ELSEVIER

Carbohydrate Polymers 37 (1998) 71–78

Carbohydrate Polymers

Polyacrylamide containing sugar residues: synthesis, characterization and cell compatibility studies

Raman Bahulekar[a], Takayoshi Tokiwa[a], Junko Kano[a], Toshiharu Matsumura[b], Isao Kojima[c], Makoto Kodama[a,*]

[a]*Bionic Design Group, National Institute for Advanced Interdisciplinary Research, 1-1-4 Higashi, Tsukuba, Ibaraki 305, Japan*
[b]*Cell Technology Center, Meiji Institute of Health Science, Meiji Milk Products Co. Ltd, 540 Naruda, Odawara, Kanagawa 250, Japan*
[c]*National Institute of Material and Chemical Research, 1-1-4 Higashi, Tsukuba, Ibaraki 305, Japan*

Received 12 February 1997; revised 17 July 1997; accepted 1 August 1997

Abstract

Hydrophilic polyacrylamide compounds having glucose (PAAm-glucose) and galactose (PAAm-galactose) as pendent groups were synthesized from poly(acryloyl chloride) by polymer analogous reactions. The degree of glucose and galactose substitution was 53.2 and 41.6%, respectively. Tissue culture polystyrene (TCPS) plates coated with these polymers showed increased surface wettability. FTIR-ATR spectra of coated plates showed characteristic bands at 1658, 1716 and 1734 cm^{-1} due to the carbonyl groups of polyacrylamide, PAAm-glucose and PAAm-galactose, respectively. Coating of polyacrylamide, PAAm-glucose and PAAm-galactose onto TCPS plates was also confirmed by X-ray photoelectron spectroscopic (XPS) characterization. Rat hepatocytes in primary culture attached to the surfaces of PAAm-glucose and PAAm-galactose, but did not attach to those of polyacrylamide (PAAm). Only small differences were found between PAAm-glucose, PAAm-galactose and PAAm in the attachment of L929 and CHO-K1 cells. Cell growth rate behavior of the latter two cell lines on PAAm-glucose, PAAm-galactose and PAAm was almost identical.

Keywords: Cell adhesion; Glycopolymers; Polymer substrates; Surface characterization; Polymer analogous reactions

1. Introduction

Glycotechnology has been widely accepted as one of the most important tools for a variety of biomedical applications (Kochetkov, 1984; Duncan et al., 1989). It has been appreciated that synthetic polymers bearing sugar residues recognize cells through the marker molecules present on the cell surface (Schnaar, 1994; Weigel et al., 1979). In cultures, such polymers also offer a good surface for cell attachment.

Much attention has been paid in recent years to synthesizing polymers having sugar residues for cell attachment. Most of the methods previously reported involved preparing a functional sugar monomer by introducing an olefinic polymerizable group (with or without spacer) and subsequent polymerization of this monomer using a free radical initiator. We investigated the synthesis and characterization of polyacrylamides having simple sugar residues such as glucose and galactose by polymer analogous reactions. Preliminary results on cell attachment to these polymer surfaces using rat hepatocytes in primary culture as well as L929 and CHO-K1 cells are presented in this communication.

2. Materials and methods

2.1. Synthesis of polymers

2.1.1. Polymer analogous reaction

Glucosamine and galactosamine hydrochloride were obtained from Wako Pure Chemical Industries Co. Ltd, Japan and were used without any further purification. The polymers were generated (Fig. 1) by first polymerizing distilled acryloyl chloride to form poly(acryloyl chloride) in dry 1,4-dioxane under N_2 at 65°C for 24 h. 2,2′-azobisisobutyronitrile (AIBN, 1 mole% of monomer) was used as the initiator. Glucosamine and galactosamine hydrochloride in an alkaline medium such as bicarbonate buffer, are then reacted with poly(acryloyl chloride) solution to form polyacrylamide, having glucose (PAAm-glucose) and galactose (PAAm-galactose) residues, respectively. The reaction

* Corresponding author.

