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OLIGOSACCHARIDE-CARRYING STYRENE-TYPE MACROMERS. POLYMERIZATION AND SPECIFIC INTERACTIONS BETWEEN THE POLYMERS AND LIVER CELLS

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ABSTRACT

Polystyrene derivatives with lactose, glucose, maltose, maltotriose, maltopentaose, and maltoheptaose on each benzene ring were prepared by the radical polymerization of a new class of macromers synthesized by coupling the corresponding oligosaccharide lactones with *p*-vinylbenzylamine. These polymers consisting of amphiphilic structural units were water-soluble, and organic solutes were bound to hydrophobic microenvironments of the polymers in water. α-D-Glucopyranose-carrying polymers were recognized and precipitated by concanavalin A. Cultivation of liver cells (hepatocytes) was attempted using culture dishes whose surface was coated with lactose-, glucose-, maltose-, and maltotriose-carrying polystyrenes. It has been found that a lactose-carrying polystyrene (PVLA) is a useful surface material for hepatocyte culture. 1) Highly specific adhesion of hepatocytes was attained for PVLA-coated dishes with or without serum supplement. 2) The cell adhesion was a threshold

phenomenon with respect to the PVLA concentration on the dish. 3) The cell adhesion was effectively inhibited when hepatocytes were treated with PVLA molecules in the medium prior to culture. 4) The adhesion was not inhibited by albumin, an adhesion-inhibitory protein in serum. These findings suggest that pendent galactose residues of a PLVA molecule functioned as a strong recognition determinant for hepatocytes. We assume that multi-antennary, high-density galactose residues of PVLA are attributed to the specific adhesion of hepatocytes.

INTRODUCTION

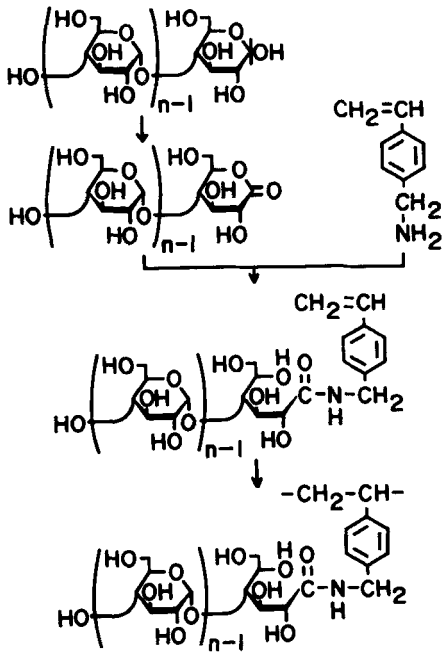
Carbohydrates comprise the most abundant group of naturally occurring substances. Recent developments in the field of molecular cell biology have clarified that oligosaccharide chains play basic roles in information, recognition, and regulation of living organisms [1-3]. Oligosaccharide chains of glycolipids and glycoproteins protrude from the surface of cell membranes and function as recognition markers for enzymes, lectins, viruses, bacteria, and cells. Synthetic polymers endowed with informational oligosaccharides, even commercially available simple ones, are of interest and importance in connection with pharmacological and biomedical applications [4].

We have developed a convenient high-yield synthetic method for oligosaccharide-substituted styrene-type macromers and their polymerizates [5-7]. These oligosaccharide-carrying styrene polymers functioned as specific recognition markers for lectins and liver cells (hepatocytes) [6, 8-10]. Lactose-carrying polystyrene has been found to be a useful biomedical material for the culture of hepatocytes. The polymer strongly enhanced adhesion and viability of hepatocytes in culture, which was attributed to the galactose-specific interaction between the polymer molecules and hepatocytes.

SYNTHESIS AND POLYMERIZATION OF OLIGOSACCHARIDE-CARRYING MACROMERS

Synthesis of Oligosaccharide-Carrying Polystyrenes

The synthetic route is shown in Scheme 1. The reducing end of oligosaccharide was oxidized with potassium hypoiodite to the corresponding lactone, which was then coupled with *p*-vinylbenzylamine in refluxing methanol. The resulting well-defined macromer was polymerized with a radical initiator in water and dimethylsulfoxide (DMSO). This simple method has several advantages [5-7]:



SCHEME 1. Synthetic route to oligosaccharide-carrying styrene homopolymers.

