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Grafting of Polypeptide on Cellulose Derivatives

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Celluose-g-polypeptide graft copolymers, which are soluble in common organic solvents, were prepared by polymerizing N-carboxy-γ-methyl-L-glutamate (γ-BLG NCA) using aminated ethylcellulose (EC) and hydroxypropyl cellulose (HPC) as macroinitiator (EC-g-PBLG and HPC-g-PBLG). Primary amino groups were successfully introduced to EC and HPC by cyanoethylation followed by reduction of the cyano groups with a borane-THF complex in homogeneous phase, where the content of primary amino groups introduced per anhydroglucose unit, DS_{NH_2}, was 0.05. Graft copolymerization was carried out at 20°C in dioxane under a variety of conditions. Graft copolymers thus obtained were characterized by means of solubility test, gel permeation chromatography (GPC), IR spectroscopy, and electron microscopy. It was found that (i) monomer conversions were higher that 90% for all the polymerization runs, (ii) all of the substrates and the polypeptides formed were grafted, (iii) the polydispersity of graft copolymers obtained was not so high, and (iv) graft copolymers in the solid state assumed microheterophase strucutres, although the structure was rather coarse.

KEY WORDS: Ethylcellulose-g-poly(γ-benzyl-L-glutamate)/ Hydroxypropyl-cellulose-g-poly(γ-benzyl-L-glutamate)/ Solubility/ Aminated cellulose derivatives/ Domain structure/

INTRODUCTION

Cellulose and polypeptide are characterized by an inherent property that their hydrophilic-hydrophobic nature can be arbitrarily varied by rather simple chemical modifications. Furthermore, both polymers possess the ability to form liquid crystalline phases.[1,2] Thus block and graft copolymers composed of these polymeric segments may be expected to be very attractive composite materials from both a scientific and a practical point of view. One of the feasible methods for preparing such materials is graft-copolymerization of amino acid N-carboxyanhydrides (NCAs) onto cellulose, which is initiated by primary amino groups introduced to cellulose.[3]

A series of preliminary experiments on such a grafting have been made with N-carboxy-γ-benzyl-L-glutamate (γ-BLG NCA) and a commercially available amino-ethyl cellulose having a degree of substitution (DS) of 0.05 as the monomer and macroinitiator, respectively.[3,4] Although it was confirmed that the copolymerization effectively proceeded without producing the homopolymer and that the products showed quite favorable characteristics as a biomedical material, there remained a drawback that the number of solvents for them is extremely limited.

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In the present study, further attempts were made to prepare other graft copolymers having less solvent problem by using ethyl cellulose (EC) and hydroxypropyl cellulose (HPC) as the substrate. The primary amino groups were introduced to these cellulose derivatives by cyanoethylation followed by reduction of the cyano groups with a borane-THF complex.[5] Aminated EC and HPC were used as the macroinitiator for γ-BLG NCA to synthesize the graft copolymers relevant to each, i.e., EC-g-PBLG and HPC-g-PBLG.

EXPERIMENTAL

Materials

Dioxane was dried over metallic sodium and fractionally distilled from sodium benzophenone solution. Dimethyl sulfoxide (DMSO) was dried over calcium hydride and then distilled under reduced nitrogen atmosphere immediately before use. The other reagents used as solvents were refined, respectively, according to the relevant procedures.

The nomomer, γ-BLG NCA, was prepared and purified as described[3]. EC having DS=2.4 was purchased from Dow chemical Co., USA. A commercial HPC was purchased from Nacalai Tesque Inc., Kyoto. The DS and molar substitution (MS) values of HPC were estimated by ^{1}H and ^{13}C-NMR methods and found to be 2.4 and 3.5, respectively.

Amination of Cellulose Derivatives

Aminated EC and HPC (A-EC and A-HPC) were prepared from cyanoethylated EC and HPC, respectively. Cyanoethylation of EC and HPC with acrylonitrile was carried out in acetone-10% NaOH aqueous solution (10 :1 by volume) and 2% aqueous NaOH solution, respectively, in order to perform the reaction in homogeneous phase. The reaction temperature was controlled at 20°C to prevent the homopolymerization of acrylonitrile.

The reduction of cyanoethylated EC and HPC to 3-aminopropyl derivatives was carried out in tetrahydrofuran (THF) with a borane-THF complex according to the method of Daly and Munir.[5] The A-EC sample was precipitated into water and purified by three further preciptations. In the case of A-HPC, purification was carried out using THF as solvent and petroleum ether as precipitant. The extent of reduction was estimated form IR spectra: no evidence of residual cyano groups (2250 cm^{-1}) was detected in the reduced products. The content of NH_2 groups per anhydroglucose unit, DS_{NH_2}, was estimated by elementary analysis.

