Synthesis of Polymethacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer and Their Application to Control Adhesion of Hepatocytes Cancer Cells on the Materials

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ABSTRACT: Polymers having a sugar moiety in the side group have been utilized as artificial matrices for cell adhesion in tissue engineering. In this study, methacrylamide - based polymers having lactose and maltose derivative structures in the side group with various aliphatic hydrocarbon spacers were synthesized, and their cell adhesion properties were examined. Methacrylamide monomers were prepared by two step amidation of a spacer diamine, first with a sugar lactone and then with a methacrylic anhydride. These monomers were radically polymerized in aqueous media using 4,4′-azobis(4-cyanovaleric acid) (ACVA) as radical initiator to give the corresponding polymethacrylamide. Specific interaction between these polymers and animal cell was investigated by adhesion of proliferated human liver cancer cell (WRL) to the polymethacrylamides. WRL interacted with polymers having a lactose structure with a hexamethylene or 1,4-cyclohexylene spacer by a specific manner and was promoted typical spheroid formation, while it did not interacted with polymers having a maltose structure. © 2013 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2013**, *51*, 4003–4010

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INTRODUCTION Saccharides–protein interactions on the cell surfaces play important roles in cell–cell communication, immune response and pathogen invasion.[1] Although, monovalent saccharide–protein interactions are generally considered weak, the interaction can be amplified by the clustering effect. Therefore, polymers having a sugar moiety in the repeating unit[2] are expected to be useful as functional biomaterials,[3] because of their strong clustering effect of the sugar moieties.[4,5] In addition, such synthetic polymers are considered as potentially important materials to study fundamentals of sugar–protein interactions. Actually, such polymers with a sugar moiety (glyco-polymer) have been utilized for recognition of protein,[6,7] toxin,[8,9] application of immunochromatography,[10] and culture of cells.[11,12]

Glyco-polymer have been applied to tissue engineering and worked as extracellular matrices (ECMs). Cell attachment, cell growth, and cell behavior to these sugar carrying polymers are regulated by their sugar groups, and these cellular effects are completely different from natural ECM interaction. Akaike and Kobayashi, as pioneers of such styrene-based glyco-polymers, synthesized fascinating polymers showing cluster effect of carbohydrate, which are applicable to cell culture materials.[13] Their polymers such as poly(*p*-*N*-vinylbenzyl-D-lactonamide) (PVLA) [Fig. 1(a)], which have a monovalent sugar moiety in each unit, showed strong affinity to cells specifically by the clustering effect. Hepatocytes attached to a PVLA-coated surface through the specific interaction between galactose groups in PVLA and cell surface receptors (ASGP-R), which recognize galactose.

As PVLA has unique amphiphilic structural units composed of polystyrene backbone as a hydrophobic part and an oligosaccharide residue in the side chain as a hydrophilic part,[14] PVLA is strongly adsorbed to hydrophobic materials such as polystyrene and polyurethane.[15] Besides that, PLVA has high biocompatibility and bioresorbability, so it is supposed to be widely employed as scaffolds in tissue engineering. However, PVLA has two problems to be solved. One is toxicity of a vinylbenzylamine intermediate used in the preparation, and the other is

Additional Supporting Information may be found in the online version of this article.

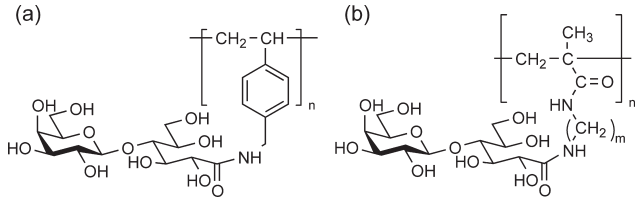


FIGURE 1 Chemical structures of (a) PVLA and (b) methacrylamide-based glycol-polymers.

the cumbersome synthetic process. They prepared the polymers by radical polymerization of a carbohydrate modified styrene monomer. According to their methodology, at least five step reactions were required to obtain the polymer.

In the most case, however, synthetic methods of such glycopolymers require protecting group chemistry and multiple-step reactions, and purifications. These chemical strategies are both time consuming and costly.

We consider that novel glyco-polymers are needed which can be simply synthesized without protection of the sugar hydroxyl groups, and without using a toxic intermediate.

Because many amines can easily add to sugar-derived lactones[16,17] and methacrylic anhydride[18] under mild conditions, various methacrylamide-based monomers with both sugar moiety and hydrocarbon spacer can be readily synthesized, by using aliphatic diamines as linking compounds.

We report the facile synthesis of polymethacrylamides having a sugar moiety with an aliphatic hydrocarbon spacer, trimethylene, hexamethylene, or 1,4-cyclohexylene, without any protections and without using any toxic intermediates.

In this study, we synthesized methacrylamides having a lactose or maltose lactone derivative with an aliphatic hydrocarbon spacer and polymerized them[19] (Fig. 2) via radical polymerization [Fig. 1(b)].

We selected two kinds of sugar, lactose and maltose. Lactose, having a β-galactose moiety, can specifically interact with WRL. In contrast, Maltose, having no β-galactose or GlcNAc moiety, can't interact with WRL.

We evaluated their interaction with cells by observing effective adhesions of WRL on the polystyrene dish coated with these polymethacrylamides having a sugar derived moiety.

