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NOTES

Graft Copolymers Having Hydrophobic Backbone and Hydrophilic Branches XXI. Preparation of Galactose Surface-Accumulated Polystyrene Nanospheres and Their Interaction with Lectin

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Polymeric nanospheres that have a variety of functional groups on their surfaces or in their cores have been widely used not only biomedically such as drug carriers and cell markers, but also industrially such as toner and spacers in liquid-crystal displays. Generally, they are synthesized *via* emulsion polymerization by using hydrophobic monomers in the presence or absence of steric stabilizers such as low molecular weight detergents or amphiphilic polymers.[1] The authors have also synthesized surface modified polymeric nanospheres by using the free radical dispersion polymerization of hydrophilic macromonomers and hydrophobic comonomers in a polar solvent.[2-12] Usually, monodispersed nanospheres are obtained by this macromonomer method, and the size of them and the surface density of the hydrophilic polymeric chains derived from macromonomers are able to be controlled by polymerization conditions. To date, by the use of this macromonomer method, the authors have synthesized polystyrene-(polySt) core nanospheres that have poly(vinylpyrrolidone),[2,3] poly(ethyleneglycol),[4,10] poly(4-vinylpyridine),[5] poly(methacrylic acid) (polyMAA),[6] poly(*N*-isopropylacrylamide) (polyNIPAAm),[7] poly(*N*-vinylisobutyramide),[11] poly(*N*-vinylacetamide) (polyNVA),[12] and poly(vinylamine) (polyVAm)[12] on their surfaces. These surface modified polySt nanospheres are adequate biomaterials because their polySt core is very stable and their surface area is large enough for the immobilization of biomolecules. For example, peptides such as calcitonin were physically immobilized in polyNIPAAm coated polySt nanospheres and were administered orally in order to show the effects.[8] Lectin was chemically immobilized by a covalent bond on a polyMAA coated one and the authors were able to capture the human immunodeficiency virus-1 (HIV-1) using a nanosphere dispersion solution.[9]

Carbohydrates on the surfaces of cells play critical roles in a variety of biological functions such as cell growth, regulation, differentiation, cancer metastasis, and infection.[13] Recently, synthetic polymers that have many kinds of carbohydrates were developed in order to study the receptor functions of carbohydrates and-to utilize them in biomaterial fields.[14-17] In order to completely understand the functionality of carbohydrates that are immobilized on polymeric materials, they should be introduced onto the surfaces of polymeric nanospheres because the concentration and amount can be controlled. The number and the density of the carbohydrates to be immobilized can be precisely controlled by changing the molecular weight and the amount of macromonomers in the copolymerization with comonomers when using the macromonomer technique for preparing polymeric nanosopheres. The other researchers prepared the latex bearing sugar residue by various significant methologies.[18-20]

In this study, polySt nanospheres that have polyVAm branches that are derived from the polyNVA on their surfaces were synthesized first and then lactose was introduced onto surfaces by the lactonization of lactose. The lectin binding affinity to carbohydrates on PSt nanospheres was examined by the transmittance measurement of their aqueous dispersion solution.

EXPERIMENTAL

Materials and Apparatus

PolyNVA coated polySt nanospheres were prepared using the macromonomer method by the free radical polymerization.[12] The number-average molecular weight (M_n) of NVA oligomers and polyNVA macromonomers, which have a vinylbenzyl group at the end of their polymer chain, was measured by gel permeation chromatography (GPC) using Tosoh HLC-8120 GPC. Lactose (guaranteed reagent) and anthrone was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Triethylamine (TEA) (guaranteed reagent) and methanol, in which the extra pure reagent grade had been distilled, were obtained from Nacalai Tesque Co. (Kyoto, Japan). *Ricinus communis* Agglutinin I (RCA_{120}) was purchased from the Funakoshi Co. (Tokyo, Japan). Transmission electron microscopy (TEM) was performed by a Hitachi H-700H microscopy that was operated at an acceleration voltage of 200 kV at a magnification of 21000. Specimens were prepared by allowing a drop of particle dispersion to be supported on a collodion film which was coated with a carbon layer and supported on copper grids. The particle size of the nanospheres was measured by a submicron particle analyzer (Coulter model N4SD). The UV/Vis absorption spectra were recorded on a JASCO model V-550 recording spec-