1) The oligosaccharide needs no protection of the hydroxyl groups; 2) the yield of each step is high; 3) each structural unit of the macromer and the polymer has a hydrophilic oligosaccharide moiety and a hydrophobic vinylbenzyl main chain; 4) both hydrophilic and hydrophobic moieties are connected covalently by an amide group.

The polystyrene derivatives abbreviated to PVLA, PVGA, PVMA, PVMTA, PVM5A, and PVM7A shown in Fig. 1 were prepared, respectively, starting from lactose, glucose, maltose, maltotriose, maltopentaose, and maltoheptaose. Lactose contains the β-(1→4)-galactopyranosyl unit, whereas the others carry α-(1→4)-glucopyranosyl units of different chain length.

The homopolymerization was carried out with azobisisobutyronitrile (AIBN) as an initiator in DMSO and with potassium peroxydisulfate ($K_2S_2O_8$) in water at 60°C. The polymerization proceeded homogeneously. White powdery polymers were isolated by dialyzing and freeze-drying their aqueous solutions. They were soluble in water and DMSO. The well-defined

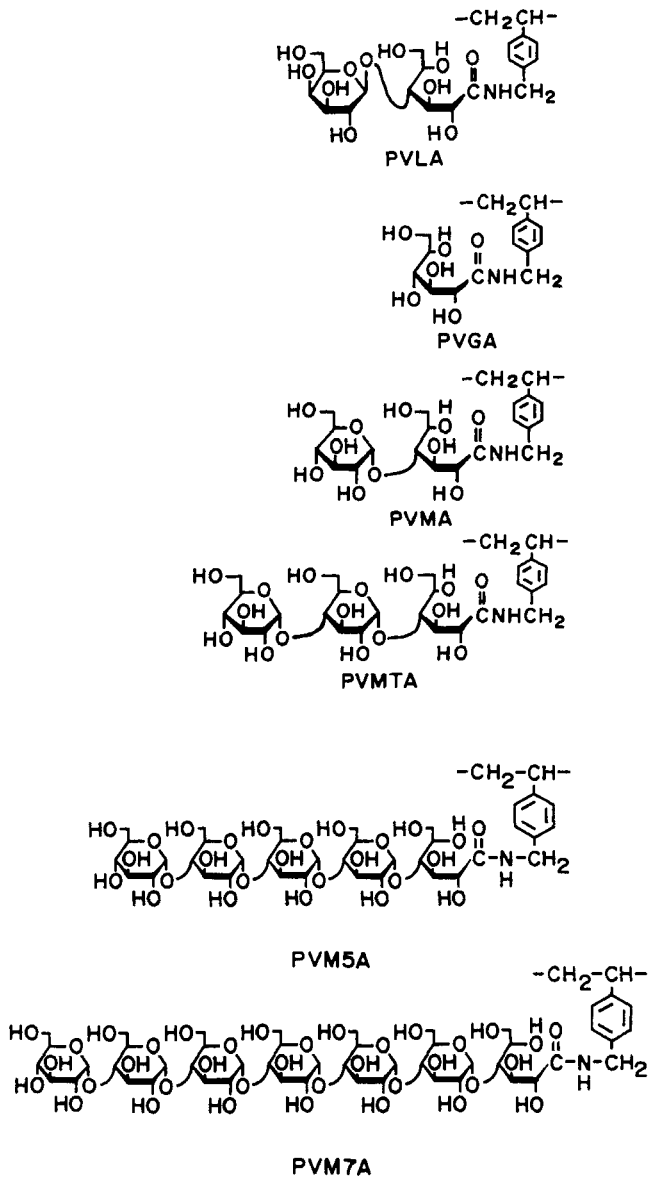


FIG. 1. Structural units of oligosaccharide-carrying styrene homopolymers.

structures of slightly different configurations and side-chain lengths were confirmed by ^{13}C-NMR spectroscopy. The anomeric C-1 signals were distinguishable as follows: 104.3 ppm, PVLA; 100.5, PVMA; 100.5 and 100.2, PVMTA; 100.3 and 100.1, each of PVM5A and PVM7A (50 MHz; solvent, DMSO-d_6).

