 DOI: 10.1002/masy.200651016

Re-examination of the Reactivity of *N*-Carboxy Amino Acid Anhydrides 1. Polymerisation of Amino Acid NCAs in Acetonitrile and in the Solid State in Hexane

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Summary: Ring-opening polymerisation of *N*-carboxy anhydrides of γ-benzyl-L-glutamate, L-alanine and L-leucine by a primary amine initiator in acetonitrile and in hexane was examined, with care taken to avoid contamination by moisture. The polymerisation of amino acid NCAs initiated by butylamine in hexane proceeded in the crystalline state (solid state) because the NCA crystals did not dissolve in hexane. Although amino acid NCAs were believed to polymerise completely in acetonitrile, polymerisation of the amino acid NCAs in acetonitrile was found to stop at around 20% conversion. As resulting polypeptides did not dissolve in acetonitrile, the polymer terminals were considered to be occluded in the polymer precipitate. On the other hand, each amino acid NCA was much more reactive in the solid state in hexane than in acetonitrile. Especially, L-leucine NCA showed remarkable reactivity in the solid state. The reactivity in the solid state was explained with reference to the crystal structure.

Keywords: amino acid NCA; crystal structure; polymerisation; solid state

Introduction

Ring-opening polymerisation of *N*-carboxy anhydrides of amino acids (amino acid NCAs) has been used extensively to prepare synthetic polypeptides for over 50 years. As good solvents that dissolve both amino acid NCAs and resulting polypeptides completely have not been obtained, only several amino acid NCAs have been polymerised in organic solvents, such as 1,4-dioxane, dimethylformamide (DMF), dichloromethane, *etc.*[1–11] On the other hand, the polymerisation of amino acid NCAs was carried out in a heterogeneous system in acetonitrile, which could dissolve amino acid NCAs but not resulting polypeptides.[12–15]

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The polymerisation of amino acid NCAs initiated by primary amines has been investigated extensively, because the molar mass of resulting polypeptides was expected to be controlled in the reaction system. However, there have been no reports of good results in the control of the molar mass of polypeptides. It was reported that the control of the molar mass of resulting polypeptides was impossible in polymerisation by a primary amine but it was successful using a nickel compound as an initiator.[16,17] Kricheldorf *et al.* reported a wide molar mass distribution of polypeptides and formation of cyclic polypeptides in the polymerisation of amino acid NCAs initiated by a primary amine in solution.[18,19]

We have been studying the polymerisation of amino acid NCAs initiated by butylamine in hexane, which cannot dissolve both amino acid NCAs and the resulting polypeptides; the polymerisation was observed to proceed in the solid state of

NCA crystals.[20–27] Polymerisation in the heterogeneous system in acetonitrile was also examined for comparison with the reactivity of amino acid NCAs in the solid state. We showed that L-leucine NCA was more reactive in the solid state than in acetonitrile, while L-alanine NCA gave the opposite result.[20–24] We have determined the crystal structure of amino acid NCAs by X-ray analysis to explain the reactivity in the solid state.[28–36]

Through many investigations of this polymerisation, we found that good reproducibility could not be obtained in the polymerisation of amino acid NCAs when highly purified NCA crystals were used. Thus, we have examined the effects of the purity of amino acid NCA crystals on reactivity and of contamination by moisture in the reaction system. The purity of amino acid NCA crystals was shown not to be determined accurately by standard titration methods or elemental analysis.[37] On the other hand, when moisture contamination in the reaction system was strictly prevented, the reactivity became much lower than that reported previously. Thus, we re-examined the polymerisation of amino acid NCAs that have been reported previously. In this article, we propose some corrections in the reactivity of amino acid NCAs in acetonitrile and in the solid state in hexane.

Experimental

Materials

Reagent-grade L-glutamic acid, L-alanine and L-leucine were supplied by Ajinomoto Co. Ltd., Tokyo, Japan. Solvents were products of Wako. Chem. Industry Ltd., Osaka, Japan. Hexane and ethyl acetate were refluxed over sodium or calcium hydride and distilled. Acetonitrile was refluxed over phosphorous pentoxide and distilled. SurfaSil Siliconizing Fluid (Pierce, Rockford, IL, USA: GL Science Co. Ltd., Tokyo, Japan) was used for silanising the walls of glass reaction tubes. Other reagents were of reagent grade and were used without further purification.