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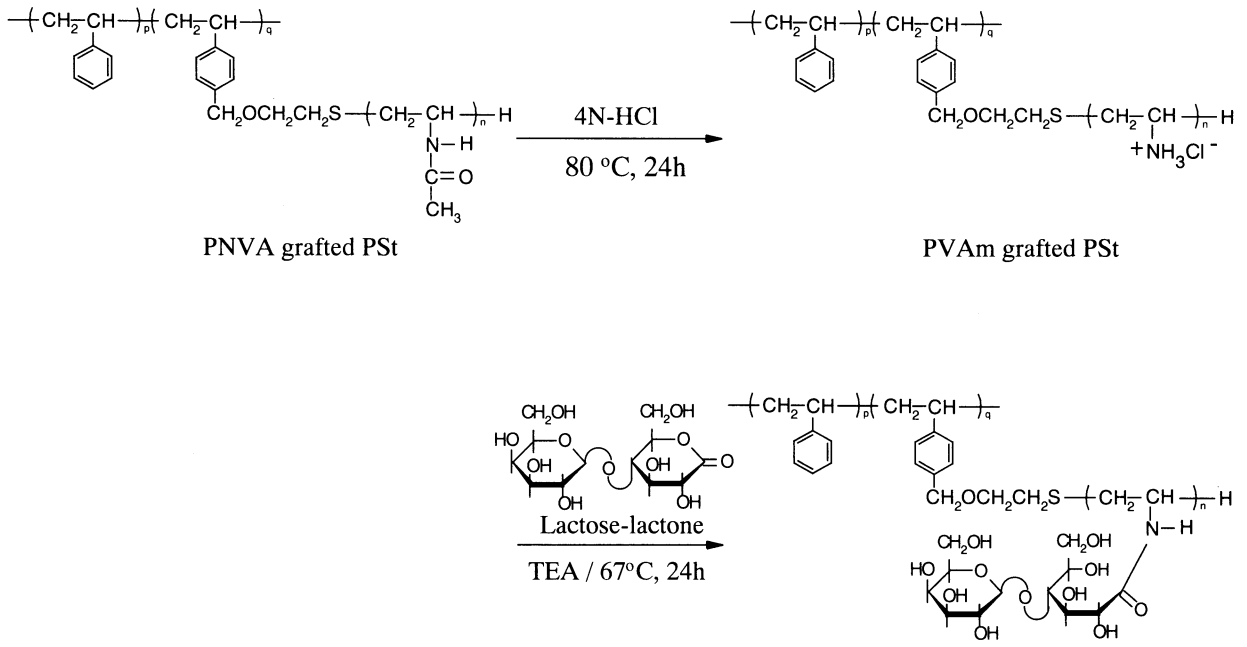

Scheme 1. Synthesis of the carbohydrate surface-accumulated PSt nanosphere.

trophotometer.

Preparation of Carbohydrate Surface-Accumulated PolySt Nanospheres

A polyVAm grafted polySt nanospheres were prepared by the hydrolysis of polyNVA grafted nanospheres under 4 *N* HCl for 24 h at 80°C. After neutralization, the latter were dialyzed in water. The lactonization of lactose was followed to the literature.[14] PolyVAm nanospheres (90 mg) were reacted with lactose lactone (1.5 mM) at 67°C for 24 h in the presence of TEA (1 mL) in methanol (100 mL). After reaction, the solvent was removed under reduced pressure. The residue was dispersed in water and dialyzed for three days. Aqueous dispersion of the nanospheres was obtained.

Determination of Carbohydrates on PolySt Nanospheres

A quantitative analysis of the carbohydrates on polySt nanospheres was done *via* the sulfuric anthrone method.[21] The dispersed carbohydrate nanospheres were placed in cold water and anthrone solutions (3 mL) were added. The mixture solution was heated in 100°C for 10 min. After being cooled with cold water, the reaction media was centrifuged with 10000 rpm for 10 min in order to remove the nanospheres, and then the absorbance was measured at 620 nm within 30 min.

Recognition of Carbohydrates on PolySt Nanospheres by Lectin

Lactose-conjugated polySt nanospheres were dispersed in phosphate buffered saline (PBS, pH 7.4) and were maintained at 37°C. After a variety of concentrations of RCA_{120} lectin was added to the nanosphere dispersion solution (77 μg ml^{-1}), the transmittance change was measured at 500 nm.