EXPERIMENTAL

Chemicals

The following reagents were used as received; 4,4′-azobis(4-cyanovaleric acid) (ACVA) (Wako Pure Chemical Industries, Oosaka, Japan), 1,3-diaminopropane, 1,6-diaminohexane, 1,4-cyclohexanediamine (TCI, Tokyo, Japan), dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), methacrylic anhydride (Sigma-Aldrich, Louisiana, MO).

The sugar lactones, lactonolactone and maltonolactone, were synthesized according to the literature. Details are described in the Supporting Information.[20,21]

Characterization

^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a JEOL AL300 NMR spectrometer in D_2O. Chemical shifts were determined using tetramethylsilane or the residual protons as the internal standards. IR spectra were taken on a Thermo Scientific Nicolet iS10 spectrometer.

Gel permeation chromatography (GPC) was carried out with a JASCO LC-Net II high-performance liquid chromatography with TOSOH TSKgel ALPHA-M and phosphate buffer saline (PBS) as eluent. Molecular weights were evaluated by a PEO standard.

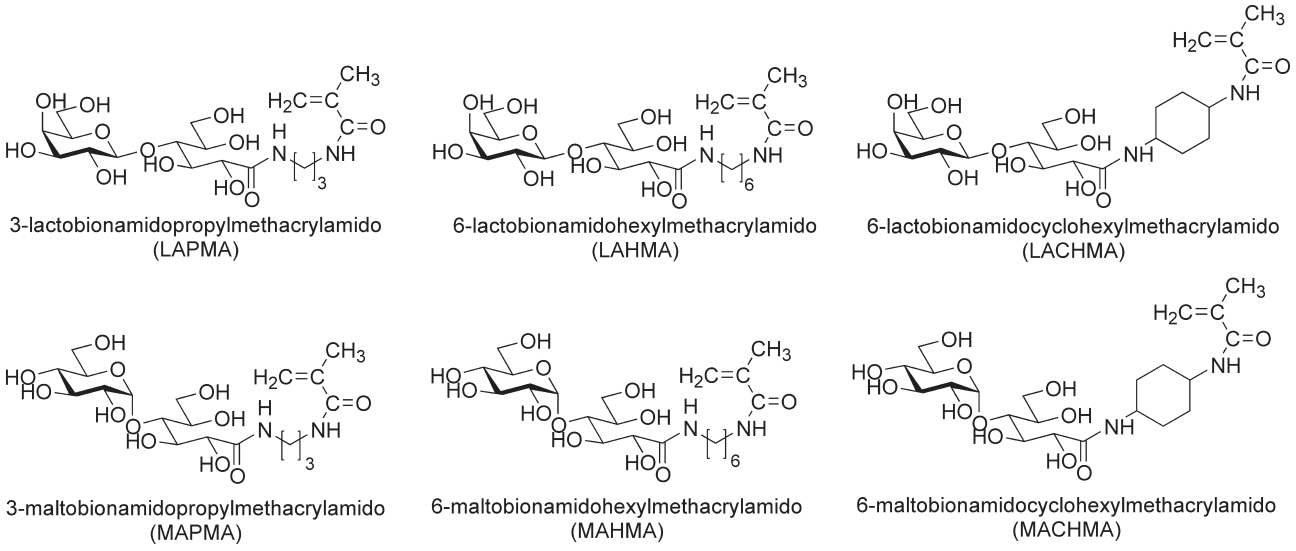


FIGURE 2 Chemical structures of methacrylamide monomers synthesized in this study.

The stained cells were observed by a fluorescence microscope on Olympus IX71.

General Procedure for Synthesis of Methacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer

A DMF solution of methylene diamine (2.8 mol/mL, 10 mL) was stirred in ice bath. A DMF solution of sugar lactone **1** (5.6×10^{-2} mol/mL, 50 mL) was added dropwise to the diamine solution over 30 min. The mixture was allowed to react for 5 h at 0 °C and then at ambient temperature overnight. The DMF was evaporated to concentrate and the residue was poured into chloroform, and an insoluble part was collected by filtration. The remaining solid was dissolved in H_2O and the solution was washed with chloroform. The aqueous solution was concentrated by evaporation to give an intermediate product (white powder) **2**, which was used for the following reactions without further purification. A DMF solution of this compound **2** (0.4 mol/mL, 100 mL) was stirred in ice bath. Methacrylic anhydride in DMF (1.2 mol/mL, 50 mL) was added dropwise to the solution over 10 min. The mixture was allowed to react for 5 h at 0 °C. The solution was poured into chloroform to give a white precipitate, which was collected by filtration. The resulting powder was dissolved in H_2O and the solution was washed with chloroform. Finally, the product was purified by reverse-phase column chromatography (LH-20 2×30 cm^2 in H_2O) to yield the desired methacrylamide **3** as a white powder.