γ-Benzyl ester of L-glutamic acid (BLG) was prepared by the method described previously.[37]

NCAs were synthesised by reaction of each of BLG, L-alanine and L-leucine with triphosgen in tetrahydrofuran in a manner similar to that described previously; triphosgen was used instead of trichloromethyl chloroformate.[20] Crude crystals of amino acid NCA were purified by repeated recrystallisation from a mixture of ethyl acetate and hexane. Crystals in the initial stage of crystallisation were removed because chlorine analysis indicated that their purity was lower than that of crystals obtained later. NCA crystals were finally crystallised from solutions in a dry box set in a refrigeration room at $-10\,^{\circ}C$ just prior to use.

The purity of amino acid NCAs was estimated by chlorine content. Sample solution for the analysis was prepared by an oxygen combustion method with amino acid NCAs, and the solution was analysed by ion chromatography (IC) as described previously.[37]

Polymerisation

Purified NCA crystals were dissolved in ethyl acetate at a high concentration in a dry box at room temperature. If the polymerisation of amino acid NCAs occurred spontaneously, the solution was not clear in this manner, as polypeptides do not dissolve in ethyl acetate. The NCA solution (volume, 2 ml) was taken into a reaction tube and the solvent was removed at reduced pressure using a vacuum pump. The dried NCA crystals obtained in this manner were weighed and the concentration of NCA in the original solution in ethyl acetate was estimated. Solvents, hexane and butylamine necessary for polymerisation were estimated from the NCA concentration. On the other hand, the chlorine content in the dried crystals was analysed by IC to estimate the purity.

A solution reaction of the NCA in acetonitrile was carried out as follows. A given amount of the ethyl acetate solution of amino acid NCA was put in a reaction

tube the inner walls of which had been silanised, and a given amount of acetonitrile was mixed in the solution (volume ratio of ethyl acetate to acetonitrile, 1/10) in a dry box at room temperature. Immediately after addition of butylamine, the reaction tube was set in a thermostat at a controlled temperature of 30 °C and the pressure change caused by carbon dioxide formed through polymerisation was measured with a digital manometer (Model PG-100; Copal Electronics Co. Ltd., Tokyo, Japan).

The concentration of NCA used for the reaction was 0.018 mol·l^{-1}; 1.00 g of BLG NCA was mixed in 20 ml of solvent in the case of BLG NCA. Molar ratio, [NCA]/[butyl amine] ([A]/[I]) was 200.

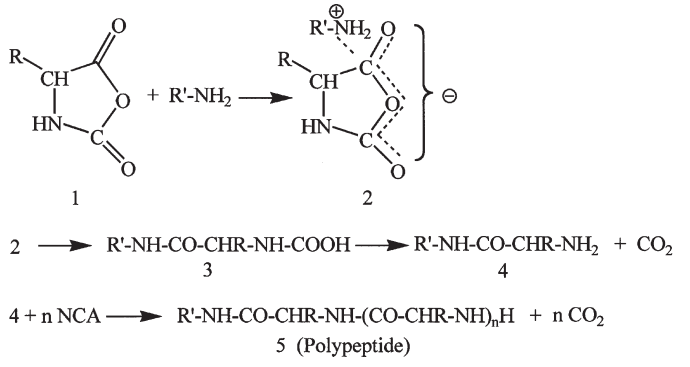
The solid-state reaction was carried out as follows. A definite amount of the ethyl acetate solution was put in the reaction tube and an excess of hexane was mixed with it to crystallise the NCA in a dry box at room temperature. The upper liquid of the mixture was removed and hexane was added in the tube to obtain the NCA crystals. This was repeated several times. Needle-like crystals with a definite size distribution (average size, $0.1 \times 0.0.1 \times 0.5$ mm) were obtained in this manner. Additional hexane necessary for polymerisation was added in the crystal mixture. After adding a definite amount of butylamine solution in hexane, the reaction tube was set in a thermostat at a controlled temperature of 30 or 40 °C. The reaction rate was followed by observing the pressure change similarly to that in acetonitrile.

