found for the sample isolated from

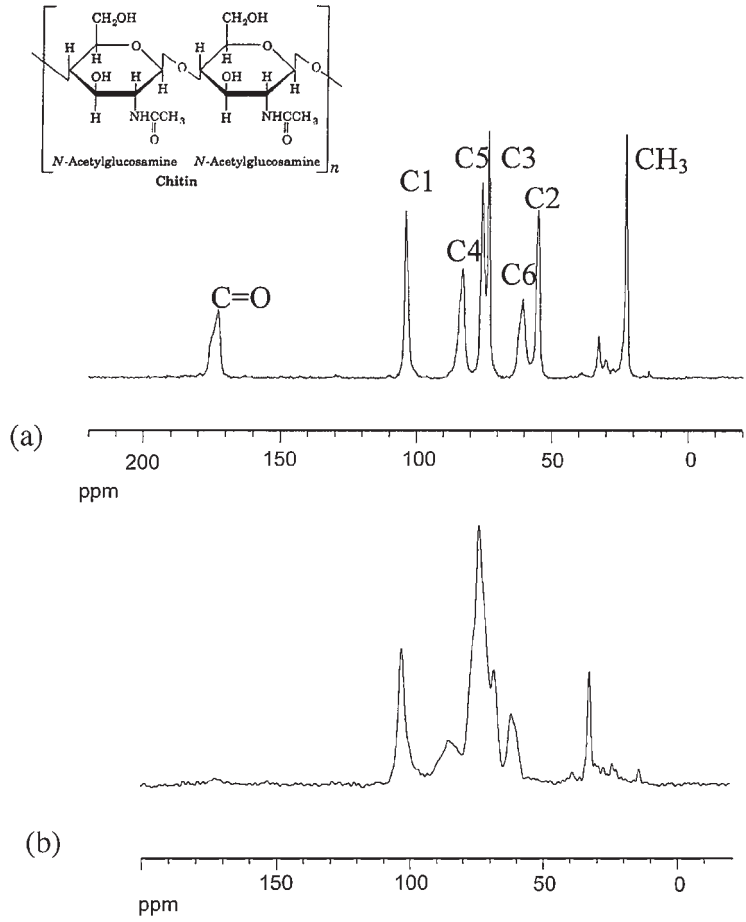

Figure 1.
^{13}C CP/MAS NMR spectra of neat α-chitin (Fluka) (a) and β-glucan isolated from yeast (b).

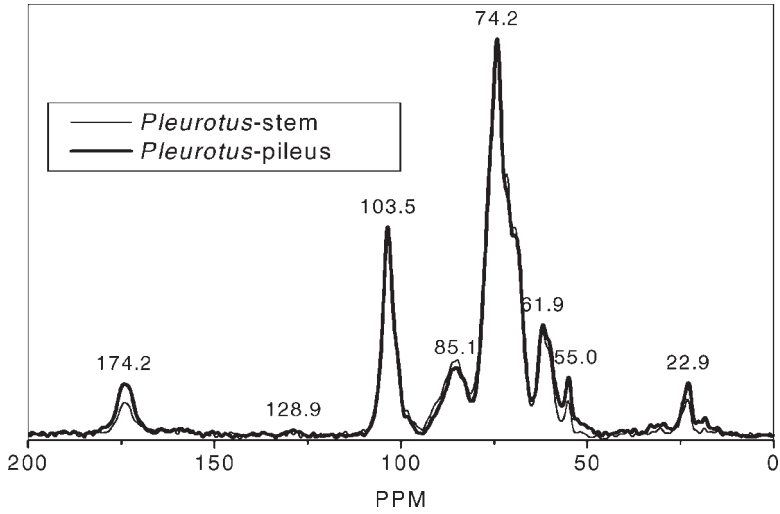

Figure 2.
^{13}C CP/MAS NMR spectra of chitin/glucan complexes isolated from the stem (thin line) and from the pileus (thick line) of the mushroom *Pleurotus* sp.

the pileus indicate a higher amount of the chitin component, i.e., a higher amount of β-glucans exists in the stem of *Pleurotus* sp. mushrooms. In the ^{13}C CP/MAS NMR spectrum of neat chitin measured with contact time 2 ms (cf. Figure 1a) the integrated intensities of all carbon types, including methyl carbons, are shown correctly. We assumed that this holds also for chitin/glucan complex and its composition was calculated using a formula

$$\text{mol\% chitin units } (N\text{–acetylglucosamine}) = [I_{CH3}/(I_{\Sigma}/6)] \times 100 \quad (1)$$

where I_{CH3} is the integrated intensity of the signal of methyl carbons at 22.9 ppm and I_{Σ} is the integrated intensity of the range 50–110 ppm where C1-C6 carbons of both components resonate. For the samples isolated from the stem and pileus, as shown in Figure 2, we obtained 12 mol% and 25 mol% of the chitin units, respectively. The finding that the content of β-glucans is higher in stems than in pilei was confirmed by ^{13}C NMR for a series of samples isolated from the mushrooms *Pleurotus* sp. and *Agaricus blazei* and it is in accord with results of enzymatic analysis (Megazyme enzymatic kit).[21]