Synthesis of 3-Lactobionamidopropyl Amine (LAPA)

A solution of 1,3-diaminopropane (2.65 g, 28 mmol, 10 equiv.) in DMF (5 mL) was stirred in ice bath. Lactonolactone (1.0 g, 2.8 mmol, 1 equiv.) in DMF (80 mL) was added dropwise to the mixture over 30 min. The product was washed with chloroform and evaporated to give LAPA (905 mg, 22 mmol) in 78.3% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.57$ (d, 1H, $J = 7.5$ Hz, H-1′), 4.40 (d, 1H, $J = 3.0$ Hz, H-2), 4.19 (t, 1H, $J = 3.0$, H-4), 3.93 (m, 3H, H-5, 2′, 5′), 3.75 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.32 (t, 2H, $J = 6.9$ Hz, —C<u>H</u>$_2$—CH_2—CH_2—NH_2), 2.67 (t, 2H, $J = 6.9$ Hz, —CH_2—CH_2—C<u>H</u>$_2$—NH_2), 1.65 (m, 2H, NH—CH_2—C<u>H</u>$_2$—CH_2—)

Synthesis of 3-Lactobionamidopropyl Methacrylamide (LAPMA)

A solution of LAPA (850 mg, 2.1 mmol, 1 equiv.) in DMF (50 mL) was stirred in ice bath. Methacrylic anhydride (388 mg, 2.5 mmol, 1.2 equiv.) in DMF (10 mL) was added dropwise to the mixture over 10 min. The product was purified by reverse-phase column (H_2O) to give LAPMA (775 mg, 1.6 mmol) in 76.0% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 5.66$ (d, 1H, $J = 0.9$ Hz, C<u>H</u>$_2$=C), 5.35 (d, 1H, $J = 0.9$ Hz, C<u>H</u>$_2$=C), 4.59 (d, 1H, $J = 7.5$ Hz, H-1′), 4.44 (d, 1H, $J = 2.7$ Hz, H-2), 4.20 (t, 1H, $J = 2.7$ Hz, H-4), 3.94 (m, 3H, H-5, 2′, 5′), 3.78 (m, 7H, H-3, 6, 3′, 4′, 6′), $J = 3.13$ (t, 2H, $J = 6.9$ Hz, —C<u>H</u>$_2$—CH_2—CH_2—NH—CO—C=C), 3.04 (t, 2H, 6.9 Hz, —CH_2—C<u>H</u>$_2$—NH—CO—C=C), 1.94 (s, 3H, C=C—C<u>H</u>$_3$), 1.80 (m, 2H, NH—CH_2—C<u>H</u>$_2$—CH_2—)

Synthesis of 6-Lactobionamidohexyl Amine (LAHA)

A solution of 1, 6-diaminohexane (3.9 g, 28 mmol, 10 equiv.) in DMF (5 mL) was stirred in ice bath. Lactonolactone (1.0 g, 2.8 mmol, 1 equiv.) in DMF (80 mL) was added dropwise to the mixture over 30 min. The product was washed with chloroform and evaporated to give LAHA (1.13 g, 25 mmol) in 89.0% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.56$ (d, 1H, $J = 7.5$ Hz, H-1′), 4.40 (d, 1H, $J = 3.0$ Hz, H-2), 4.19 (t, 1H, $J = 3.0$, H-4), 3.94 (m, 3H, H-5, 2′, 5′), 3.79 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.28 (t, 2H, $J = 6.6$ Hz, —NH—C<u>H</u>$_2$—$(CH_2)_4$—CH_2—NH_2), 2.82 (t, 2H, $J = 6.9$ Hz, —$(CH_2)_4$—C<u>H</u>$_2$—NH_2), 1.59 (m, 4H, NH—CH_2—C<u>H</u>$_2$—CH_2—), 1.38 (m, 4H, NH—CH_2—CH_2—C<u>H</u>$_2$—)

Synthesis of 6-Lactobionamidohexyl Methacrylamide (LAHMA)

A solution of LAHA (1.0 g, 2.2 mmol, 1 equiv.) in DMF (50 mL) was stirred in ice bath. Methacrylic anhydride (407 mg, 2.6 mmol, 1.2 equiv.) in DMF (10 mL) was added dropwise to the mixture over 10 min. The product was purified by reverse-phase column (H_2O) to give LAHMA (1.08 g, 2.1 mmol) in 94.1% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 5.67$ (d, 1H, $J = 0.9$ Hz, C<u>H</u>$_2$=C), 5.44 (d, 1H, $J = 0.9$ Hz, C<u>H</u>$_2$=C), 4.57 (d, 1H, $J = 7.5$ Hz, H-1′), 4.40 (d, 1H, $J = 2.7$ Hz, H-2), 4.19 (t, 1H, $J = 2.7$ Hz, H-4), 3.94 (m, 3H, H-5, 2′, 5′), 3.75 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.59 (t, 2H, $J = 7.2$ Hz, —NH—C<u>H</u>$_2$—$(CH_2)_4$—CH_2—NH—CO—C=C), 3.26 (t, 2H, 6.9 Hz, —C<u>H</u>$_2$—NH—CO—C=C), 1.94 (s, 3H, C=C—C<u>H</u>$_3$), 1.55 (m, 4H, NH—CH_2—C<u>H</u>$_2$—CH_2—), 1.36 (m, 4H, NH—CH_2—CH_2—C<u>H</u>$_2$—)

Synthesis of 6-Lactobionamidocyclohexyl Amine (LACHA)

A solution of 1,4-cyclohexanediamine (3.18 g, 28 mmol, 10 equiv.) in DMF (10 mL) was stirred in ice bath. Lactonolactone (1.0 g, 2.8 mmol, 1 equiv.) in DMF (80 mL) was added dropwise to the mixture over 30 min. The product was washed with chloroform and evaporated to give LACHA (998 mg, 22 mmol) in 78.7% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.47$ (d, 1H, $J = 7.2$ Hz, H-1′), 4.29 (d, 1H, $J = 7.5$ Hz, H-2), 4.09 (t, 1H, $J = 2.4$ Hz, H-4), 3.83 (m, 3H, H-5, 2′, 5′), 3.67 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.46 (m, 1H, —NH—C<u>H</u>—$(CH_2$—$CH_2)_2$—CH—NH_2), 2.53 (m, 1H, —C<u>H</u>—NH_2), 1.50 (m, 8H, —NH—CH—(C<u>H</u>$_2$—C<u>H</u>$_2$)$_2$—CH—NH_2)