0144–8617/98/$ - see front matter
PII S0144-8617(98)00027-7

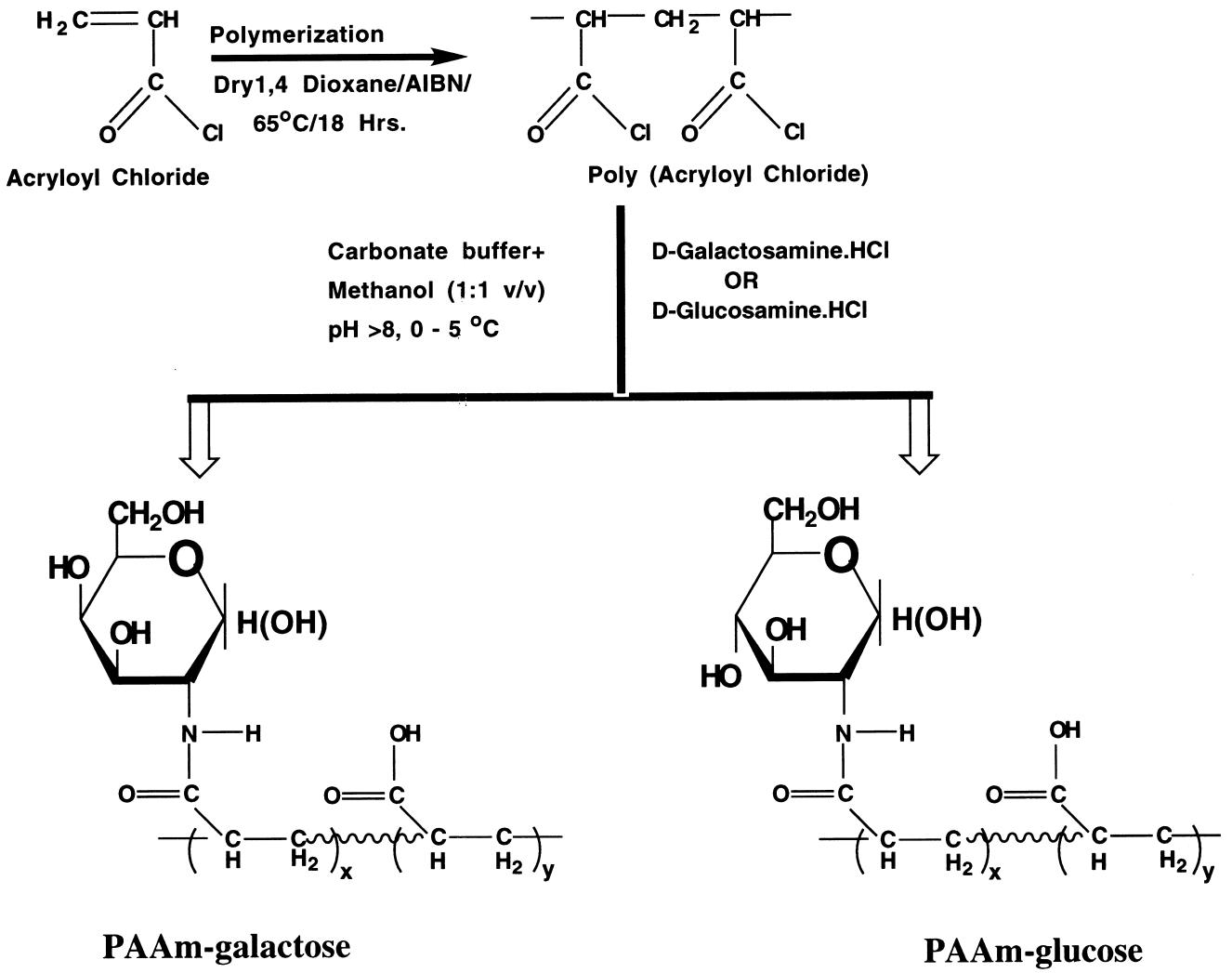

Fig. 1. Synthesis scheme for generating PAAm-glucose and PAAm-galactose by polymer analogous reaction.

mixtures were diluted with ethanol and poured into acetonitrile to precipitate the polymers. The polymers were purified by repeated dissolution and precipitation (three times) and finally freeze-dried from aqueous solution.

2.1.2. Homopolymer synthesis from functionalized monomer

Glucosamine and galactosamine hydrochloride were reacted with acryloyl chloride in bicarbonate buffer (pH $>$ 8):methanol (1:1 v/v), to form monomers (Kallin et al., 1989). The structure of the monomers was confirmed by NMR. Polymerization of *N*-acryloylated monomers was carried out in water at 30 $\pm$ 1°C for 18 h using the $(NH_4)_2S_2O_8$/TEMED initiator system. Polymers were precipitated by pouring the reaction mixture into methanol. The polymers were purified by repeated dissolution and precipitation (three times) and finally freeze-dried from aqueous solution.

2.2. Prepararation of polymer coated plates

Aqueous solutions of these polymers (1.0 ml, 1% w/v) were contacted with 35 mm tissue culture polystyrene (TCPS) plates (area = 9.62 cm^2) (Iwaki, Tokyo, Japan) for 48 h at room temperature. The plates were thoroughly dried under vacuum at room temperature. Crosslinking of adsorbed polymer was done by exposing the plates to UV radiation (UV crosslinker CL-1000, UVP Inc., Upland, USA) of 1.2×10^5 $\mu J/cm^2$ for 30 min. The polymer coating was confirmed by surface characterization techniques (FTIR-ATR, X-ray photoelectron spectroscopy (XPS) and contact angle measurements). FTIR-ATR measurements was carried out on a Jasco FT/IR-350 equipped with ATR cell ATRMax™ (Pike Technologies Inc., USA). The spectra were recorded at an angle of 45° using a trapezoidal shaped ZnSe ATR crystal and liquid nitrogen cooled MCT detector. Fifty scans were taken for each spectrum at a resolution of 4 cm^{-1}. XPS spectra were measured on an ESCAlab 220i (Fisons Instruments) with monochromatized Al/K_α X-ray radiation at an energy of 100 eV for survey spectra, 10 and 20 eV to resolve the C 1s and O 1s spectra, respectively. Water contact angle measurements were made using a FACE contact angle goniometer (Kyowa Scientific Instruments, Japan). Polyacrylamide (PAAm) was used as