Interesting information on hydrogen bonding and structures in investigated samples follows from the expanded carbonyl region of ^{13}C NMR spectra as depicted in Figure 3. Already for the neat α-chitin the asymmetric lineshape indicates the presence of two components with chemical

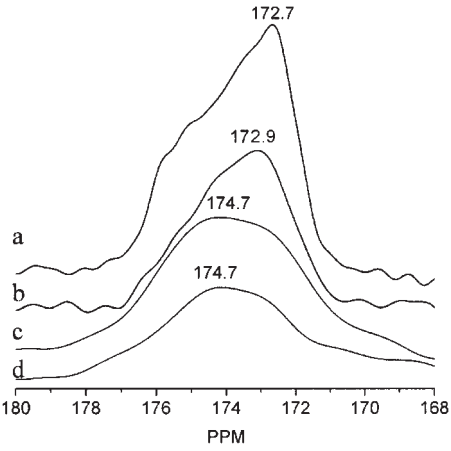

Figure 3.
Expanded carbonyl region in ^{13}C CP/MAS NMR spectra of neat α-chitin (a), chitin/glucan complex isolated from the *Aspergillus niger* mycelium (b), chitin/glucan complex isolated from the pileus (c) and stem (d) of the mushroom *Pleurotus* sp.

shifts that differ by ~2 ppm. Kameda et al. assigned these two components to carbonyl groups hydrogen bonded exclusively to the NH groups (more intensive component at 172.7 ppm) and to carbonyl groups hydrogen bonded also to hydroxy groups (less intensive component with larger chemical shift).[18] Assuming Lorentzian lineshapes, the ratio 0.38 : 0.62 was obtained by us after deconvolution for integrated intensities of both components, in accord with ref.[18] The carbonyl lineshape detected for the chitin/glucan complex isolated from the *Aspergillus niger* mycelium is similar to that for the neat chitin showing that hydrogen bonding is not significantly influenced by the presence of the glucan component. This result can indicate for this complex the grafted structure as suggested recently by Tarabukina et al.,[5] where both components are probably linked by 1,3-β-D-glucopyranoside covalent bonds, without forming mutual hydrogen bonds significantly. On the contrary for the samples of chitin/glucan complexes isolated both from the stem and pileus of the mushroom *Pleurotus* sp. the intensity of the carbonyl component with 2 ppm larger chemical shift is by 34% higher in comparison with the neat chitin; the ratio 0.51 : 0.49 was obtained for integrated intensities of both carbonyl components after deconvolution. This result suggests that in this case the carbonyl groups of the chitin component take part in the stabilization of the chitin/glucan complex by forming hydrogen bonds with hydroxy groups of the glucan component in significant amount. A larger density of hydrogen bonds between chitin and glucan also suggests a rather parallel arrangement of both components in the respective chitin/glucan complex. The obtained results indicate relatively large structural diversity of chitin/glucan complexes in fungal cells of various origin.

Biodegradable Starch/PCL Blends

In Figure 4 ^{13}C CP/MAS NMR spectra of the native starch, starch formate, starch/PCL (40/60) blend, starch formate/PCL (40/60) blend and neat PCL are shown. The signals of the starch and PCL were assigned in accord with the literature.[8–10,22,23]

The signal of C4 carbons of starch is reported to be characteristic for the amorphous glassy phase because it was not detected in hydrated, highly crystalline starch samples.[23] For neat PCL and PCL component in blends, signals of methylene carbons I and II are split into two lines corresponding to crystalline and amorphous components.[17] From integrated intensities of two signal components of PCL I and II carbons it follows that degree of crystallinity of PCL component in starch/PCL and starch formate/PCL blends is virtually the same as in neat PCL and amounts to ~63–66%. For neat PCL this value agrees well with the degree of crystallinity determined by DSC,[9] so supporting that ^{13}C CP/MAS NMR spectra show essentially correct signal intensities (cf. Experimental Part, NMR Measurements). From Figure 4 it follows that in blend samples the signals of starch and PCL components are well separated and therefore ^{1}H spin-lattice relaxation times T_1(H) and spin-lattice relaxation times in the rotating frame $T_{1\rho}$(H) can be measured via ^{13}C detection from a series of ^{13}C CP/MAS NMR spectra.