Synthesis of 6-Lactobionamidocyclohexyl Methacrylamide (LACHMA)

A solution of LACHA (980 mg, 2.2 mmol, 1 equiv.) in DMF (50 mL) was stirred in ice bath. Methacrylic anhydride (400 mg, 2.6 mmol, 1.2 equiv.) in DMF (10 mL) was added dropwise to the mixture over 10 min. The product was purified by reverse-phase column (H_2O) to give LACHMA (915 mg, 1.8 mmol) in 81.2% yield.

^{1}H—NMR (300 MHz, D_2O) $\delta = 5.55$ (d, 1H, $J = 1.2$ Hz, C<u>H</u>$_2$=C), 5.24 (d, 1H, $J = 1.2$ Hz, C<u>H</u>$_2$=C), 4.46 (d, 1H, $J = 7.5$ Hz, H-1′), 4.33 (d, 1H, $J = 2.7$ Hz, H-2), 4.10 (t, 1H, $J = 2.7$ Hz,

H-4), 3.80 (m, 3H, H-5, 2′, 5′), 3.62 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.45 (m, 1H, —NH—CH—(CH_2—CH_2)$_2$—CH—NH—CO—C≡C), 3.23 (m, 1H, —CH—NH—CO—C≡C), 1.72 (s, 3H, C≡C—CH_3), 1.50 (m, 8H, —NH—CH—(CH_2—CH_2)$_2$—CH—NH—)

Synthesis of 3-Maltobionamidopropyl Amine (MAPA)

A solution of 1,3-diaminopropane (2.65 g, 28 mmol, 10 equiv.) in DMF (10 mL) was stirred in ice bath. Maltonolactone (1.0 g, 2.8 mmol, 1 equiv.) in DMF (80 mL) was added dropwise to the mixture over 30 min. The product was washed with chloroform and evaporated to give MAPA (950 mg, 23 mmol) in 81.3% yield.

^{1}H NMR (300 MHz, D_2O) δ = 4.31 (d, 1H, J = 2.4 Hz, H-1′), 4.19 (d, 1H, J = 3.6 Hz, H-2), 4.13 (t, 1H, J = 3.0, H-4), 3.91 (m, 3H, H-5, 2′, 5′), 3.71 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.31 (t, 2H, J = 6.9 Hz, —CH_2—CH_2—CH_2—NH_2), 2.70 (t, 2H, J = 7.2 Hz, —CH_2—CH_2—CH_2—NH_2), 1.67 (m, 2H, —NH—CH_2—CH_2—CH_2—NH—)

Synthesis of 3-Maltobionamidopropyl Methacrylamide (MAPMA)

A solution of MAPA (900 mg, 1.9 mmol, 1.0 equiv.) in DMF (50 mL) was stirred in ice bath. Methacrylic anhydride (845 mg, 2.2 mmol, 1.2 equiv.) in DMF (10 mL) was added dropwise to the mixture over 10 min. The product was purified by reverse-phase column (H_2O) to give MAPMA (832 mg, 1.5 mmol) in 80.2% yield.

^{1}H NMR (300 MHz, D_2O) δ = 5.42 (d, 1H, J = 0.9 Hz, CH_2=C), 5.18 (d, 1H, J = 0.9 Hz, CH_2=C), 4.33 (d, 1H, J = 2.4 Hz, H-1′), 4.22 (d, 1H, J = 3.6 Hz, H-2), 4.14 (t, 1H, J = 3.0, H-4), 3.94 (m, 3H, H-5, 2′, 5′), 3.74 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.43 (t, 2H, J = 6.9 Hz, —CH_2—CH_2—CH_2—NH—CO—C≡C), 3.31 (t, 2H, 6.9 Hz, —CH_2—CH_2—CH_2—NH—CO—C≡C), 1.99 (s, 3H, C≡C—CH_3), 1.78 (m, 2H, —NH—CH_2—CH_2—CH_2—NH—)

Synthesis of 6-Maltobionamidohexyl Amine (MAHA)

A solution of 1,6-diaminohexane (3.9 g, 28 mmol, 10 equiv.) in DMF was stirred in ice bath. Maltonolactone (1.0 g, 2.8 mmol, 1 equiv.) in DMF (80 mL) was added dropwise to the mixture over 30 min. The product was washed with chloroform and evaporated to give MAHA (1.12 mg, 25 mmol) in 88.0% yield.