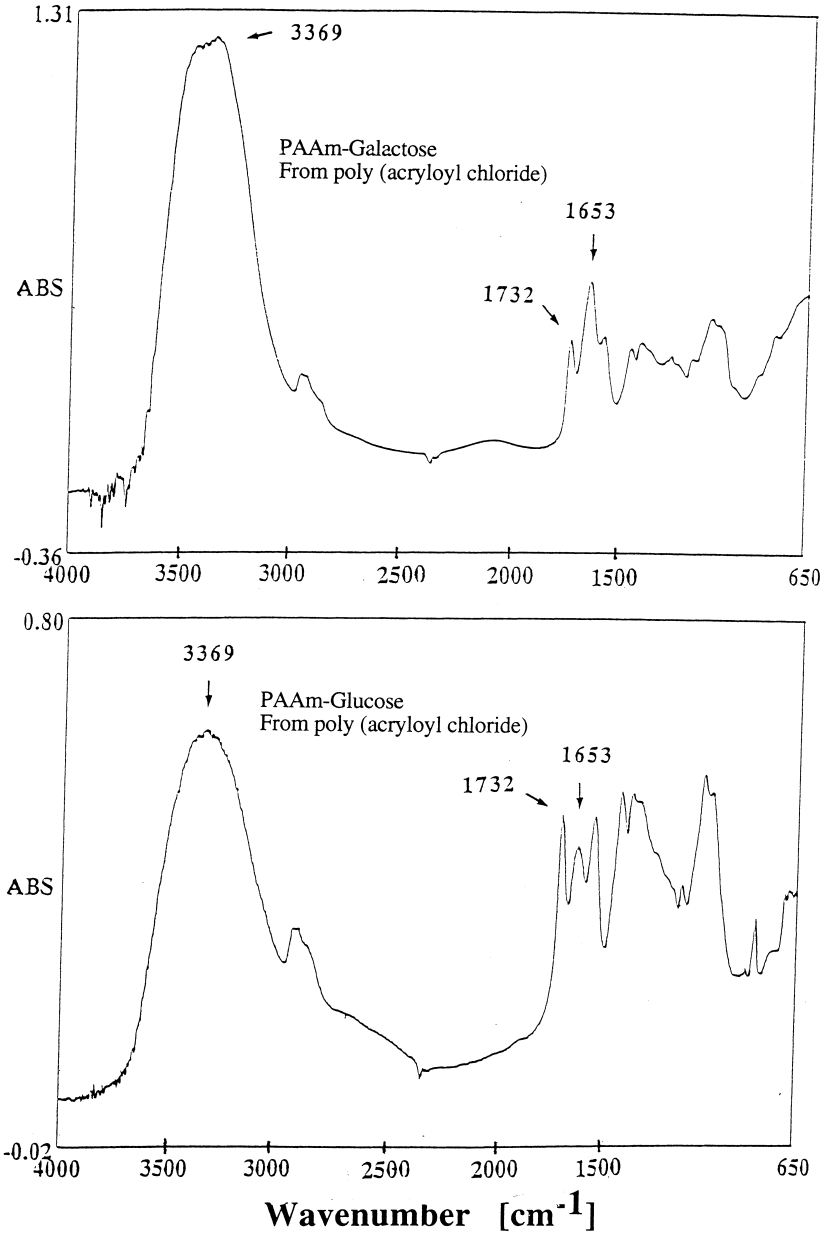

Fig. 2. FTIR spectra (KBr pellet) of PAAm-glucose and PAAm-galactose.

control. GPC analyses were made using a Viscotek system with an RI detector. Pullulan samples were used as standards. The solvent was water containing 50 mM LiCl and the column temperature was 40°C.

2.3. Preparation of hepatocytes and cell attachment test

Hepatocytes were prepared from male Sprague-Dawley rats (150–200 g) by a collagenase perfusion method (Seglen, 1976). The liver cells suspended in Hanks balanced salt solution (HBSS) were filtered through a 50 μm nylon mesh. The cell pellet was collected by centrifugation for 2 min at 50 $\times$ *g*. Cells were further purified by repeating the centrifugation. The cell pellets were suspended in Percoll™ solution (density 1.07 g/ml) and centrifuged for 10 min at 50 $\times$ *g*. More than 90% of the isolated cells were viable as measured by trypan blue dye exclusion test. The resulting parenchymal cells were suspended in Williams E medium containing 50 ng/ml EGF (R&D system, Minneapolis, USA), 0.1 μM insulin (Wako) and 1 μM dexamethasone (Sigma, St Louis, MO, USA). Isolated cells were inoculated at a concentration of 2.4×10^5 cells/plate with 2 ml of serum-free culture medium and incubated for 24 h in an incubator as mentioned above. In this study, type I collagen-coated dishes (Iwaki) were used as controls. Cell attachment was evaluated by taking the photographs of cells on a Olympus IMT-2 phase contrast microscope equipped with a camera.

2.4. Culture of cell lines and cell growth test

Mouse fibroblast L929 and CHO-K1 cell lines were obtained from RGB cell bank, RIKEN, Japan. The culture medium was minimum essential medium (MEM) supplemented with 10 mM non-essential amino acid solution (MEM NAA) and 10% fetal bovine serum (FBS). For cell growth study, inocula (2 ml) of 5×10^4 and 10^5 cells/ml of L929 and CHO-K1 cells, respectively, were seeded onto TCPS plates coated with polymers and cultured as monolayers in a humidified incubator at 37°C under 5% CO_2 in air. The medium was changed once every 2 days. For cell counting, culture medium was decanted off and culture plates were treated with 0.25% trypsin in 0.02% ethylenediamine tetraacetic acid (EDTA). The plates were incubated at 37°C for 3–5 min. Trypsin–EDTA action was quenched by adding several milliliters of culture medium. The detached cells were suspended in culture medium and counted using a hemocytometer.