Micellar Conformation of the Polymers in Water

The intrinsic viscosities determined in DMSO were higher than 0.9; i.e., high molecular weight polymers were obtained. The intrinsic viscosities in water, however, were only about 25-50% of those in DMSO. We assumed that the low viscosity in water reflected a tightly coiled micellar conformation of the polymer, which is attributable to their amphiphilic structure.

The micellar characteristics of the polymers in water were also suggested by study of their interactions with organic solutes [5, 6]. Binding of methyl orange and magnesium 1-anilino-8-naphthalenesulfonate (ANS) to the polymers (PVGA, PVMA, PVLA, and PVMTA) in water was investigated by difference absorption spectroscopy and spectrofluorometry, respectively. The polymers induced a blue shift of the main 464 nm methyl orange absorption by 15-20 nm and also of the 525-nm ANS fluorescence by 45 nm. These shifts suggested that both solutes were bound to hydrophobic regions of the polymers in a micelle-like conformation. The vinylbenzyl residues aggregated to form hydrophobic regions surrounded by hydrated carbohydrate residues.

INTERACTION BETWEEN A LECTIN (CONCANAVALIN A) AND THE POLYMERS

Lectins are highly specific carbohydrate-binding proteins that agglutinate cells and/or precipitate glycoconjugates [11]. Concanavalin A (Con A), isolated from jack bean, is a tetramer with four carbohydrate binding sites. It specifically binds to α-D-mannopyranosyl or α-D-glucopyranosyl residues at the nonreducing termini of oligo- and polysaccharides.

When PVMA and PVMTA were mixed with Con A in a buffer solution, the turbidity of the solution increased, and soon a precipitate was deposited. The precipitate, however, was dissolved by the addition of a monosaccharide D-mannose, and a clear solution was restored. The precipitation can be rationalized on the basis of crosslinkages among the tetrameric Con A molecules and α-D-glucose-carrying multivalent polymer molecules. The more sensitive monovalent ligand D-mannose expelled the glucose residues from the binding sites, resulting in dissociation of the multivalent interactions. PVGA and

PVLA did not show such a specific interaction with Con A [6]. The oligosaccharide chains of the polymers could be distinguished by Con A specifically.

THE GALACTOSE-SPECIFIC INTERACTIONS BETWEEN THE POLYMERS AND HEPATOCYTES

Significance of Hepatocyte Culture

A hepatocyte carries on hundreds of metabolic activities, and a surge of cell divisions occurs when the liver is injured. *In vitro,* however, the hepatocyte loses its cellular functions rapidly and has only limited viability. If hepatocytes could survive and proliferate *in vitro* over long periods of time, such a culture system would have a bright prospect as a biosimulator, a bioreactor, and also an artificial organ [12]. The most essential requirement of hepatocytes to survive is a solid surface to which they can anchor and adhere. We were interested in applying oligosaccharide-carrying polymers to substrata of culture dishes, since it is known that a hepatocyte has characteristic recognition abilities for oligosaccharide chains [13-18].

Experimental Method of Adhesion of Hepatocytes

Figure 2 shows the outline of the procedure of cell culture using PVLA, PVGA, PVMA, and PVMTA as substrata [9]. An aqueous solution of each homopolymer (0.1 mg/mL) was placed in polystyrene culture dishes at room temperature to adsorb the polymer on the surface of dishes. After the solution was decanted, a suspension of rat hepatocytes [19] was fed into the polymer-coated dishes and maintained at 37°C in a humidified incubator for a prescribed time. Nonadhering cells were collected and counted.

Highly Specific Adhesion of Hepatocytes to Dishes Coated with Lactose-Carrying Styrene Homopolymer (PVLA)

The most effective cultivation of hepatocytes was attained when the culture dishes were coated with lactose-carrying polystyrene (PVLA) in serum-supplemented Williams' medium E, as shown in Fig. 3 [9]. The adhesion occurred rapidly within 1 h, then increased gradually and reached a maximum (92%) after 12 h. Thereafter, some cells were detached slowly, but more than 50% of the cells still adhered and survived even after 5 days' incubation.