The approach used to characterize the molecular morphology (domain size) of investigated blend samples is based on the ^{1}H spin-diffusion. The maximum diffusive path length L by spin-diffusion in three dimensions for a time T_i is approximately given as[24]

$$L = (6DT_i)^{1/2} \quad (2)$$

where D is the spin-diffusion coefficient and T_i is either $T_{1\rho}$(H) or T_1(H) according to the relaxation experiment. For the spin-diffusion coefficient a value of 8×10^{-16} m^{2}s^{-1} has been found in poly(methyl methacrylate)/polystyrene block copolymers; this value is expected to be typical for rigid organic systems.[25] For the spin-diffusion coefficient in mobile amorphous PCL (in rubbery state at room temperature) a lower value can be expected.[24,26] A value $D = 0.5 \times 10^{-16}$ m^{2}s^{-1} is probably a reasonable estimate.[27]

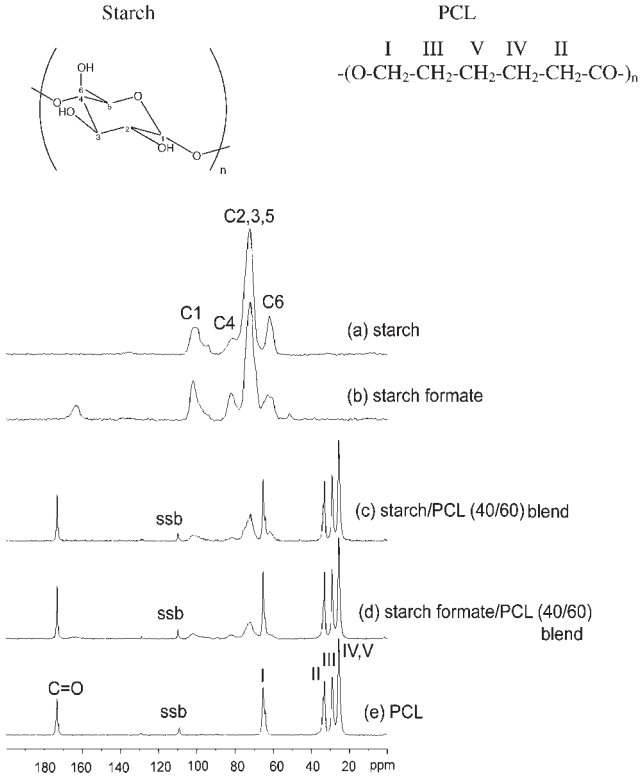

Figure 4.
^{13}C CP/MAS NMR spectra of the native starch (a), starch formate (b), starch/PCL (40/60) blend (c), starch formate/PCL (40/60) blend (d) and neat PCL (e); ssb means spinning sideband.[6]

In Table 1 the values of spin-lattice relaxation times in the rotating frame $T_{1\rho}$(H) are shown. We always could fit the relaxation curves by one component. $T_{1\rho}$(H) values determined from an analysis of various carbon lines of the starch or starch formate were equal within experimental error due to the spin-diffusion mechanism[6] and therefore only the mean values as obtained for the signals of C1, C2,3,5, C4 and C6 carbons are shown in Table 1. From Table 1 it follows that in blends the $T_{1\rho}$(H) values of the starch formate component (and to a certain extent also of the starch component) are shorter in comparison with values in neat samples. This indicates that molecular mobility of the starch formate (and perhaps also of the starch) is somewhat higher in blends in comparison with neat samples. For PCL carbons only the mean $T_{1\rho}$(H) values as obtained for III and IV,V carbons are shown because for I and II carbons, where separated signals were detected for the crystalline and amorphous

Table 1.
^{1}H spin-lattice relaxation times in the rotating frame $T_{1\rho}$(H) of starch (or starch formate) and PCL (or ester oligomers) in investigated blends and neat components

Sample	$T_{1\rho}$(H)[a] (ms)	
	Starch	PCL
starch	6.9	–
starch formate	4.7	–
PCL	–	24.5
ester oligomers	–	46.5
starch/PCL (60/40)	5.3	29.2
starch/PCL (40/60)	4.9	24.4
starch formate/PCL (60/40)	2.5	29.0
starch formate/PCL (40/60)	2.3	28.3
starch/ester oligomers (40/60)	4.8	34.0
starch formate/ester oligomers (40/60)	2.6	37.1

[a] Estimated error ±10%.