3. Results and discussion

It has been reported previously that 2,2′-azobisisobutyronitile initiated acryloyl chloride polymerization in 1,4-dioxane yields soluble linear poly(acryloyl chloride) (Schulz et al., 1960). However, there have been very few reports on the utilization of poly(acryloyl chloride) in polymer analogous reactions (Strohriegl, 1993). Two polyacrylamide derivatives bearing glucose and galactose sugar moieties were synthesized from poly(acryloyl chloride) and amino sugars such as glucosamine and galactosamine hydrochloride. The two sugars are isomers of each other. These polyacrylamide-sugar derivatives are hydrophilic and miscible with water in all proportions. The transmission FT-IR spectra of PAAm-glucose and PAAm-galactose in KBr pellets (Fig. 2) shows 1732 and 1653 cm^{-1} bands, characteristic of acid (or ester) and amide carbonyl groups, respectively. It should be noted that there are some inherent shortcomings associated with polymer analogous reactions. The most important one is structural units formed by side reactions and complete substitution of reactive carbonyl chloride groups in the prepolymer. In the present case, a substitution reaction between amino sugars (glucosamine and galactosamine hydrochloride) and reactive carbonyl chloride groups is carried out in bicarbonate buffer (pH $>$ 8.0) and methanol (1:1 v/v) solvent. It has been reported previously that *N*-acryloylation of glucosamine proceeds best above pH 8 in bicarbonate:methanol (1:1 v/v) solvent (Kallin et al., 1989). Other factors, such as solubility of glucosamine or galactosamine hydrochloride in organic solvents, also restrict the choice of the solvents. As a result a bicarbonate–methanol reaction medium, side reactions leading to the formation of acid and/or ester groups cannot be avoided. An aqueous 1% (w/v) solution was used to coat the TCPS plates. The polymer layer was adsorbed strongly onto TCPS plates. The polymer coating was found to be quite stable under surface characterization and cell culture experimental conditions. The water contact angle decreased substantially after coating. TCPS plates had a contact angle of 71.8°. TCPS plate coated with polyacrylamide had a contact angle of 24.3°. The contact angle of TCPS plates coated with PAAm-glucose and PAAm-galactose was below 20°. FTIR-ATR spectra of bare TCPS plate and TCPS plates coated with polyacrylamide, PAAm-glucose and PAAm-galactose are presented in Fig. 3. The coated TCPS plates showed bands at 1658, 1716 and 1734 cm^{-1} caused by the carbonyl group present in polyacrylamide, PAAm-glucose and PAAm-galactose, respectively. PAAm and PAAm-galactose coated TCPS plates showed additional broad bands at 3332 and 3306 cm^{-1} due to N–H and OH stretching vibrations. In contrast, the TCPS plate coated with PAAm-glucose showed a very weak and broad hump in the same region though the transmission spectra (Fig. 2) shows the presence of strong –OH (3369 cm^{-1}) stretching vibrations in PAAm-glucose as well as in PAAm-galactose. This may be caused by the difference in adsorption behavior of these polymers on TCPS plates. XPS survey spectra (Fig. 4) of TCPS plates coated with polycrylamide, PAAm-glucose and PAAm-galactose showed N 1s peaks at 399.68, 399.52 and 399.69 eV, respectively. An additional peak due to Na was also noted in these samples. The presence of Na in PAAm-glucose and PAAm-galactose is probably due to the sodium carbonate/bicarbonate buffer system used as the reaction medium for the glucosamine or galactosamine substitution reaction. The bare TCPS plate shows two peaks. One is due to C 1s at 284.96 eV and the other due to O 1s at 533.18 eV. The latter peak in commercial TCPS plates is probably due to the oxygen containing additives as it is known that tissue culture polystyrene is not a pure polystyrene (Steele et al., 1993). The C 1s peak in PAAm-glucose and PAAm-galactose (Fig. 5) showed shoulder peaks towards higher binding energy with main peaks at 285.02 and 284.99 eV, respectively. The presence of acid and/or ester carbonyl species contributes to this shoulder formation (Briggs, 1982). This is also reflected in O 1s peaks of PAAm-glucose and PAAm-galactose (Fig. 6) which are wide and slightly deformed. XPS data were also used to obtain a rough estimation of glycoside content in PAAm-glucose and PAAm-galactose. Homopolymers of PAAm-glucose and PAAm-galactose synthesized from pure monomers were coated onto TCPS plates under identical conditions. The N atom percentage was selected for calculating glycoside content since there is only one type of N (coming from the initial glucosamine or galactosamine sugar) present in PAAm-glucose or PAAm-galactose synthesized either from monomer or from the poly(acryloyl chloride) method. The N atom percentage is listed in Table 1. Theoretical N atom percentages for homopolymers based on their repeat unit, are in fairly good agreement with the XPS data. The N atom percentages for homopolymers were taken as 100% glycoside content as they were synthesized

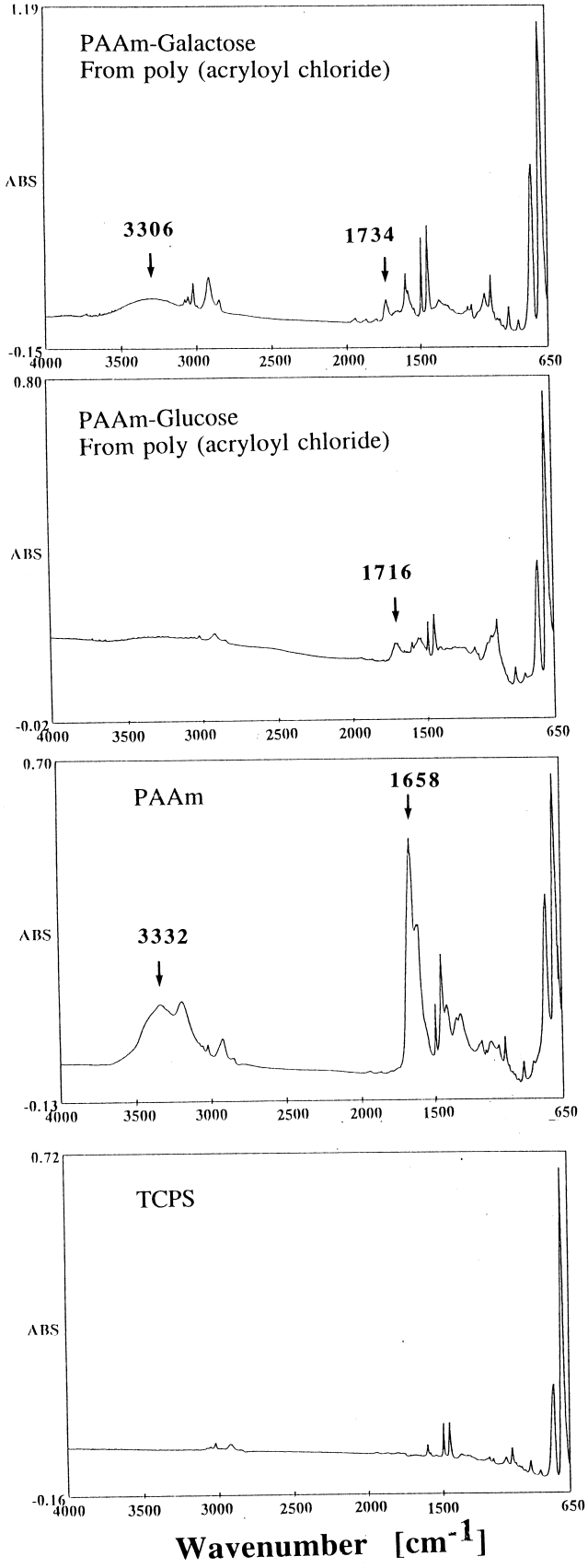

Fig. 3. FTIR-ATR spectra of tissue culture PS (TCPS) plate, TCPS coated with polyacrylamide, PAAm-glucose and PAAm-galactose.