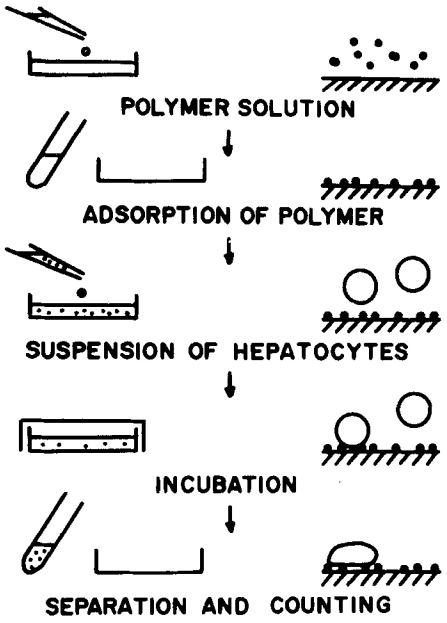


FIG. 2. Experimental procedure for cultivation of hepatocytes in polymer-coated dishes.

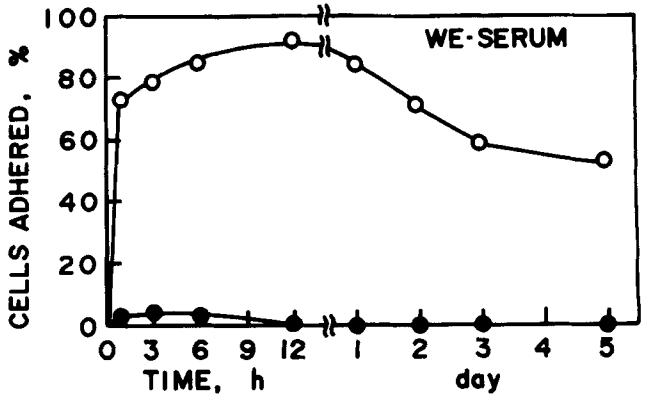


FIG. 3. Adhesion of hepatocytes to polymer-coated dishes in serum-supplemented Williams' medium E. (○): PVLA; (●): PVGA, PVMA, and PVMTA.

On the other hand, little adhesion of hepatocytes was induced in those dishes coated with PVGA, PVMA, and PVMTA.

The marked specificity for PVLA can be explained on the basis of the participation of galactose-specific lectins which are located on the surface of a rat hepatocyte [13, 14]. It is supposed that the lectins are bound to the galactose terminals which protrude numerously from a PVLA molecule but cannot be bound to the open-chain gluconamide residue of PVGA and the α-D-glucopyranose residue of PVMA and PVMTA.

Serum-Free Culture of Hepatocytes in PVLA-Coated Dishes

PVLA-coated dishes were effective even under serum-free conditions as shown in Fig. 4 [9]. About 75% adhesion in 12 h and 30% survival after 5 d were attained. Serum contains poorly defined biological substances which are responsible for the complex and ambiguous nature of the cultivation [20]. It is also expensive and, hence, efforts were made to replace the serum by well-defined hormones and growth factors. As the composition of the medium is simplified, the viability of the cells becomes sensitive to the properties of the solid surface to which the cells are attached directly. The following study on interactions between PVLA molecules and hepatocytes was carried out under serum-free conditions.

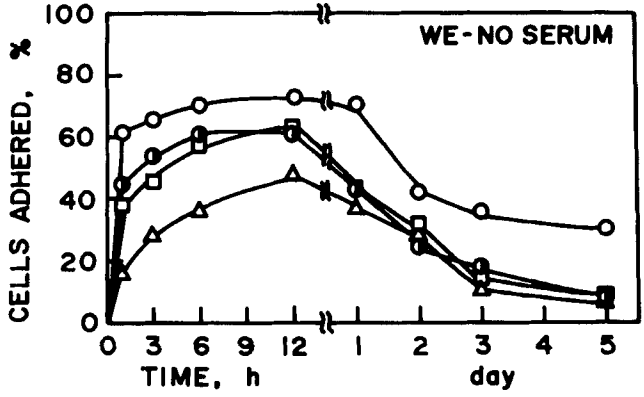


FIG. 4. Adhesion of hepatocytes to polymer-coated dishes in serum-free Williams' medium E. (○): PVLA; (◑): PVGA; (□): PVMA; (△): PVMTA.