Graft Copolymerization

Graft copolymerization was carried out for 48 h under nitrogen at room temperature using dioxane as solvent, unless otherwise stated. EC-g-PBLG graft copolymers were recovered from the reaction mixture by pouring it into a tenfold excess of cold methanol except for the graft products with PBLG contents lower than 20% which were precipitated with petroleum ether. In the case of HPC-g-PBLG, the graft products

with low PBLG contents were recovered by precipitation with petroleum ether and dried in vacuo. The relative amount of γ-BLG NCA to A-EC or A-HPC was calculated to obtain the desired degree of polymerization (DP) of the polypeptide side chains by DP=[NCA]/[NH_2], where [NCA] and [NH_2] denote the mole concentration of γ-BLG NCA and amino groups in substrate, respectively.

The polypeptide content of graft copolymers was determined by analysis of the nitrogen content of the copolymer.

Aminolysis of EG-g-PBLG

The PBLG side chains in EC-g-PBLG graft copolymers were converted into poly(N^5-2-hydroxyethyl-L-glutamine) (PHEG) by aminolysis with 2-amino-1-ethanol:[6] the reaction with 2-amino-1-ethanol was carried out at 56°C for 24 h in dioxane, and care was taken to avoid a rise in temperature during the reaction.[3] The complete aminolysis was confirmed by IR spectroscopy using the C=0 band around 1730 cm^{-1}.

Characterization of Graft Copolymers

The characterization of graft copolymers was made by infrared (IR) spectroscopy, gel permeation chromatography (GPC), and viscometry.

IR spectra were obtained with a Perkin-Elmer Medel 521 spectrophotometer in order to confirm the completion of reduction of cyano groups into amino groups. The sample films were prepared by solvent casting method.

GPC chromatograms were recorded on a Waters High-Speed GPC model ALG/GPC-202/R401. Four columns of microstyragel® of 10^6, 10^5, 10^4, and 10^3 Å nominal pore size were used. The carrier solvent was N, N'-dimethylformamide (DMF). The flow rate was set to 1 ml/min, and the injections were usually 0.3 ml of 0.5% stock solutions.

Intrinsic viscosities [η] were determined in DMF at 25°C.

Transmission Electron Microscopy

The sample films were stained with osmium tetraoxide vapor. The stained specimens were examined in a JEOL JEM-1200EX transmission electron microscope.

RESULTS AND DISCUSSION

Preparation of Aminated Cellulose Derivatives

First we intended to prepare EC-g-PBLG graft copolymers using anthranilated EC[7] as macroinitiator. During the experiments, we noticed that the aromatic amino groups cannot polymerize amino acid NCAs because of low basicity of aromatic amino groups.[8] Thus, we attempted to prepare cellulose deivatives having primary amino groups, which can initiate the polymerization of amino acid NCAs.[9,10]

Cellulose derivatives containing primary amino groups are very promising not only as macroinitiator of amino acid NCAs but also as functional materials and/or intermediates. However, there are only a few methods avaliable for preparing such derivatives.[5,11-13] One of the methods is the introduction of primary amino groups to

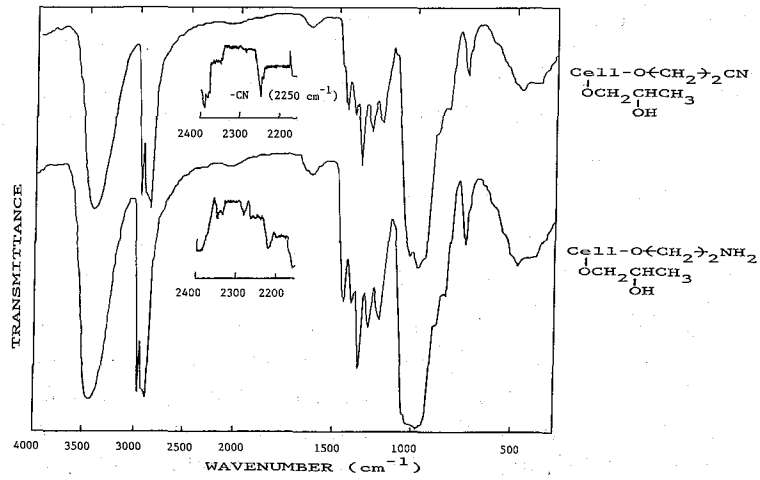

Fig. 1. IR spectra of cyanoethylated HPC and its reduced HPC.