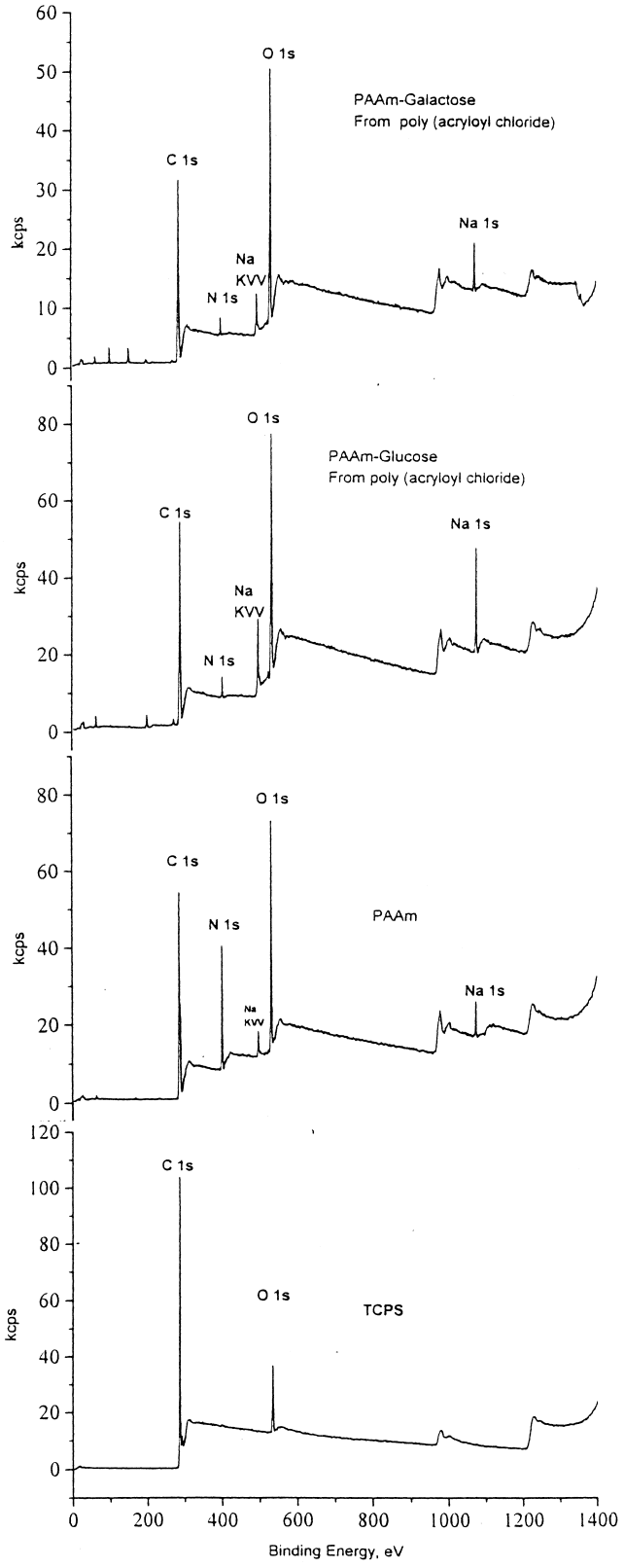

Fig. 4. XPS spectra of tissue culture PS (TCPS) plate and TCPS coated with polyacrylamide, PAAm-glucose and PAAm-galactose.