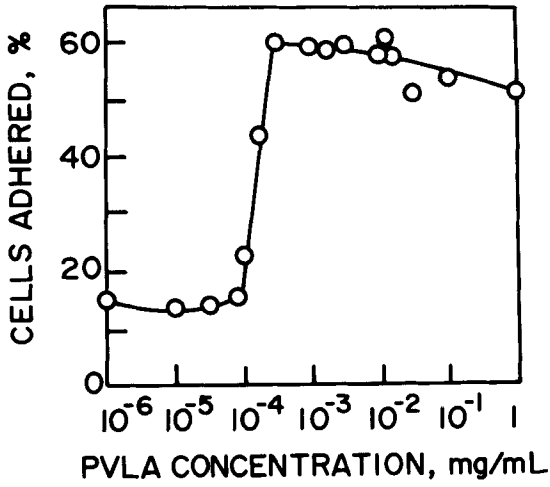


FIG. 5. Threshold response of hepatocyte adhesion to PVLA-coated dishes. The abscissa is PVLA concentration used for coating polystyrene dishes. Cell culture was carried out in a serum-free Hanks' solution for 60 min.

Threshold Phenomenon of Hepatocyte Adhesion to PVLA-Coated Dishes

Figure 5 shows that the percent cell-adhesion depended upon the PVLA concentration of the diluted aqueous solution that was used to absorb the polymer on the surface of the dishes [8]. There was little adhesion until a critical PVLA concentration (10^{-4} mg/mL) was reached, and an increase of the concentration above this value permitted maximal adhesion, i.e., the cell-adhesion was a threshold phenomenon [15, 16].

The amount of adsorbed PVLA has not been determined, but the following estimation was made [10]. When 0.30 mL aqueous PVLA solution (0.10 mg/mL) was eluted through a micro flow cell packed with monodisperse polystyrene microspheres, the amount of equilibrium adsorption of PVLA was 59 ± 4 ng/cm^2. If 1 mL aqueous PVLA solution (10^{-4} mg/mL) is placed on a 35-mm diameter dish and all of the PVLA molecules are adsorbed, the amount of adsorption will be 11 ng/cm^2. These two values are of the same order of magnitude. We assumed that the equilibrium PVLA adsorption was attained when PVLA was adsorbed monomolecularly on the surface of the dish, and that the adhesion threshold occurred at this stage.

TABLE 1. Inhibition Effect of Carbohydrate Derivatives on Hepatocyte Adhesion to PVLA-Coated Dishes[a]

Carbohydrate	Concentration, mmol/L)	Adhesion, %
None	–	78
Galactose	10	71
Lactose	10	74
VLA[b]	0.2	74
PVMA	0.2	70
PVLA	0.2	18

[a]3×10^5 cells/mL; serum-free Williams' medium E; hepatocytes were treated with carbohydrate derivatives for 60 min and then incubated for 60 min.

[b]Lactose-substituted styrene monomer.

Inhibition of Hepatocyte-Adhesion to PVLA-Coated Dishes by Pre-treating Hepatocytes with PVLA Solution

The addition effect of some related carbohydrates on hepatocyte adhesion was examined and is summarized in Table 1 [10]. The hepatocyte suspension was treated with an aqueous carbohydrate solution for 60 min with occasional shaking and then incubated in PVLA-coated dishes. The hepatocyte adhesion was effectively inhibited by treating the hepatocytes with PVLA molecules in a culture medium prior to adhesion. However, little effect was observed on the addition of free galactose, lactose, lactose-carrying styrene monomer (VLA), and maltose-carrying styrene polymer (PVMA).

The galactose-specific interactions of hepatocytes and lectins has been investigated using naturally occurring and synthetic oligosaccharides which carry galactose terminals [15-18]. It was reported that the galactose density is the most dominant factor for recognition: Multiantennary or highly branched oligosaccharides were bound very strongly to the receptors [17, 18]. In this respect, PVLA is expected to exhibit very strong binding abilities for hepatocytes. PVLA has multiantennary and high-density galactose residues because numerous galactose terminals protrude from a PVLA molecule. Hepatocytes could interact with PVLA in solution as well as on the solid surface, and, hence, not only adhesion of hepatocytes but also their inhibition were effected [10].