phase, the different $T_{1\rho}$(H) values (~35 ms and 12 ms) were found for the crystalline and amorphous phase, respectively, both for neat PCL and PCL component in blends.[6] This means that the sizes of the crystalline and amorphous domains of PCL are larger than the maximum diffusive path length L and therefore the $T_{1\rho}$(H) values are not equilibrated over the whole system. For the $T_{1\rho}$(H) experiment a scaling factor 1/3 should appear in parenthesis in Eq. (2), due to spin-lock field and faster spinning[28], and therefore $L \cong 8$ nm and 1 nm for the crystalline and amorphous PCL phase, respectively. Similar situation follows from Table 1 for domain size in investigated blend samples. From Table 1 it follows that $T_{1\rho}$(H) values are markedly different when determined from analysis of signals of starch (or starch formate) carbons in comparison with those determined from PCL (or ester oligomers) carbon signals. These results show that in all investigated blends both components are separated into domains of which the length scale exceeds the maximum diffusive path length $L \approx 1$–9 nm.

To investigate the molecular miscibility on the larger scale, the spin-lattice relaxation times T_1(H) were measured, again using a selective carbon detection. Also in this case the relaxation curves were well fitted by one component. The values T_1(H) as determined from relaxation curves of various starch or starch formate carbons do not significantly differ; the same holds for T_1(H) determined from signals of various PCL (or ester oligomers) carbons[6] showing that the spin-diffusion process is effective within domains formed by single components of the blends. Therefore only the mean T_1(H) values as obtained for starch (starch formate) C1-C6 carbons and PCL (ester oligomers) I–V carbons are shown in Table 2. From Table 2 it follows that for the blends starch/PCL, starch/ester oligomers and starch formate/ester oligomers T_1(H) of both components differ from one another. These results suggest that in these blends both components are phase separated even on the larger scale of 20–110 nm (cf. Eq. (2)). A different behaviour was found from

Table 2.
^{1}H spin-lattice relaxation times T_1(H) of starch (or starch formate) and PCL (or ester oligomers) in investigated blends and neat components

Sample	T_1(H)[a] (s)	
	Starch	PCL
starch	1.4	–
starch formate	2.6	–
PCL	–	1.1
ester oligomers	–	1.5
starch/PCL (40/60)	1.6	1.1
starch formate/PCL (40/60)	1.6	1.6
starch/ester oligomers (40/60)	1.8	1.5
starch formate/ester oligomers (40/60)	2.5	1.6

[a] Estimated error ±10%.

T_1(H) values for starch formate/PCL blend. From Table 2 it follows that in this blend the same T_1(H) values were determined for starch formate and PCL components. At the same time, the T_1(H) value found from signals of starch formate carbons is shorter in comparison with the neat starch formate and the value found from signals of PCL carbons is longer in comparison with the neat PCL, showing that the spin-diffusion process is sufficient to equilibrate T_1(H) values. Therefore it is possible to conclude that in starch formate/PCL blend both components are homogeneously mixed on the scale 20–90 nm and that formylation of starch significantly improves its miscibility with PCL.

Conclusion

Two polysaccharide systems were studied by solid-state NMR methods which we applied to investigate their composition and interactions on the one hand (chitin/glucan complexes) and phase structure on the other hand (biodegradable starch/PCL blends):

Chitin/glucan complexes. The ^{13}C CP/MAS NMR spectra enabled us to characterize the composition and structure of the complexes. In samples isolated from the mushroom *Pleurotus* sp., the glucan content was always higher in stems than in pilei. Analysis of the carbonyl region of the chitin

component in complex isolated from the *Aspergillus niger* mycelium corresponds to the grafted structure where both components might be linked virtually only by covalent bonds.[5] By contrast, analysis of the same region in samples isolated from the mushroom *Pleurotus* sp. shows higher amounts of hydrogen bonding between carbonyl groups of chitin and hydroxy groups of glucan, indicating a rather parallel arrangement of both components in the complex.

Biodegradable starch/PCL blends. ^{1}H spin-lattice relaxation times $T_1(H)$ and spin-lattice relaxation times in the rotating frame $T_{1\rho}(H)$ were measured via ^{13}C detection from ^{13}C CP/MAS NMR spectra to characterize the blend morphology (domain size). From the $T_{1\rho}(H)$ and $T_1(H)$ values it follows that blends starch/PCL, starch/ester oligomers and starch formate/ester oligomers are phase-separated even on the scale 20–110 nm. On the contrary, starch formate/PCL blend is phase-separated on the scale 1–9 nm but homogeneously mixed on the scale 20–90 nm. Therefore formylation of starch significantly improves its miscibility with PCL.

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