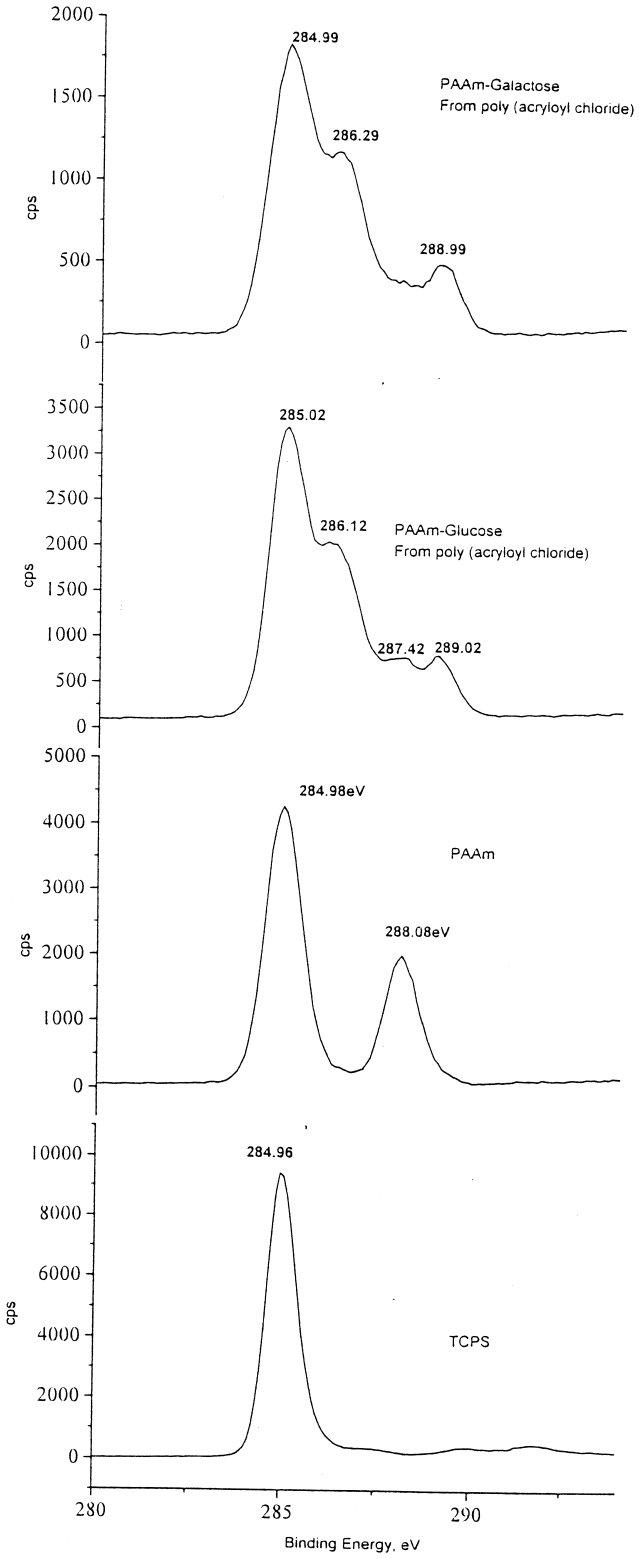

Fig. 5. C 1s XPS spectra of tissue culture PS (TCPS) plate and TCPS coated with polyacrylamide, PAAm-glucose and PAAm-galactose.

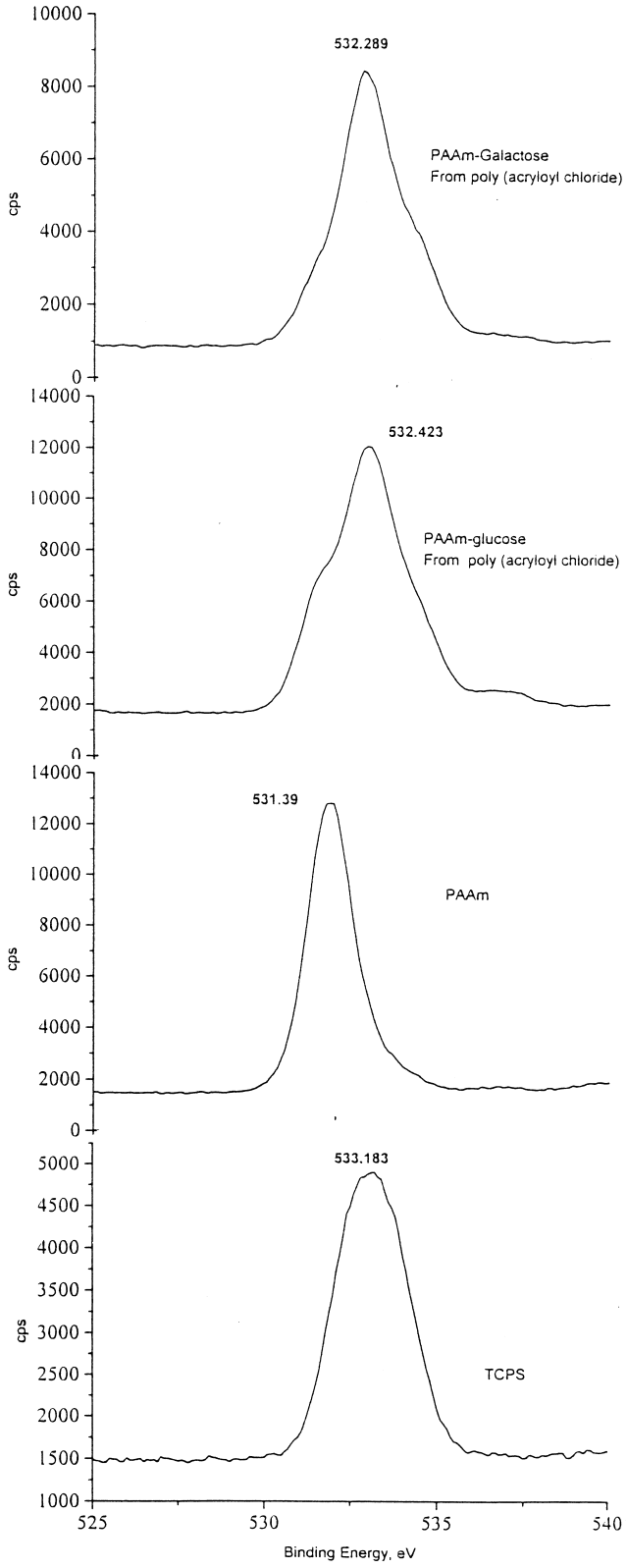

Fig. 6. O 1s XPS spectra of tissue culture PS (TCPS) plate and TCPS coated with polyacrylamide, PAAm-glucose and PAAm-galactose.