^{1}H NMR (300 MHz, D_2O) δ = 4.30 (d, 1H, J = 2.4 Hz, H-1′), 4.22 (d, 1H, J = 3.6 Hz, H-2), 4.09 (t, 1H, J = 2.4 Hz, H-4), 3.88 (m, 3H, H-5, 2′, 5′), 3.74 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.36 (m, 1H, —NH—CH_2—(CH_2—CH_2)$_2$—CH_2—NH_2), 2.42 (m, 1H, —CH_2—NH_2), 1.60 (m, 8H, —NH—CH_2—(CH_2—CH_2)$_2$—CH_2—NH_2)

Synthesis of 6-Maltobionamidohexyl Methacrylamide (MAHMA)

A solution of MAHA (1.0 g, 1.9 mmol, 1 equiv.) in DMF (50 mL) was stirred in ice bath. Methacrylic anhydride (354 mg, 2.3 mmol, 1.2 equiv.) in DMF (5 mL) was added dropwise to the mixture over 10 min. The product was purified by reverse-phase column (H_2O) to give MAHMA (977 mg, 1.6 mmol) in 85.3% yield.

^{1}H NMR (300 MHz, D_2O) δ =5.45 (d, 1H, J = 0.9 Hz, CH_2=C), 5.20 (d, 1H, J = 0.9 Hz, CH_2=C), 4.32 (d, 1H, J = 2.4 Hz, H-1′), 4.23 (d, 1H, J = 3.6 Hz, H-2), 4.10 (t, 1H, J = 2.4 Hz, H-4), 3.90 (m, 3H, H-5, 2′, 5′), 3.75 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.42 (t, 2H, J = 7.2 Hz, —NH—CH_2—(CH_2)$_4$—CH_2—NH—CO—C≡C), 3.26 (t, 2H, 6.9 Hz, —CH_2—NH—CO—C≡C), 1.94 (s, 3H, C≡C—CH_3), 1.55 (m, 4H, NH—CH_2—CH_2—CH_2—), 1.36 (m, 4H, NH—CH_2—CH_2—CH_2—)

Synthesis of 6-Maltobionamidocyclohexyl Amine (MACHA)

A solution of 1,4-cyclohexanediamine (3.18 g, 28 mmol, 10 equiv.) in DMF was stirred in ice bath. Maltonolactone (1.0 g, 2.8 mmol, 1 equiv.) in DMF (80 mL) was added dropwise to the mixture over 30 min. The product was washed with chloroform and evaporated to give MACHA (1.0 g, 22 mmol) in 79.5% yield.

^{1}H NMR (300 MHz, D_2O) δ = 4.30 (d, 1H, J = 1.8 Hz, H-1′), 4.12 (m, 2H, H-2, 4), 3.95 (m, 3H, H-5, 2′, 5′), 3.77 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.46 (m, 1H, —NH—CH—(CH_2—CH_2)$_2$—CH—NH_2), 2.24 (m, 1H, —CH—NH_2), 1.60 (m, 8H, —NH—CH—(CH_2—CH_2)$_2$—CH—NH_2)

Synthesis of 6-Maltobionamidocyclohexyl Methacrylamide (MACHMA)

A solution of MACHA (900 mg, 1.9 mmol, 1 equiv.) in DMF (50 mL) was stirred in ice bath. Methacrylic anhydride (345 mg, 2.2 mmol, 1.2 equiv.) in DMF (5 mL) was added to the mixture dropwise over 10 min. The product was purified by reverse-phase column (H_2O) to give MACHMA (898 mg, 1.8 mmol) in 82.2% yield.

^{1}H NMR (300 MHz, D_2O) δ = 5.55 (d, 1H, J = 1.2 Hz, CH_2=C), 5.24 (d, 1H, J = 1.2 Hz, CH_2=C), 4.46 (d, 1H, J = 7.5 Hz, H-1′), 4.10 (m, 2H, H-2, 4), 3.90 (m, 3H, H-5, 2′, 5′), 3.70 (m, 7H, H-3, 6, 3′, 4′, 6′), 3.45 (m, 1H, —NH—CH—(CH_2—CH_2)$_2$—CH—NH—CO—C≡C), 3.23 (m, 1H, —CH—NH—CO—C≡C), 1.72 (s, 3H, C≡C—CH_3), 1.50 (m, 8H, —NH—CH—(CH_2—CH_2)$_2$—CH—NH—)

General Procedure for Synthesis of Polymethacrylamides Having Sugar Moieties with Aliphatic Hydrocarbon Spacers

A mixture of monomers **3**, an initiator (ACVA), and water was charged in a schlenk flask and degassed by three freeze-pump-thaw cycles. The flask was sealed under vacuum and heated at 60 °C with occasional agitation. The polymerization was stopped by cooling the tube, and the products were precipitated in acetone and collected by centrifugal sedimentation (10,000 G for 30 min). The polymeric products were purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000. Freeze drying of the solution by FD510 gave the desired polymer as a white solid powder.

Polymerization of LAPMA

LAPMA (100 mg, 207 mmol) was polymerized using 2.0 mol % of initiator of ACVA (1.2 mg, 4.15 mmol) in water (830

μL, 0.25 mol/L) at 60 °C. The product was precipitated in acetone, collected by centrifugal sedimentation, and purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000 to give polyLAPMA (78 mg) in 78% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.40–2.80$ (lactose part), 2.35–1.60 (spacer and linear), 1.55–0.70 (—CH_2—C(CH_3)—)

Polymerization of LAHMA

LAHMA (100 mg, 191 mmol) was polymerized using 1.0 mol % of ACVA (0.54 mg, 1.91 mmol) in water (770 μL, 0.25 mol/L) at 60 °C. The product was precipitated in acetone, collected by centrifugal sedimentation, and purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000 to give polyLAHMA (75 mg) in 75% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.50–2.80$ (lactose part), 2.20–1.50 (spacer and linear), 1.40–0.75 (—CH_2—C(CH_3)—)