When the polymerisation seemed to have finished in acetonitrile or in hexane, the reaction mixture was filtered and the filtrate was washed with ethyl acetate and methanol. The obtained polymer was dried *in vacuo* at 60 °C and weighed. Time conversion curves were obtained from the relation between polymer weight and observed pressure at each time point.

Results and Discussion

The polymerisation mechanism of amino acid NCA initiated by a primary amine is considered to be as follows. A primary amine (R'NH_2) attacks an amino acid NCA nucleophilically, and an intermediate 2 is ring-opened to form a compound 4 and carbon dioxide. The compound 4 can react with another NCA, and a polypeptide 5 is produced.

With use of high purity NCA crystals in air, it was impossible to avoid a slight polymerisation caused by moisture adsorbed on the crystals. When NCA crystals covered with trace amounts of polymer were used in the reaction in acetonitrile or in some solutions, the reaction rate increased because of polymerisation with polymers and water together with an initiator. However, the solid-state polymerisation of amino acid NCA crystals covered with a trace of polymer is sometimes less reactive, because the contact of the initiator with the crystal surface is prevented by the polymer on the crystals.



The purity of amino acid NCA was often determined by titration methods, such as Volhard's and Mohr's methods in the typical references of the polymerization of the NCAs.[4,12] It was proposed in the references that NCAs with chlorine contents less than 0.02 wt% should be used. However, it was impossible to determine such low values by these methods because the endpoint of the titration could not be seen clearly. For example, the value of 0 wt.% of chlorine content in amino acid NCAs was sometimes obtained by these titration methods, while the chlorine content of the same NCA was determined as 0.2 wt.% by IC. On the other hand, elemental analysis and spectroscopy also did not provide sufficient accuracy for determination of low chlorine contents in amino acid NCAs. Thus, we determined the chlorine contents of amino acid NCAs by combination of an oxygen combustion method and IC. As crystallisation is caused through nucleus formation by impurities, it is impossible to obtain amino acid NCA crystals purified completely by crystallisation in solution.

As the reactivity of amino acid NCAs was found to be largely affected by the purity of the compound,[37] the chlorine content is given for each polymerisation result.

Figure 1 shows the results of polymerisation of BLG NCA in acetonitrile at 30 °C and in the solid state in hexane at 30 °C and 40 °C. Figure 2 shows the results of polymerisation of L-alanine NCA in acetonitrile at 30 °C and in the solid state in hexane at 30 °C and 40 °C and those of L-leucine NCA in acetonitrile at 30 °C and in the solid state in hexane at 30 °C. Chlorine contents of BLG NCA, L-alanine NCA and L-leucine NCA were 0.024%, 0.034% and 0.032%, respectively. Amino acid NCAs dissolved in acetonitrile but the resulting polypeptides did not dissolve. As a result, the polymerisation had been considered to occur between the precipitated polymer and amino acid NCA dissolved in acetonitrile.[11–14] The polymerisation of