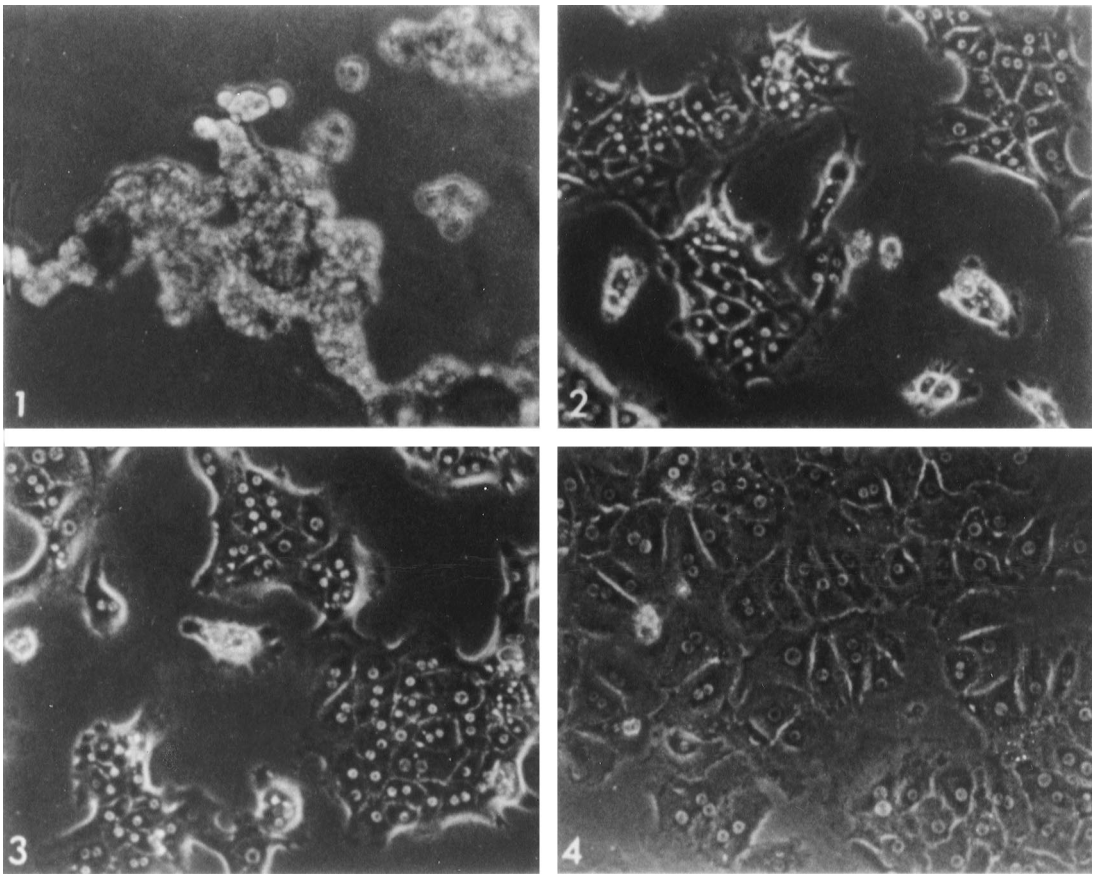

Fig. 7. Rat hepatocytes at 24 h of primary culture on the surfaces of: (1) polyacrylamide; (2) PAAm-glucose; (3) PAAm-galactose; and (4) type I collagen (phase contrast × 100).

from pure monomers. From this the glycoside content of PAAm-glucose and PAAm-galactose synthesized from poly(acryloyl chloride) was estimated. This data showed 53.2 and 41.6% of glucose and galactose in PAAm-glucose and PAAm-galactose, respectively, synthesized from poly(-acryloyl chloride) by polymer analogous reaction. The FTIR-ATR, XPS and contact angle measurements indicate a firm coating of polyacrylamide, PAAm-glucose and PAAm-galactose on TCPS plates.

Phase contrast microscope photographs of hepatocytes cultured on the surface of PAAm-glucose and PAAm-galactose in a serum-free medium are presented in Fig. 7. Hepatocytes rarely attached onto polyacrylamide surfaces and formed non-spheroidal aggregates. In this condition, albumin levels were highly maintained for more than 2 weeks in culture (Tokiwa et al., 1996). Amide groups in polyacrylamide may be responsible for poor cell attachment and the formation of non-spheroidal aggregates. In contrast, hepatocytes attached well to the surfaces of PAAm-glucose and PAAm-galactose, though attachment efficiencies to these polymer surfaces were low as compared with those on type I collagen coated plates.

L929 and CHO-K1 cell growth curves are presented in Figs. 8 and 9, respectively. A small but clear difference

Table 1
XPS (ESCA) and GPC data of PAAm-glucose, PAAm-galactose, homopolymers PAAm-glucose and PAAm-galactose

Sample code	N atom % (from ESCA)	Theoretical[a] N atom (%)	Sugar content (%) (from ESCA)	Mol. wt (from GPC)		
				M_w	M_n	PDI (M_w/M_n)
PAAm-glucose	3.2	—	53.2 ± 3.5	64 500	46 000	1.40
PAAm-galactose	2.5	—	41.6 ± 8.5	118 000	69 100	1.71
Homopolymer PAAm-glucose	5.8	6.01	100	567 000	211 000	2.69
Homopolymer PAAm-galactose	5.5	6.01	100	18 100	12 200	1.48

[a]Calculated from polymer repeat unit.

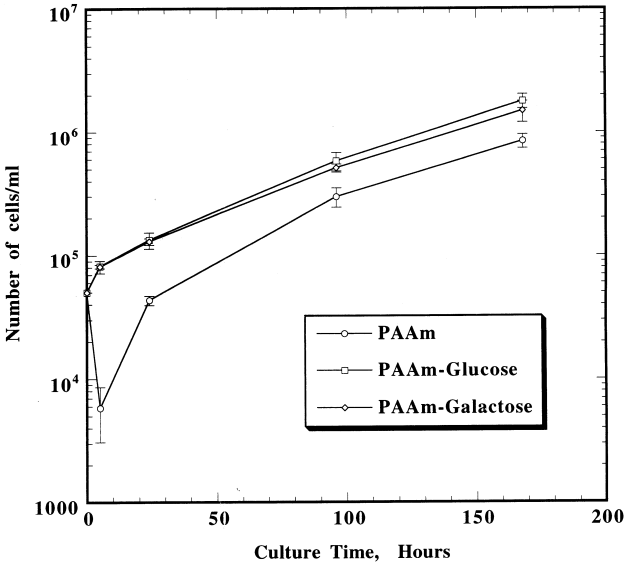

Fig. 8. Growth of L929 cells on the surfaces of polyacrylamide, PAAm-glucose and PAAm-galactose. Bars indicate the mean ± SD of three experiments.