RESULTS AND DISCUSSION

Preparation of PolyVAm Grafted PolySt Nanospheres

The synthetic route for polySt nanospheres that have carbohydrates is shown in Scheme 1. The polymerization conditions for the dispersion copolymerization of polyNVA macromonomers and St in ethanol at 60°C and the characterization of the resulting nanospheres are shown in Table I. PolyNVA appears to be very soluble in ethanol, but polySt is not; therefore polyNVA graft chains accumulate on polySt core nanospheres as well as on other hydrophilic polymer chains that have coated polySt nanospheres.[2,3,5-9,11,12] PolyNVA coated PSt nanospheres were characterized as similarly as those described in previous studies.[2-7,10-12] In this study, nanospheres ranging in size from 300 to 600 nm were prepared by changing the molar concentration of the monomers. Usually, highly monodispersed nanospheres are obtained by this macromonomer method. Though the dispersity of this polyNVA grafted nanosphers in the present study was low, we used the nanospheres without further purification. The size distribution was increased with an increase in the size, as is shown in Table I. In this study, their nanospheres with run 4 in Table I were used for lactose conjugation because of its smaller size distribution.

The synthesized polyNVA-grafted polySt nanospheres were hydrolyzed in a 4 *N* HCl solution to give polyVAm-grafted polySt nanospheres quantitatively. The precise hydrolysis behavior on the nanosphere surface will be reported elsewhere.[12] The mean size of the polyVAm-grafted nanosphere was 743 nm, which was larger than polyNVA-grafted ones, as is shown in Table II. The size may have been affected by the electrostatic repulsion of polyVAm chains on the nanosphere.

Lactose Conjugation on Nanospheres

The well-known lactone method is useful for the immobilization of lactose lactone to polyVAm nano-

Table I. Copolymerization of NVA macromonomer (M_1) with styrene (M_2)[a].

Run	M_n[b]	M_1	M_2	St/macromonomer	dm[c]	S.D.[d]	C.V.[e]
		mmol	mmol		nm	nm	%
1	7000	0.007	0.29	40	638	305	48
2	7000	0.014	0.57	40	539	215	40
3	7000	0.021	0.86	40	344	75	22
4	7000	0.029	1.14	40	421	99	24

[a] Initiator, AIBN (1 mol% to total monomer); polymerization time, 24 h; temp, 60°C; solvent, ethanol. [b] Estimated by GPC. [c] dm = mean diameter. [d] S.D. = standard deviation. [e] C.V. = S.D./dm, coefficient of variation.

Table II. Particle size of nanosphere

	dm[a]	S.D.[b]	C.V[c]
	nm	nm	%
PNVA nanosphere	421	99	24
PVAm nanosphere	743	83	12
Lactose conjugated nanosphere	690	135	20

[a] dm = mean diameter. [b] S.D. = standard deviation. [c] C.V. = S.D./dm, coefficient of variation.

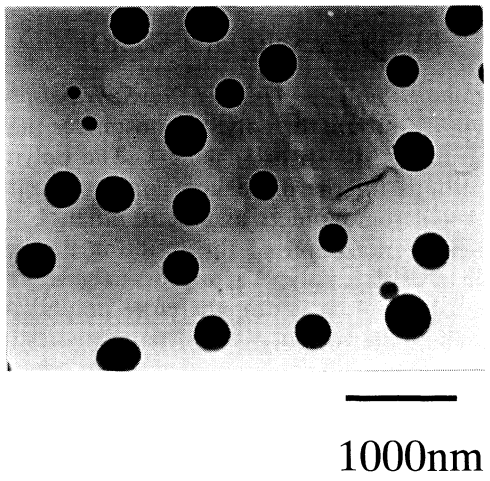

Figure 1. Transmission electron microscopy (TEM) image of nanospheres obtained by the dispersion copolymerization of PNVA macromonomer with styrene in ethanol.

spheres. Amide bonds are formed by the nucleophilic reaction of amine to carbonyl carbon; Kitano *et al.* prepared galactose lipid using this method.[22] In this study, lactose was conjugated on polyVAm chains that had accumulated on the polySt nanospheres by amide linkage. The amount of lactose conjugated as determined by the sulfuric anthrone method was 0.61 μg lactose residues per surface area of the nanospheres (1 cm^2), which was calculated by assuming that the density of the nanospheres was 1. That corresponded to fact that there are 3.2×10^6 lactose residues on the surface of one nanosphere. The authors estimate that 15% of the amino groups of the polyVAm on the nanospheres were conjugated by lactose. In fact, we did not fully understand where the carbohydrates locate at the surface or the core of the nanosphere. However, authors had clarified that the polyVAm exist on nanosphere surface by means of XPS analysis.[12] Therefore, hydrophilic polymeric chains having carbohydrates could be favorably located on the nanosphere surface. Figure 1 shows a TEM image of