Hepatocyte-PVLA Specific Interactions Are Not Inhibited by Bovine Serum Albumin

As mentioned above, hepatocyte adhesion to PVLA-coated dishes was enhanced by the addition of serum. However, time-adhesion profiles on the other polymer-coated dishes were distinct from those on PVLA-coated dishes [9]. Under serum-free conditions, a considerable amount of hepatocytes adhered to the dishes coated with PVGA, PVMA, and PVMTA (Fig. 4), whereas very little adhesion was observed under serum-supplemented conditions (Fig. 3). It is known that serum contains adhesion-inhibitory proteins as well as stimulatory factors. It is possible that the sum of these interactions increased the adhesion of hepatocytes to the PVLA-coated dishes, but decreased their adhesion to the other polymer-coated dishes.

One of the inhibitory proteins in serum is albumin [20]. Addition effects of bovine serum albumin (BSA) on the hepatocyte adhesion are shown in Fig. 6. In the experiments represented by solid lines, hepatocytes were treated with BSA in a serum-free medium and then cultured on the polymer-coated dishes. The treatment of hepatocytes with BSA enhanced their adhesion to PVLA-coated dishes but inhibited their adhesion to the other polymer-coated dishes. It is reasonable to assume that the interactions between hepatocytes and PVLA molecules were highly specific and strong enough not to be hindered by albumin. It is also possible that the surface of hepatocyte modified by BSA accelerated their interactions with PVLA. In contrast, the adhesion

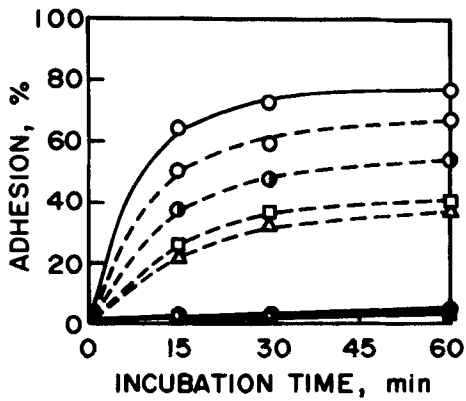


FIG. 6. Effect of bovine serum albumin (BSA) on hepatocyte adhesion. (—): Hepatocyte suspension in Williams' medium E was treated with BSA (0.1 mmol/L); (– –): control experiment; (○): PVLA; (◑): PVGA; (□): PVMA; (△): PVMTA.

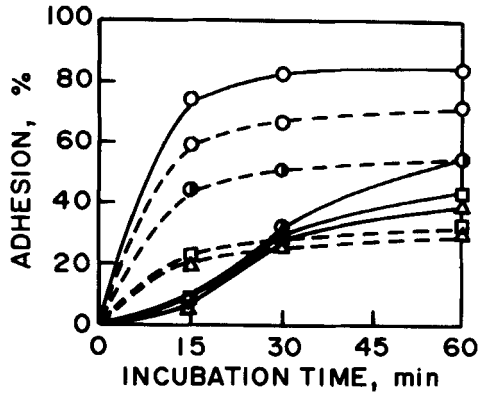


FIG. 7. Effect of bovine serum albumin (BSA) on hepatocyte adhesion. (——): The culture dishes were treated at first with the polymers in Williams' medium E and then BSA; (– –): control experiment; (○): PVLA; (◑): PVGA; (□): PVMA; (△): PVMTA.

of hepatocytes to the glucose-type polymer-coated dishes was mediated by nonspecific interactions, and these weak interactions were hindered by albumin.

In the experiments represented by solid lines in Fig. 7, the culture dishes were first coated with the polymers and then with BSA. These polymer- and BSA-coated dishes were compared with polymer-coated but BSA-noncoated dishes (broken lines). The adhesion to PVLA-coated dishes was enhanced by treatment with BSA; this tendency was similar to the addition effect of BSA observed in Fig. 6. The strong specific interactions were not hindered by BSA. When other polymer-coated dishes were treated with BSA, the adhesion was retarded but soon recovered. We assume that the hepatocyte adhesion was inhibited because albumin was adsorbed on the oligosaccharide-containing polymer molecules. However, as the small amount of the adsorbed hydrophilic protein was gradually dissolved into the medium, adhesion was improved. When the culture dishes were coated with the polymers and also with fetal calf serum, the results were similar to the polymer- and BSA-coated dishes.

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