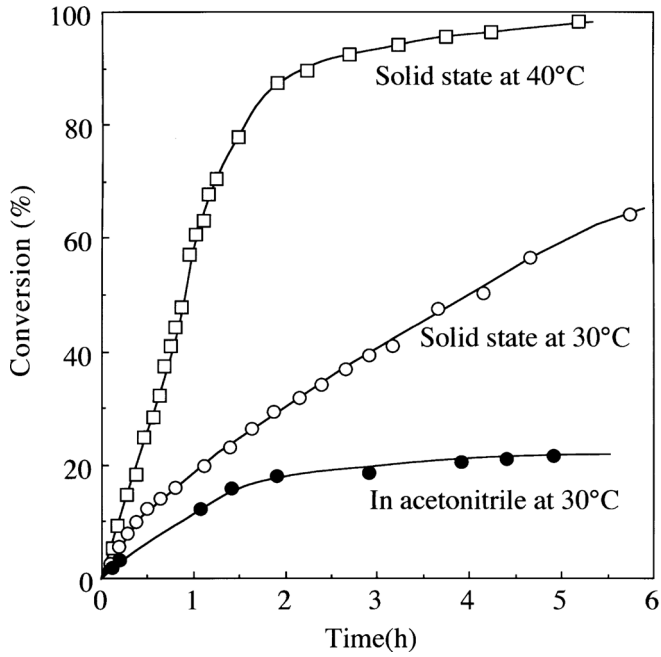


Figure 1.
Polymerisation of BLG NCA in acetonitrile and in the solid state in hexane; acetonitrile contained 10 volume% of ethyl acetate and [NCA]/[butylamine] ratio was 200.

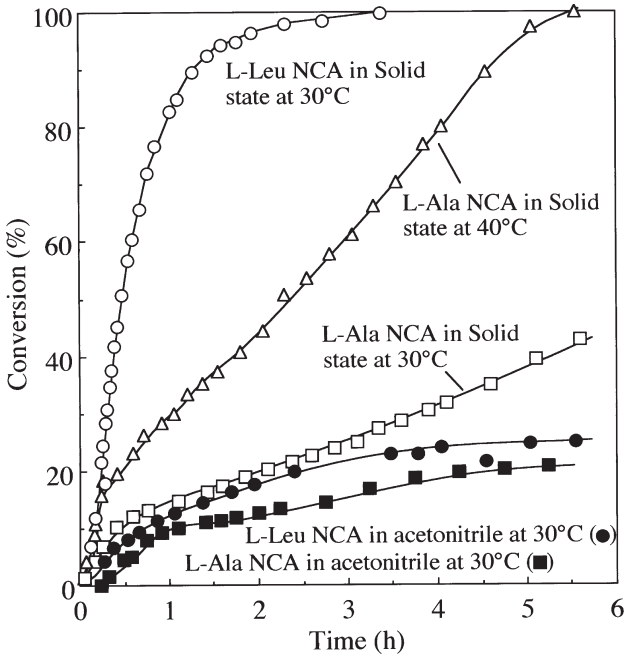


Figure 2.
Polymerisation of L-alanine NCA and L-leucine NCA in acetonitrile and in the solid state in hexane; [NCA]/[butylamine] ratio was 200.

L-alanine NCA and DL-alanine NCA initiated by butylamine in acetonitrile was investigated in detail; L-alanine NCA was very reactive even at 8 °C.[13] We also reported similar polymerisation of various amino acid NCAs in acetonitrile at 30 °C as compared with the solid-state polymerisation and observed that L-alanine NCA was the most reactive of the NCAs examined in acetonitrile.[20–24] However, none of the present three amino acid NCAs were so reactive in acetonitrile and polymer conversion did not result in an increase after about 20% as shown in Figs. 1 and 2.

When NCA crystals prepared in air at room temperature were used for polymerisation with butylamine initiator in acetonitrile, the polymerisation rate was more reactive than the present results. Thus, we considered that the polymerisation of amino acid NCAs in acetonitrile reported to date may have been accelerated by moisture adsorbed on the NCA crystals.

Figure 3 shows the polymerisation of BLG NCA in acetonitrile. The solution of NCA in acetonitrile was clear at the initial stage, but polymer precipitate, which was seen as a white material in Fig. 3, was formed after initiation. In this case, we considered that the polymer terminals were occluded in the polymer precipitate and

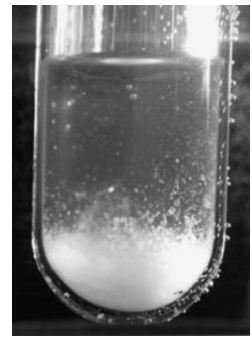


Figure 3.
Polymerisation of BLG NCA in acetonitrile.

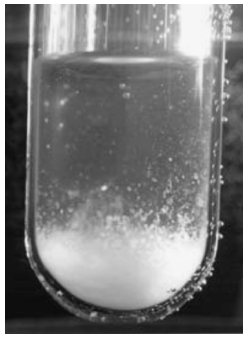


Figure 4.
Polymerisation of BLG NCA in hexane.