cellulose by cyanoethylation followed by reduction of the cyano grups, which has been reported recently by Daly and Munir.[5] In the present study, we attempted to prepare aminated cellulose derivatives by applying their method to EC and HPC. The DS value of NH_2 introduced (DS_{NH_2}) was controlled to become 0.05; every cellulose molecule possesses one possible grafting site per 20 anhydroglucose units. This is suitable for the preparation of the graft copolymers with a relatively low degree of grafting substitution.[3]

As described in Experimental Section, cyanoethylation of EC and HPC with acrylonitrile was carried out in homogeneous phase in order to uniformly introduce the cyano groups along the cellulose chain. Homogeneous reaction also requires complete reduction of cyano groups with a borane-THF complex.[14] Fortunately, cyanoethylated EC having a DS_{CN} of 0.05 was soluble in THF and cyanoethylated HPC ($DS_{CN}=0.06$) was insoluble, though highly swollen, in THF. The reduction of cyanoethylated EC and HPC was carried out in THF. Figure 1 shows the IR spectra of cyanoethylated HPC and its reduced HPC (A-HPC). The magnified spectrum of the former shows a characteristic absorption band at 2250 cm^{-1}, which may be assigned to the nitrile groups, while the absorption band at 2250 cm^{-1} is not found in that of the latter. The presence of primary amino groups was checked by ninhydrin test, that is, it was confirmed by the formation of a dark blue complex with ninhydrin. The results on the reduction of cyanoethylated EC and HPC in THF with a borane-THF complex are shown in Table 1. It can be seen that the efficiency of amination was almost 100%.

Preparation and Characterization of Graft Copolymers

In the polymerization of NCA by macroinitiator with amine functions, two types of initiation mechanisms are known to coexist: one is the primary amine type leading to the block or graft copolymers, and the other is the tertiary amine type leading to a homopolypeptide. The priority in the two mechanisms is determined not only by a

Table I. Efficiency of Aminaion of CE-HPC and CE-EC with a Borane-THF Complex

Starting material	DS_{CN}	DS_{NH_2}[a)]	Efficiency (%)
HPC	0.06	0.06	100
EC	0.05	0.05	100

[a)] Determined by elementary analysis.

Table II. Effect of Solvent on Copolymerization of γ-BLG NCA onto Aminated EC.[a)]

Run	Solvent	NCA/EC[b)] (wt. ratio)	Conv. of NCA (%)	PBLG content (wt %)[c)]	$[\eta]$ (cm^3/g)[d)]
1	DMSO	5/6	90	45	110
2	Dioxane	5/6	100	52	106
3	DMF	5/6	95	49	120

[a)] Polymerization conditions: 1 g A-EC/200 ml solvent; reaction time=72 h; temperature=20°C.
[b)] Aminated ethyl cellulose with a DS_{NH_2} of 0.05.
[c)] Determined from the N-content.
[d)] In DMF at 25°C.

balance between the nucleophilicity and basicity of the amine but also by the nature of the polymerization solvent employed.[9,10] To examine the effect of polymerization solvent on the graft efficiency and the polydispersity of the graft copolymers, three polymerization runs of γ-BLG NCA by aminated EC were made at 20°C for 72 h using DMSO, dioxane, and DMF as polymerization solvent. Table 2 summarizes the polymerization data.

EC was found considerably swollen in DMSO but not soluble, and the graft copolymerization proceeded heterogeneously. Dioxane is a good solvent for both EC and PBLG, but gel was formed as the graft copolymerization proceeded. In DMF, on the other hand, the reaction mixture was homogeneous during the whole polymerization process. The results show that the total conversion of γ-BLG NCA monomer to polymer was very high for all the solvents examined. In the present case, there exists no unreacted EC because the EC sample having a DS_{NH_2} of 0.05 was used as substrate which possessed several possible grafting sites on each molecule. The problems are the graft efficiency and the polydispersity of the graft copolymers obtained. At this stage, there is no selective solvent with which homo-PBLG is extracted from the graft product. In the present study, the graft products were washed with DMSO to eliminate the unreacted monomer NCA and low-molecular-weight PBLG. The results suggest that the graft efficiency is very high for all the solvent systems. In order to further examine the graft efficiency, the graft products were subjected to aminolysis with 2-amino-1-ethanol, and the resultant graft products were washed with water to eliminate the homo-PHEG which is soluble in water. The recovery of graft products

after the aminolysis was found to be 90–100%, showing that the graft efficiency was approximately 100% for all the solvents examined. These results also indicate the occurrence of a nearly prefect aminolysis without side reaction such as the chain scission of polypeptide.