Polymerization of LACHMA

LACHMA (100 mg, 191 mmol) was polymerized using 3.0 mol % of initiator of ACVA (1.6 mg, 5.74 mmol) in water (830 μL, 0.25 mol/L) at 60 °C. The product was precipitated in acetone, collected by centrifugal sedimentation, and purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000 to give polyLACHMA (70 mg) in 70% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.50–2.90$ (lactose part), 2.35–1.45 (spacer and linear), 1.30–0.75 (—CH_2—C(CH_3)—)

Polymerization of MAPMA

MAPMA (100 mg, 207 mmol) was polymerized using 2.0 mol % of initiator of ACVA (1.2 mg, 4.15 mmol) in water (830 μL, 0.25 mol/L) at 60 °C. The product was precipitated in acetone, collected by centrifugal sedimentation, and purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000 to give polyMAPMA (80 mg) in 80% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.45–2.80$ (maltose part), 2.35–1.60 (spacer and linear), 1.55–0.70 (—CH_2—C(CH_3)—)

Polymerization of MAHMA

MAHMA (100 mg, 191 mmol) was polymerized using 3.0 mol % of initiator of ACVA (1.6 mg, 5.74 mmol) in water (770 μL, 0.25 mol/L) at 60 °C. The product was precipitated in acetone, collected by centrifugal sedimentation, and purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000 to give polyMAHMA (70 mg) in 70% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.45–2.80$ (maltose part), 2.35–1.50 (spacer and linear), 1.40–0.70 (—CH_2—C(CH_3)—)

Polymerization of MACHMA

LACHMA (100 mg, 191 mmol) was polymerized using 2.0 mol % of initiator of ACVA (1.1 mg, 3.83 mmol) in water (830 μL, 0.25 mol/L) at 60 °C. The product was precipitated in acetone, collected by centrifugal sedimentation, and purified by dialysis in cellulose tubes with a molecular weight cutoff of 2000 to give polyMACHMA (72 mg) in 72% yield.

^{1}H NMR (300 MHz, D_2O) $\delta = 4.45–2.80$ (maltose part), 2.30–1.45 (spacer and linear), 1.20–0.70 (—CH_2—C(CH_3)—)

Preparation of Polystyrene Substrates Coated with Polymethacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer

Polymethacrylamides having a lactose and maltose derived moiety with an aliphatic hydrocarbon spacer were diluted to 0.01% (w/v) concentration with by ultrapure water. One milliliter of the solution was put on a nontreated six-well cell culture plate and the water was evaporated under atmospheric conditions for overnight and then rinsed twice with PBS buffer.

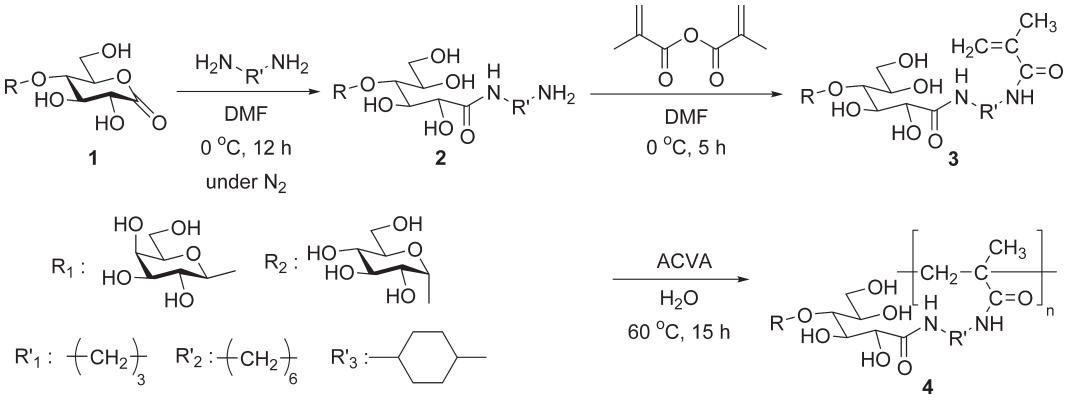
Culture of Cells

1.5 mL of hepatic cancer cell line WRL cells (1.5×10^4 cell/mL) in Dulbecco's modified essential medium (DMEM) were seeded for each polymer coated six wells plate and then cultured in 5% CO_2 at 37 °C for 4 h. After 4 h incubation, the medium was removed to separate nonadherent cells and 1.5 mL of 10% fetal bovine serum (FBS) containing DMEM was added. The cell morphology was observed at 4, 24, 48, and 72 h after seeding the cells.

RESULTS AND DISCUSSION

Syntheses of Polymethacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer

As shown in Scheme 1, methacrylamides having a sugar lactone derivative of lactonolactone or maltonolactone with an



SCHEME 1 Synthesis of polymethacrylamides having a sugar moiety with an aliphatic hydrocarbon spacer.

appropriate spacer were synthesized in good yields by an amidation of the corresponding sugar lactone with an amine in DMF at 0 °C, followed by a further amidation with methacrylic anhydride. These monomers were obtained as a white powder over 60% yields. The chemical structures were identified and analyzed by their ^{1}H NMR and IR spectroscopy.