between polyacrylamide and PAAm-glucose/PAAm-galactose in the cell attachment was noted. Namely, in both L929 and CHO K1 cells, fewer cell numbers were counted at 5 h of inoculation on the surface of polyacrylamide than on that of PAAm-glucose and PAAm-galactose, indicating poor cell attachment efficiency in the former. A significant difference in cell growth rate was not noted among the cells growing on polyacrylamide and PAAm-glucose/PAAm-galactose surfaces. These data suggest that PAAm-glucose/PAAm-galactose affect cell attachment, but not cell growth. PAAm-glucose, PAAm-galactose generated from poly(acryloyl chloride) by polymer analogous reaction, also contain other chemical species such as acids and/or esters. Such species might be contributing to the cell attachment along with sugar residues in the polymer. Effective cell attachment in the present case may be attributed to the cumulative effect of sugar and other chemical species present in the polymer. The results presented here also demonstrate that the polymer analogous reactions offer an easy way to synthesize the functional polymers for cell culture studies.

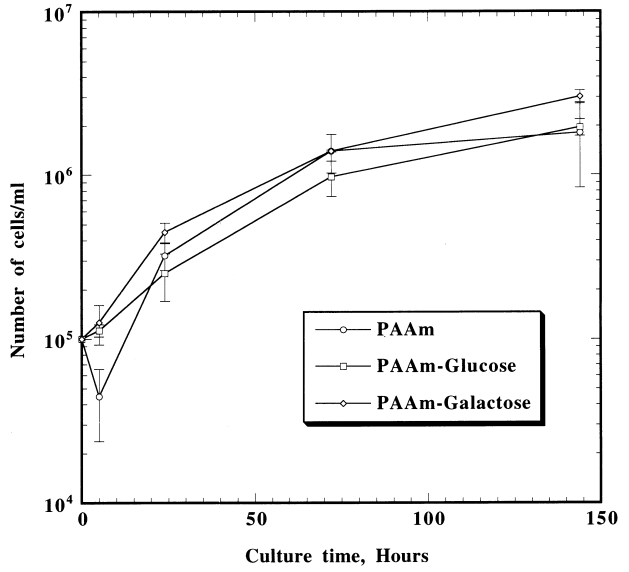

Fig. 9. Growth of CHO-K1 cells on the surfaces of polycrylamide, PAAm-glucose and PAAm-galactose. Bars indicate the mean ± SD of three experiments.

4. Conclusions

TCPS plates coated with PAAm-galactose and PAAm-glucose offer a good surface for the attachment of rat hepatocytes in primary culture as compared to those coated with PAAm. A small but noticeable difference was found among PAAm-glucose, PAAm-galactose and PAAm in the attachment of L929 and CHO-K1 cells. Cell growth rate behaviors of the latter two cell lines were almost identical on PAAm-glucose, PAAm-galactose and PAAm.

Acknowledgements

The authors thank Ms. Meng for her technical assistance. This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO).

References

Briggs, D. (1982). In D. M. Brewis (Ed.) *Surface Analysis and Pretreatment of Plastics and Metals.* London: Macmillan.

Duncan, R., Hume, I. C., Kopeckova, P., Ulbrich, K., Strihalm, J., & Kopecek, J. (1989). Anticancer agents coupled to *N*-(2-hydroxypropyl) methacrylamide copolymers 3. Evaluation of adriamycin conjugates against mouse leukemia L 1210 in vivo. *J. Control. Rel., 10*, 51–63.

Kallin, E., Lonn, H., Norberg, T., & Elofsson, M. (1989). Derivatization procedures for reducing oligosaccharides, Part 3: Preparation of oligosaccharide glycosylamines and their conversion into oligosaccharide-acrylamide copolymers. *J. Carbohydrate. Chem., 8*, 597–611.

Kochetkov, N. K. (1984). Synthesis of fragments of bacterial polysaccharides and their application for the preparation of synthetic antigens. *Pure. Appl. Chem., 56*, 923–938.

Schnaar, R. L. (1994). Immobilized glycoconjugates for cell recognition. In Y. C. Lee & R. T. Lee (Eds.), *Neoglycoconjugates: Preparation and Applications*. New York: Academic Press, pp. 425–443.

Schulz, R. C., Elzer, P., & Kern, W. (1960). Über die polymerisation von acrylsäure chlorid. *Makromol. Chem., 42*, 189–196.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol., 13*, 29–83.

Steele, J. G., Dalton, B. A., Johnson, G., & Underwood, P. A. (1993). Polystyrene chemistry affects vitronectin activity: An explanation for cell attachment to tissue culture polystyrene but not to unmodified polystyrene. *J. Biomed. Mater. Res., 27*, 927–940.

Strohriegl, P. (1993). Esterification and amidation of polymeric acyl chlorides. A new route to polymethacrylates and polymethacylamides with a variety of different side groups. *Makromol. Chem., 194*, 363–384.

Tokiwa, T., Kano, J., Meng, X., Bahulekar, R., & Kodama, M. (1996). Formation of non-spheroidal aggregates by adult rat hepatocytes on polyacrylamide coated surfaces. *Biotech. Tech., 10*, 845–848.

Weigel, P. H., Schnaar, R. L., Kuhelnschmidet, M. S., Schmell, E., Lee, R. T., Lee, Y. C., & Roseman, S. (1979). Adhesion of hepatocytes to immobilized sugars: A threshold phenomenon. *J. Biol. Chem., 254*, 10830–10838.