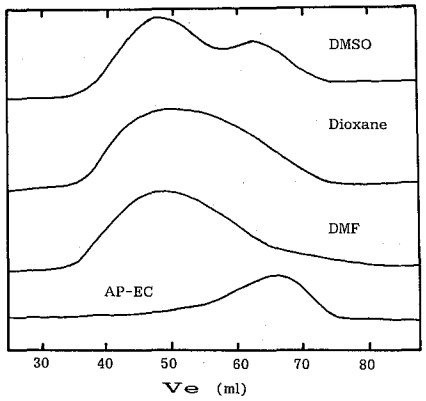

Fig. 2. GPC chromatograms of EC-g-PBLG graft copolymers obtained using AP-EC with different polymerization solvent.

Figure 2 shows the GPC chromatograms, together with that of A-EC employed as substrate. It can be seen that the graft copolymer obtained in DMF has the best polydispersity. Since it is known that the homo-PBLG results from traces of amines present in DMF, it is absolutely necessary to purify DMF carefully in order to obtain the graft product whose chromatogram is shown in Fig. 2. During the preliminary experiments using DMF as solvent, the formation of homo-PBLG was sometimes observed. It was very difficult to reproduce the graft copolymers having a polydisperesity similar to that shown in Fig. 2. The chromotograms also indicate that a satisfactory result is obtained in dioxame solvent, in spite of the fact that the gelation occures as the graft copolymerization proceeds. On the other hand, in DMSO where the graft copolymerization proceeds heterogeneously, the molecular weight distribution of the graft product obtained was very broad. Taking the results obtained here and the ease of solvent purification into consideration, the subsequent polymerization runs were carried out using dioxane as solvent.

The results on graft copolymerization in dioxane are summarized in Table 3, from which one finds that the total conversion of NCA monomer to polymer were very high. The DP of grafted PBLG denotes the apparent degree of polymerization estimated from DS_{NH_2} value of substrate and PBLG content of graft copolymers. The samples with DP less than ca. 5–6 are cellulose derivatives with oligopeptides as substituents, because such peptide sequences cannot assume a helical conformation characteristic of PBLG.

To check the molecular weight heterogeneity of the graft copolymers obtained, the samples were examined by GPC. Figure 3 shows the GPC chromatograms of HPC-g-PBLG graft copolymers in DMF solution. Although the molecular weight distribution of the samples is rather broad, the chromatograms exhibit a single peak comparable to

Table III. Polymerization Data of HPC-g-PBLG and EC-g-PBLG Graft Copolymerization

Sample code	Conv. of NCA (%)	PBLG content (wt %)	DP of grafted PBLG[b)]
HPC-g-PBLG-10	100	10	3–4
HPC-g-PBLG-20	85	17	6–7
HPC-g-PBLG-50	90	45	27
HPC-g-PBLG-70	100	75	70
EC-g-PBLG-20	100	21	5
EC-g-PBLG-80	100	52	22
EC-g-PBLG-80	100	77	85

a) Polymerization conditions: total weight of (substrate+NCA)=2–5 g/100 ml dioxane; reaction time=72 h; temperature=25°C.
b) Apparent degree of polymerization calculated from DS_{NH_2} value of substrate and average composition of graft copolymer.

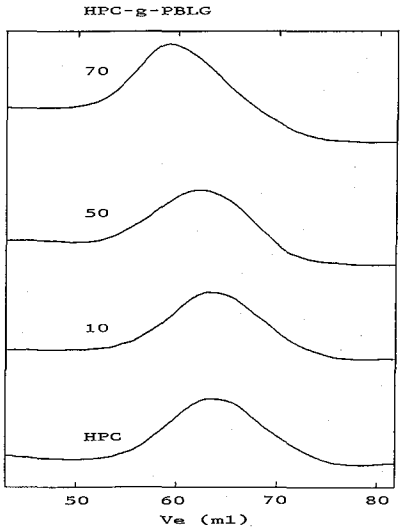

Fig. 3. GPC chromatograms of HPC-g-PBLB graft copolymers with different PBLG contents and HPC itself.

that of the substrate HPC, indicating that the graft copolymers obtained are sufficiently homologous with respect to both molecular weight and composition.