We selected three kinds of aliphatic hydrocarbon spacers, trimethylene, hexamethylene, and 1,4-cyclohexylene. We made a comparative review of polymer character, that trimethylene and hexamethylene are difference in carbon number, and hexamethylene and 1,4-cyclohexylene are difference in structure.

Homopolymerizations of these monomers were conducted in water using ACVA as an initiator via free radical polymerization (Scheme 1). As shown in Table 1, the homopolymerizations smoothly proceeded with high conversions (over 90%) in water. When the [initiator] / [monomer] ratio was decreased from 3.0 to 1.0 mol %, the molecular weight of the polymer increased. High molecular weight polymers were obtained when DMF or DMAc was used as a solvent, however the obtained polymers were insoluble in water.

These polymethacrylamides synthesized here were subjected to the examination of culture of cells.

Modification of Polystyrene Substrates with Polymethacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer

Polystyrene substrates were coated by an addition of 0.01 wt % aqueous solution of the polymethacrylamides with lactose or maltose derived structure. Water contact angles of some of the substrates thus prepared were significantly reduced by the modification. As shown in Table 2, contact angles of substrates modified with PLAHMA, PMAHMA, and PLACHMA reduced less than 40° from the control contact angle of 87.7°. Conversely, the contact angle of the substrates were almost unchanged (>71.5°) by the modification with PLAPMA, PMAPMA and PMACHMA.

The polymethacrylamides with lactose or maltose—derived structure on polystyrene substrate were further investigated by XPS analysis. Typical XPS wide scans of substrates modified with PLACHMA and PMACHMA are shown in Figure 3. The substrate modified with PLACHMA showed a main emission peak at 530.0 eV, which can be assigned to O1s. It also exhibited a peak at 397.0 eV, which can be assigned to N1s. These observations suggest the presence of sugar moiety on polystyrene surface. In contrast, PMACHMA modified substrate didn't show any peaks related to sugar moieties. Substrates modified with PLAHMA and PMAHMA also showed emission peaks of sugar moieties, while substrates modified with PLAPMA or PMAPMA did not show them (Supporting Information).

TABLE 2 Contact Angles of Polystyrene Surfaces Modified with Polymethacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer

Polymer	Contact Angle (°)
PLAPMA	78.6
PLAHMA	27.1
PLACHMA	30.3
PMAPMA	81.6
PMAHMA	39.9
PMACHMA	71.5
Control	87.7

The polymethacrylamides prepared in this study have both hydrophilic saccharide moieties and hydrophobic spacer in the side groups. The saccharide moieties increase polymer's solubility toward water in addition to the ability of cellular recognition, while the hydrophobic spacer parts should be strongly absorbed to the polystyrene substrate. Therefore, the hydrophilic—hydrophobic balance, in sugar moiety[22] and spacer part of these polymers are quite important not only for cellular recognition but also for efficient coating of the substrates. In the case of PLACHMA, PLAHMA, and PMAHMA, substrates modified with these polymers showed much smaller contact angle than that of the control experiment, although the polymers had hydrophobic hexamethylene or 1, 4-cyclohexylene spacers. We consider that these polymers had enough hydrophobicity to be absorbed on polystyrenes

TABLE 1 Polymerization of Lactose or Maltose Derivative Monomers with Various Aliphatic Hydrocarbon Spacers[a]

Run No.	Monomer	[Initiator]/[Monomer]	Conv. (%)[b]	Yield (%)[c]	M_n (g/mol)[d]	M_w/M_n[d]
1	LAPMA	2.0 mol %	94	78	38,000	4.9
2	LAHMA	1.0 mol %	97	75	120,000	3.3
3	LACHMA	3.0 mol %	96	70	82,000	2.7
4	MAPMA	2.0 mol %	95	80	14,000	3.0
5	MAHMA	3.0 mol %	97	70	20,000	8.1
6	MACHMA	2.0 mol %	92	72	14,000	4.5

[a] Concentration: 0.25 mol/L, solvent: H_2O, 60 °C, reaction time: 15 h.
[b] Determined by ^{1}H NMR.
[c] Acetone insoluble part was dialyzed (under cut 2,000).
[d] Estimated by GPC (Eluent : PBS(-), poly (ethylene oxide) standards).

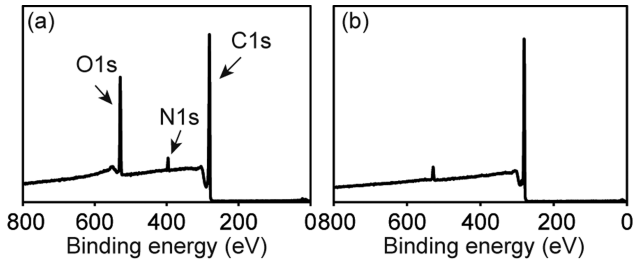


FIGURE 3 Typical XPS spectra of substrates modified with (a) PLACHMA and (b) PMACHMA.

substrates. As a result, the contact angles of the substrates modified with these polymers may become smaller. Conversely, in the case of PLAPMA, PMAPMA, and PMACHMA, the hydrophobic—hydrophilic balance might not good to be absorbed on a polystyrene substrate.