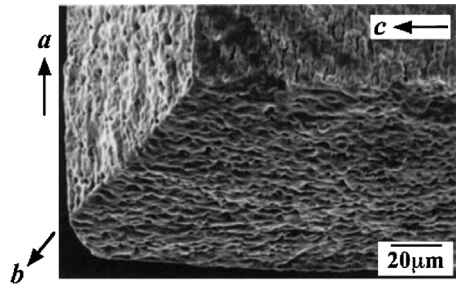


Figure 5.
SEM of a polymerised BLG NCA crystal.

thus further polymerisation could not proceed easily.

On the other hand, each of three NCAs was more reactive in the solid state than in acetonitrile. Although the chlorine contents of the three NCAs were slightly different to each other, it is remarkable that the reactivity of L-leucine NCA was much higher than those of the other two NCAs. In addition, BLG NCA seemed more reactive than L-alanine NCA in the solid state.

Figure 4 shows the appearance of BLG NCA crystals polymerising in hexane. The white materials are BLG NCA crystals and some crystals were blown off explosively by generated carbon dioxide. As the NCA crystals in hexane showed mutual repulsion because of static electricity, the volume of crystals seemed large. If crystals with adsorbed moisture were used, no such volume increase was seen.

When amino acid NCA crystals are allowed to stand in air at room temperature, polymerisation is initiated by moisture and it proceeds in the solid state. It is believed that moisture decomposes amino acid NCAs to amino acids and the amino groups of the amino acids can initiate the polymerisation of amino acid NCAs similarly to primary amines. Even if the initiation mechanisms by moisture and primary amines are not similar to each other, the polymerisation initiated by moisture in air and that by butylamine would proceed similarly in the solid state. Thus, microscopic observation of amino acid NCA polymerised by moisture in the solid state often provides significant information. Figure 5 shows a SEM of BLG NCA polymerised by moisture. Voids formed by carbon dioxide through polymerisation were observed on the *a*-*b* and *b*-*c* planes as compared with the *a*-*c* planes.

Crystal data of three NCAs are shown in Table 1. Part of the crystal structure of BLG is shown in Figure 6. Five-membered NCA rings are in a layer and side chains seem to be in another layer. These two layers stacks alternately and form a sandwich structure. This structure suggests that the crystals break parallel to the *a*-*c* plane, which is considered to be caused by the layer of side chains.

Table 1.
Crystal data of three amino acid NCAs.

NCA	Space Group	*a*/Å	*b*/Å	*c*/Å	*Z*	*d* g cm^{-3}
L-Ala	$P2_12_12_1$	7.749(2)	10.699(3)	6.063(2)	4	1.52
L-Leu	$P2_12_12_1$	6.518(4)	23.983(4)	5.531(5)	4	1.21
BLG	$P2_12_12_1$	7.766(1)	22.470(4)	5.948(1)	4	1.4

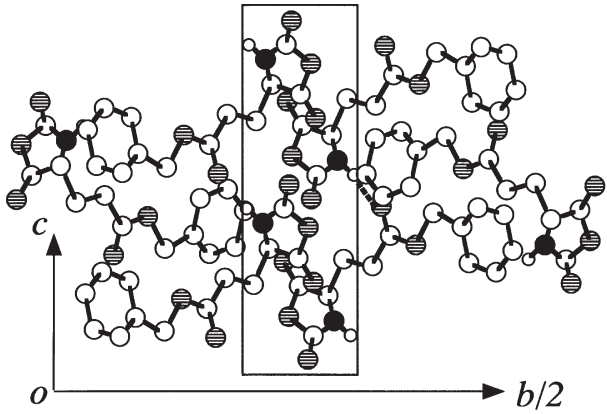


Figure 6.
Crystal structure of BLG NCA.

Figure 5 and the crystal structure suggest that the amino acid NCAs react preferentially in the layer of NCA rings parallel to the *a-c* plane.