Table 4 shows the solubility of HPC-g-PBLG copolymers. The graft copolymers were insoluble in selective solvents for HPC, such as water, giving the direct evidences that grafting was effectively taken place in HPC and that most of HPC were grafted. The solubility test results on EC-g-PBLG and EC-g-PHEG graft copolymers are shown in Table 5. It can be seen that the solubility behavior of graft copolymers is mainly determined by grafted polypeptide side chains. Figure 4 shows the IR spectra of the samples EC-g-PBLG-50 and EC-g-PHEG-50. The spectra of the former shows a characteristic absorption band at 1730 cm^{-1} assigned to the carbonyl group in PBLG, while the carbonyl band is not found in that of the latter, proving that the aminolysis

Table IV. Solubility of HPC, PBLG and HPC-g-PBLG Graft Copolymer

Solvent	Solubility[a]		
	HPC	PBLG	HPC-g-PBLG-50
Water	O	X	X
Ethanol	O	X	X[b]
$CHCl_3$	O	O	O
DMF	O	O	O

[a] O, soluble; X, insoluble.
[b] HPC-g-PBLG-20, soluble.

Table V. Solubility of EC, PBEG, EC-g-PBLG and EC-g-PHEG Graft Copolymers

Solvent	Solubility[a]				
	EC	PBLG	EC-g-PBLG-50HPC	PBLG	EC-g-PHEG-50
Water	X	X	X	O	X
Ethanol	O	X	X	X	X
$CHCl_3$	O	O	O	X	X
DMF	O	O	O	X	X
TFE[b]	O	O	O	O	O

[a] O, soluble; X, insoluble.
[b] Trifluoroethanol.

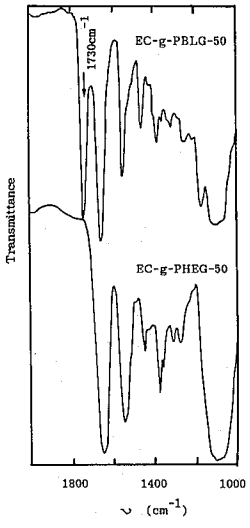

Fig. 4. IR spectra of unoriented solid films of EC-g-PBLG-50 and EC-g-PHEG-50 samples cast from chloroform solution.

was practically complete.

Last to be mentioned in this section is the initiation efficiency of amino groups in A-EC and A-HPC. In the case of cellulose graft copolymers prepared by using aminoethylcellulose (AE-Cell) with a low DS as macroinitator, the initiation efficiency can be estimated from the characterization of PBLG side chains isolated by selective degradation of cellulose backbone with cellulase.[15] It is reported that the initiation

efficiency of amino groups in AE-Cell is sufficiently high.[15] Unfortunately, the selective degradation method with cellulase cannot be applied to the EC and HPC graft copolymers.[16] The results on initiation efficiency of primary amino groups in AE-Cell suggest strongly that the initiation efficiency in A-EC and A-HPC prepared here is as high as that in AE-Cell.

Domain Structure of Graft Copolymers

In order to elucidate the domain structure of the graft copolymers, the morphology of the EC-g-PBLG graft copolymers was examined by electron microscopy. Figure 5 shows an electron micrograph of the sample EC-g-PBLG-50. The dark portion in the photograph corresponds to the domain composed of ethylcellulose portions stained with osmiun tetraoxide. A microheterophase structure can be observed, although the strucutre is rather coarse. A similar electron micrograph was also obtained from the section of ruthenium tetraoxide (RuO_4) stained EC-g-PBLG graft copolymers.

An attempt was also made to take the electron micrographs of HPC-g-PBLG graft copolymers but was unsuccessful because of the difficulty in ultrasectioning the specimens with an ultamicrotome. These graft copolymers, however, are considered to assume the phase separated strucutres similsr to those of EC-g-PBLG graft copolymers.

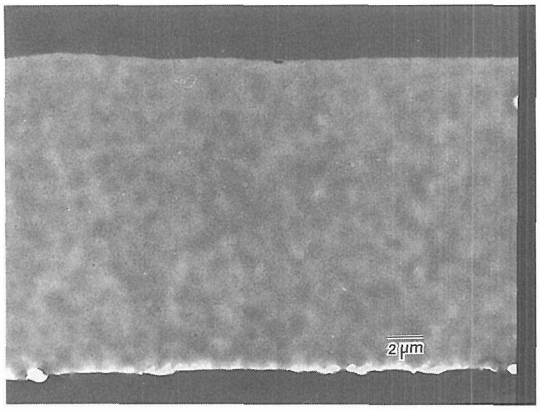

Fig. 5. Transmission electron micrograph of EC-g-PBLG graft copolymer. Black portions correspond to EC domains stained with OsO_4.

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