Cell Culture

To investigate specific interactions between polymers having sugar-derived structures with various aliphatic hydrogen carbon spacers and cell, the cell adhesion experiments were carried out on a polystyrene dish coated with these polymers. The human hepatic cancer cell line WRL specifically recognized glycoproteins having terminal galactose/GalNAc moieties. The WRL exclusively adhered onto the polystyrene surface coated with 0.01% (w/v) solution of PLAHMA and PLACHMA as shown in Figure 4. As expected, WRL interacted with polymers having lactose derived moieties with aliphatic hydrocarbon spacers, PLAHMA and PLACHMA, which possess terminal galactose moieties. On the PLAHMA and PLACHMA, WRL cells became round shape at 4 h after seeding the cell and then formed spheroids at 24 h after seeding, which is well-known as a phenomenon for keeping high hepatic function. When hepatocytes were cultured by these polymers, adhesion and proliferation of the cells were almost same as the aggregates cultured on PVLA coated surface (Supporting Information).[23,24]

TABLE 3 The Number of WRL Recognized on Polystyrene Surface Modified with Polymethacrylamides Having a Sugar Moiety with an Aliphatic Hydrocarbon Spacer

Polymer	The Number of Cells (cells/nm^2)
PLAPMA	21
PLAHMA	70
PLACHMA	68
PMAPMA	20
PMAHMA	5
PMACHMA	22
Control	24

Conversely, WRL did not interact with MAHMA, which has glucose moieties at the end of the side groups. These results suggested that PLAHMA and PLACHMA specifically interacted with the WRL by β-galactose recognition, while in the case of PLAPMA, PMAPMA, and PMACHMA, WRL interacted onto the surface by a nonspecific way similar to the control experiment.

WRL adhesion with surface modified sugar polymer was compared in term of number of cells at 4 h after seeding the cell (Table 3). The numbers of adhesion cells for PLAHMA and PLACHMA were 70 (cells/nm^2) and 68 (cells/nm^2), respectively, whereas that for PMAHMA was 5 (cells/nm^2), and those for the other polymers ranged from 20 to 22 (cells/nm^2).

It is also worth noting that the hydrophobicity of the spacer was important for successful coating of the polystyrene dish with the polymers with sugar moieties and good cell recognition.

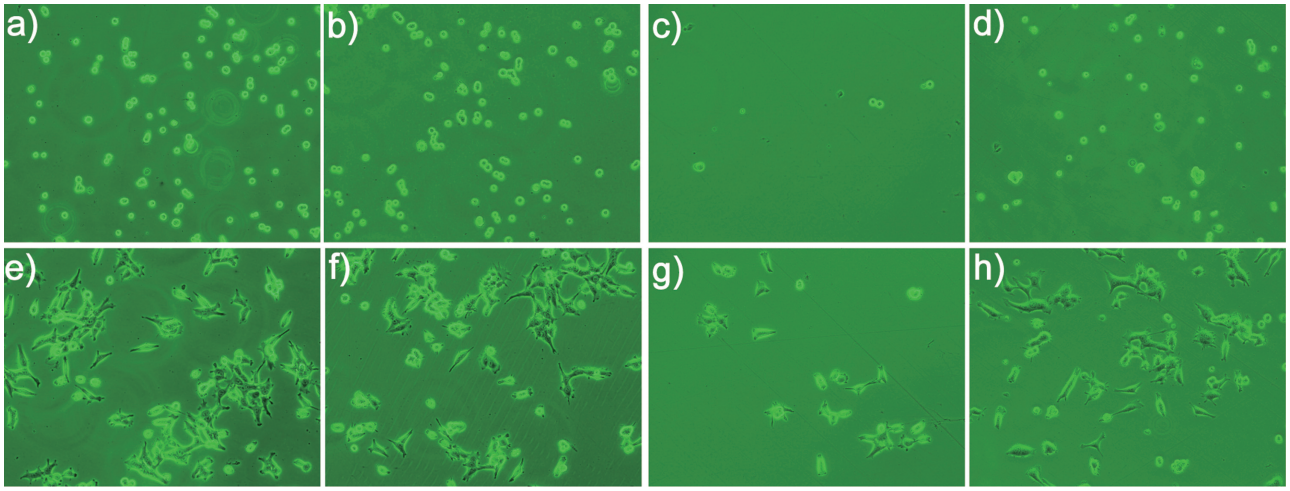


FIGURE 4 Morphology of WRL cultured on polystyrene dishes with various polymethacrylamides having a sugar moiety. WRL were cultured for 4 h (a) PLAHMA, (b) PLACHMA, (c) PMAHMA, (d) control PS dish, and cultured for 24 h (e) PLAHMA, (f) PLACHMA, (g) PMAHMA, (h) control PS dish.

In the case of polymers with a trimethylene spacer, coating efficiency was not enough to attach the WRL cells onto the corresponding polymers.

CONCLUSIONS

In this study, we synthesized methacrylamides having a sugar moiety with an aliphatic hydrocarbon spacer by two steps starting from sugar lactones. These monomers were easily polymerized via radical polymerization using ACVA as an initiator.

Polymethacrylamides having strong hydrophobic part (LAHMA, LACHMA and MAHMA) are well absorbed onto hydrophobic surfaces such as the polystyrenes dish, exposing the hydrophilic carbohydrate moiety in an aqueous phase. Conversely, other polymers having weak hydrophobic part could not be absorbed onto hydrophobic surfaces. The exposed carbohydrates worked well as matrix ligands for WRL recognition.

We demonstrated that a specific function could be achieved by culturing hepatocytes cancer cells in the PLAHMA and PLACHMA. These polymethacrylamides may offer various possibilities to be used in cell biological studies of liver functions on polystyrene substrates.

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