Figures 7 and 8 show the crystal structures of L-leucine NCA and L-alanine NCA. The sandwich structure similar to BLG NCA can also be seen clearly in the L-leucine NCA crystal. As described previously,[21–24] polymerised L-leucine NCA showed clear cleavage perpendicular to the *b* axis of the crystal. The polymerisation of L-leucine NCA was considered to proceed preferentially along the *c* axis of the crystal, as seen in the X-ray analysis of NCA crystals that were polymerising.[21]

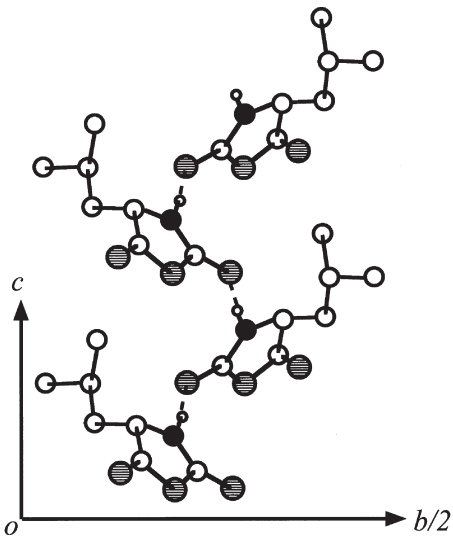


Figure 7.
Crystal structure of L-leucine NCA.

On the other hand, polymerised L-alanine NCA showed rather irregular deformation of the original crystal.[21–24] Although the NCA rings seems to be packed in a relatively thin layer, N–H groups seem to be embedded in the layer as shown in Fig. 8. Thus, the sandwich structure similar to the other NCAs was not seen clearly in the crystal. This may be the reason why L-alanine NCA in the solid sate is less reactive than the other two NCAs. These results suggest that low density of a crystal is one of the requirements for high reactivity. However, amino acid NCAs that were reactive but did not have high density were found in other investigations.

As polymerisation of amino acid NCAs cannot proceed rapidly in acetonitrile without moisture contamination, the present solid-state polymerisation is useful for preparation of polypeptides using all amino acid NCAs that have no organic solvents.

As the polymerisation was carried out under strict conditions in the present study, gel permeation chromatography showed that the obtained polypeptide had a high molar mass and a narrow molar mass distribution as compared with those obtained previously.[16,18,38] For example, the polypeptide of BLG obtained in poly-

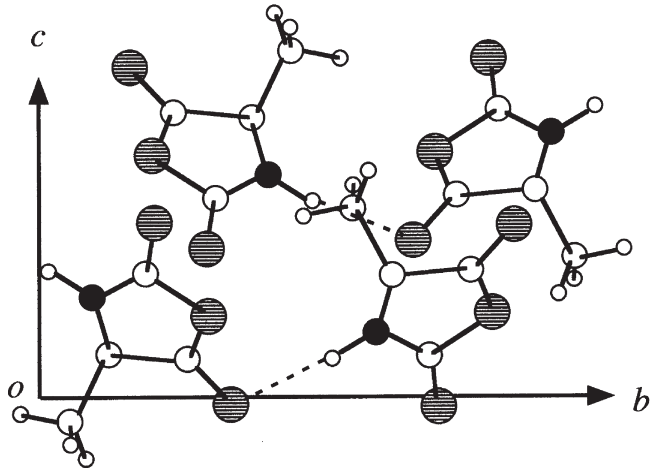


Figure 8.
Crystal structure of L-alanine NCA.

merisation of the NCA initiated by butylamine in acetonitrile gave the value of $M_w/M_n = 1.05$. We are currently investigating the molar mass of the polypeptide and the results will be reported in the near future.

Conclusion

We carried out the purification of amino acid NCAs very cautiously and carried out the polymerisation under strict conditions. As a result, we found that the polymerisation of amino acid NCAs in acetonitrile stopped at low polymer conversion, which was very different from the results reported previously. On the other hand, the polymerisation of each amino acid NCA by butylamine in hexane was observed to proceed in the solid state and was much more reactive than that in acetonitrile. The reactivity in the solid state was considered to depend on the crystal structure. It is necessary to re-examine the reactivity of amino acid NCAs under strict conditions.

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