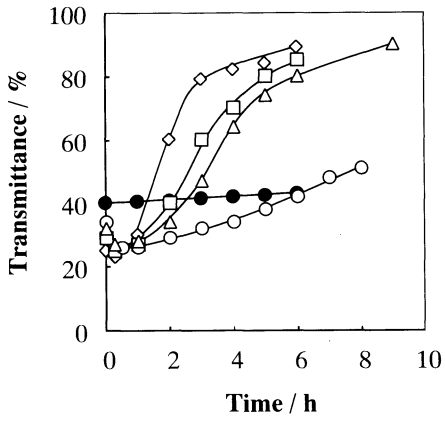

Figure 2. Nanosphere aggregation upon the addition of RCA_{120} lectin. Lectin concentration (○) 0.1 μM, (△) 0.3 μM, (□) 0.5 μM, (◇) 1 μM, (●) PVAm grafted PSt nanospheres.

a lactose-conjugated nanosphere. This nanosphere was spherical in form and there was no transformation in the form after lactose conjugation. The mean size of the lactose-conjugated nanospheres was 690 nm, which was between that of the polyNVA-grafted and polyVAm-grafted ones, as is also shown in Table II. That is feasible if the electrostatic repulsion of polyVAm chains was reduced by lactose conjugation.

Interaction of Lactose with Lectin

Figure 2 shows the time dependence of the transmittance of lactose-conjugated nanosphere dispersion, when RCA_{120} lectin, which interacts with galactose, was added at suitable concentrations. In all examples, the transmittance was decreased at first and subsequently increased gradually. The authors predicted that the decrease in the transmittance change might be shown by a carbohydrate-lectin interaction for the appearance of the lectin to the surface of the nanospheres. This increase in transmittance also leads to the precipitation of the aggregated nanospheres. These behavior was dependent on lectin concentration, *e.g.*, this was clearly observed at a lectin concentration of 1 μM, and not at 0.1 μM. The sedimentation velocity increased with the lectin concentration because the nanosphere binding to the lectin was too strong to make it a Brownian motion. When polyVAm-grafted nanospheres were used in this study, these phenomenon were not observed, as is also shown in Figure 2. When the lactose-conjugated nanospheres were remained alone, actually we observed slight precipitation which was smaller than that by adding lectin (data are not shown). The interaction between lectin and carbohydrate of the nanosphere surface

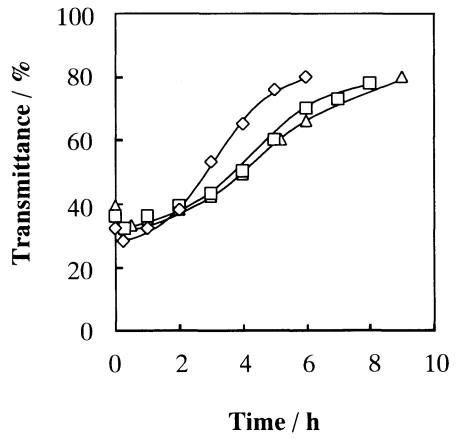

Figure 3. Nanosphere aggregation upon the addition of RCA_{120} lectin (1 μM) in the presence of lactose. (◇) no inhibitor, (△) lactose concentration 11 mg mL^{-1}, (□) lactose concentration 3 mg mL^{-1}.

might be inhibited by electric repulsion of the remained amino functions. We will discuss in detail after blocking the amino functions by acetyl group in the near future. The carbohydrate nanospheres showed transmittance changes by adding lectin, and also illustrated the possibility of the recognition of carbohydrates.

Next, the authors analyzed lectin interaction is inhibited by the addition of free lactose. Figure 3 shows the time dependence of the transmittance of the lactose-conjugated nanosphere dispersion when both RCA_{120} lectin and free lactose were added. The transmittance change became slightly gentle when lactose was added in the dispersion as compared to that without the inhibitor. In addition, inhibition was increased with an increase in the lactose concentration. Consequently, we concluded that the lectin recognized the lactose on the nanosphere. Although a slight inhibition was observed on the addition of free lactose, a much stronger inhibition must be observed when other detection systems were used. In this paper, it was found that the carbohydrates, which were conjugated on the surface of nanospheres, could be recognized by a carbohydrate binding protein.

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